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REVIEW

Genotypes and viral variants in chronic hepatitis B: A review of epidemiology and clinical relevance

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Abstract

The Hepatitis B Virus (HBV) has a worldwide distribution and is endemic in many populations. It is constantly evolving and 10 genotypic strains have been identified with varying prevalences in different geographic regions. Numerous stable mutations in the core gene and in the surface gene of the HBV have also been identified in untreated HBV populations. The genotypes and viral variants have been associated with certain clinical features of HBV related liver disease and Hepatocellular carcinoma. For example Genotype C is associated with later hepatitis B e antigen (HBeAg) seroconversion, and more advanced liver disease. Genotype A is associated with a greater risk of progression to chronicity in adult acquired HBV infections. Genotype D is particularly associated with the precore mutation and HBeAg negative chronic hepatitis B (CHB). The genotypes prevalent in parts of West Africa, Central and South America, E, F and H respectively, are less well studied. Viral variants especially the Basal Core Promotor mutation is associated with increased risk of fibrosis and cancer of the liver. Although not currently part of routine clinical care, evaluation of genotype and viral variants may provide useful adjunctive information in predicting risk about liver related morbidity in patients with CHB.

Key words: Chronic hepatitis B; Genotype; Pre-core; Basal core promotor; Mutations

Core tip: Chronic hepatitis B (CHB) is a major global cause of liver related morbidity and mortality. Genotypes of the Hepatitis B virus have distinct geographical distributions and are known to influence a number of clinical features of disease and response to treatment. Certain well recognised viral mutations are also known to influence clinical risk of cirrhosis and hepatocellular carcinoma but in addition may have implications for vaccination programs and screening of blood for donation. This review examines the current state of knowledge about genotype and viral variants of CHB and their utility in the management of this disease.

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INTRODUCTION

Chronic hepatitis B (CHB) is a global health problem and a leading cause of cirrhosis and hepatocellular carcinoma (HCC) worldwide. The Hepatitis B virus (HBV) is a hepatotropic virus of the family hepadnaviridae. It comprises a central icosahedral core protein (HBcAg) which contains the viral DNA and HBV viral polymerase. This core (also called the nucleocapsid) is surrounded by a lipid membrane studded with viral proteins which are the small, medium and large HBV surface proteins. The entire virion is 42 nmol/L and was originally referred to as the Dane particle following its discovery by an English pathologist DS Dane[1]. In the past, HBV was divided into serotypes which were subgroups based on the antigenic determinants of the hepatitis B surface antigen (HBsAg) and 4 subtypes were known, adr, adw, ayr and ayw[2].

GENOTYPES IN CHB

In 1988 it was first suggested by Okamoto *et al*[3] that HBV could be divided into 4 genotypes based on a divergence of $\geqslant$ 8% in the complete genomic sequence and genotypes A,B,C and D were identified. The relationship between serotypes and genotypes is not clearly known and the same serotype may be classified into different genotypes[3]. Genotyping may be performed by a number of different techniques including restriction fragment length polymorphism, line probe assay, the enzyme-linked immunosorbent assay or genotype specific polymerase chain reaction[4]. Direct sequencing can also be used and for commercial purposes, genotype can usually be determined through a partial sequence especially of the S gene since it is usually more conserved than other parts of the HBV genome. Following the initial description of genotypes A-D, Norder *et al*[5] also proposed genotypes E and F which differed by more than 4% in the *S* gene from the other genotype groups and this has become an alternative criterion for classification of distinct genotypes. Genotype G is the least common of the genotypes and was reported in 2000 from samples of French and American patients[6] but its geographic origin is still unknown[7]. The precore and core regions of genotype G are aberrant with a 36-nucleotide insertion within the core gene making it the longest of the HBV genotypes[8]. Stop codons in the precore region are also present and some have suggested it is not able to produce hepatitis B e antigen (HBeAg)[8] although others report high HBeAg levels in HIV/HBV coinfected patients with genotype G[9]. However, this may be due to coinfection with genotype A[10]. It is thought that genotype G requires the presence of another genotype, most commonly genotype A2 to enhance its viral replication[11]. Although monoinfection has been reported, this was transient[12]. Genotype H has been shown to be prevalent in central America and the Amazon region and is closely related to genotype F HBV. It is thought to perhaps have split off from genotype F within the "New World"[13]. It is particularly common in Mexico[14].

Genotype I described in Vietnam[15] may not meet the criteria for a novel genotype since the diversity in its complete genome sequence is only 7% from that of its closest neighbour, genotype C[16]. Genotype J is a novel variant described in a Japanese patient who had previously travelled to Borneo. It is thought to be phylogenetically positioned between human and primate HBV variants being close to strains which had been previously found in orang-utans and gibbons[17].

This genetic variability in the HBV may come about through natural mutation or by recombination. Natural mutation rates are high in HBV. The HBV is error prone since its reverse transcriptase lacks proof reading ability and it is estimated that the rate of nucleotide substitutions per site per year is approximately $1.4\text{-}3.2 \times 10^{-5}$[18] which is 10 times higher than other DNA viruses and 100 times higher than the human genome[19]. Recombination, in which DNA exchange or cross over of parts of a gene sequence occurs between two different viruses, is thought to be another potential mechanism for the development of divergent strains of HBV[20] and has been described between numerous different genotypes[21,22]. Recent evidence suggests that the core gene may be a preferred site for recombination to take place as noted in West African patients with A/E recombinant strains of HBV[23].

SUBGENOTYPES

Subgenotypes are also described if there is a divergence of > 4% (but less than 7.5%) of the nucleotide sequence in the complete genomic sequence[19]. There have been numerous (up to 40) subgenotypes reported amongst genotypes A-D and F. Geographic distribution varies for subgenotypes and certain clinical outcomes have also been attributed to some of them however confounding factors are difficult to control for in many of these studies. Divergence of < 4% between subgenotypes are referred to as "clades"[24]. There has been concern raised by experts in the field about the accuracy of classification of some of the newly reported subgenotypes[25]. Pourkarim *et al*[25] suggest that applying phylogenetic analysis over a full length genome sequence rather than partial sequence only is critical to avoid misclassification. They also suggest that recombinant strains should not necessarily be introduced as independent subgenotypes and propose the term "recombino-subgenotype" to identify HBV strains that show strong evidence of recombination in their nucleotide divergence.

GEOGRAPHICAL DISTRIBUTION OF GENOTYPES

The geographical distribution of the different geno-

Table 1 Geographic distribution of genotypes by continent

	Region	Genotype distribution
Africa	Subsaharan Africa (Egypt, Algeria, Libya)	Genotype D
	West Africa (Guinea Bissau, Ghana, Cameroon)	Genotype E and also A
	Central Africa	Genotype E and also A
	East Africa (Malawi, Tanzania)	Genotype A
	South Africa	Genotype A
Europe	Mediterranean Basin (Greece, Italy, Spain)	Genotype D in majority. Gentype A also seen in Spain
	Western Europe	Mixtures of A-D from various migrant groups
	Eastern Europe	A (Czech republic, Poland) and D (Russia, Croatia, Romania)
Americas	North America	Mixtures of A-D from various migrant populations
		Genotype F and B in Alaskan natives
	Central America	Genotype H (Mexico)
		Genotype F (Costa Rica)
	South America	Genotype F predominant and Genotypes A and D in Brazil/Argentina
Asia	Western Asia (Iran, Yemen, Saudi Arabia, Turkey)	Genotype D
	Central Asia (Uzbekistan, Tajikistan, Afghanistan, Pakistan)	Genotype D
	South Asia (India and Pakistan)	Genotype D in India but also Gentoype A

types is quite varied and for most of the older known genotypes and many regions, is well documented. Many parts of the world have dominant genotypes although frequently there are at least 2 prevalent genotypes.

Patients may also be coinfected with more than one genotype since in many parts of the world 2 or more genotypes are commonly found eg B and C in Asia[26]. Genotype G also, as mentioned, appears to require the presence of genotype A[10] or H for chronic infection[27]. The distribution of HBV genotypes in different continents are detailed in Table 1.

AFRICA

In East Africa (including Malawi and Tanzania) genotype A is found in the vast majority (> 90%) of patients[28,29]. In South Africa also, the dominant genotype is A[30]. In other parts of Africa, for example West Africa, Genotype E is prevalent[31,32] and this appears to stretch into parts of central Africa also[33]. There has been some work suggesting that HBV genotype E introduction and expansion in West Africa has been a relatively recent phenomenon[34,35] . In sub-Saharan (or Northern) Africa including Egypt, Algeria and Libya) which forms part of the Mediterranean Basin, genotype D predominates in up to 80% of patients[36,37].

EUROPE

In the European countries of the Mediterranean basin, in particular Greece[38], Italy[39], and Spain, the predominant genotype is Genotype D[40]. Genotype D is also found in about 50% of cases in Eastern Europe, with genotype A in approximately 30%[26]. Although some countries have a higher proportion of genotype A, *e.g.,* 86% in Poland and 67% in Czech republic, Genotype D is found in the majority of Russian (93%), Romanian (67%) and Croatian (80%) patients[41,42]. The proportions of genotypes A and D are similar at about 30%-40% in the remainder of Europe (the EU and northern Europe) with smaller contributions from other genotypes including B and C, most likely due to migration[43].

ASIA

In Western Asia, *e.g.,* Turkey[44] and the Middle East including Iran[45], the prevalent genotype is D. Central Asian countries of Uzbekistan and Tajikistan also have a preponderance of genotype D infection of up to 88%[46,47]. Southern Asian countries similarly show predominantly genotype D infection, *e.g.,* 95% of cases in Afghanistan[48] and in a majority of Indian patients[49-51] and 65% of Pakistani patients[52]. However genotype A is also seen in India[53] and a recent study from Eastern India highlights a shift in the prevalences of genotypes with an increase in Genotypes A and C along with a decrease in that of genotype D in East India[54].

Moving further east into South East Asia and China, genotypes B and C start to predominate. The relative prevalences of genotypes in many countries of south east Asia and regions of China are set out in Table 2[55-79] . In brief however, genotype C is seen in the majority of patients of Cambodian, Thai, Laotian and Myanmar ethnicity. Genotype B is predominant in Vietnamese cohorts and some parts of Indonesia and Malaysia. In China, Genotype C is prevalent in most areas although in other parts, Genotype B is seen frequently. Japan has a predominance of genotype C (82% in a study of 1271 patients)[80]. This study also reported an increase in the prevalence of genotype A from 1.7% to 3.5% from the period 2001-2006 which is thought to be due to persistence of sexually acquired acute HBV in adulthood. In Korea genotype C2 predominates[81].

AMERICAS

Among Indigenous populations living in the Arctic, and northern Canada and Greenland genotype B

Table 2 Prevalence of different hepatitis B virus genotypes in Southeast Asian countries and China

	No. in study	Genotype distribution	Notes	Ref.
Laos	386	42.2% B 55.4% C 2.4% not typable ? I	Cohort of patients from Vientiane city and central provinces. 19 patients did not group into genotype A-H ? genotype I	[55]
Cambodia	12	67% C , 33% B (subtype 4)		[56]
	22	72% C 28% B		[57]
Vietnam	76	51% B, 48.7% C	Chronic cohort	[58]
	40	75% B 18% C 2.5% B + C, 5% not determined	Based in Hanoi	[59]
Indonesia	54	76% B 24% C		[60]
	54	100% B	Surabaya	[61]
	27	85% C 7.4% B 7.4% D	Papua	[62]
Malaysia	86	60% B 34% C 2% D	Genotype B 80% in ethnic Chinese Genotypes B and C equal prevalence in Ethnic Malays Gentoype D in Indian patients	[63]
	51	56.9% B 31.4% C 7.8% B + C 2% each D and E		[64]
Thailand	224	86.6% C (Subgeno C1) 11.2% B 0.44% each of A and D 3 suspected recombinations	Myanmar ethnicity 97.5% genotype C Laos ethnicity 71% C 26% B, Cambodia 84% C, 12% B	[65]
	216	89.3% C 7.4% B, 1.9% B + C, 0.5% A	Northern Thailand adult voluntary blood donors	[66]
	53	90.6% C 7.5% B 1.9% B + C	Children in Chiang Mai	[67]
	332	73.2% C 20.8% B 3.3% A 2.7% unclassified	Cohort included CHB and HCC patients and found that genotype B was not associated with HCC in younger patients	[68]
Philippines	100	51% A 22% B 27% C		[69]
	50	28% A 12% B 26% C 6% Mixed A + C/A + B + C 28% Non typable		[70]
China	101	36% B 64% C	Hong Kong 42% B Shnaghai 39% B Beijing 20% B	[71]
	121	33% B 63.6% C 1.7% B/C 1.7% D	From Beijing China	[72]
	126	38.1% B 54.8% C 0.8% D, 3.2% unknown 1.6% B/C, 1.6% A/C	Yunnan China	[73]
	142	9.2% B 88% C 2.8% D	Northern China (Harbin University China)	[74]
	142	4.2% A 14.1% B 78.9% C 1.4% D	Southern China (Nanning)	[75]
	786	63.23% B 34.99% C 0.89% A and D each	Southern China (Guizhou)	[76]
	220	1.4% A 17.2% B 81.4% C	Shanghai China	[77]
China (Hong Kong)	776	1.5% A 32.5% B 62.6% C 3.4% Mixed	Hong Kong	[78]
Tibet	26	96% C/D recombinant 4% C	Sequences based on surface Ag gene showed that 25 clustered with genotype D and 1 clustered with genotype C. However based on core gene all clustered with genotype C	[79]

(subgenotype B6) has been found to be prevalent[82]. Genotype F has also been shown to be predominant in Alaskan native Inuit populations. Genotype F is also found in South and central America and is thought to be the most prevalent genotype in most of these countries[83] although in Brazil, and Argentina genotypes (plural) A and D are also seen[84]. In Central America, genotype H HBV is prevalent, being found in approximately 75% of patients in a small study in Mexico[85].

In the United States, CHB is found primarily in migrant populations where the mix of different genotypes reflects the various immigrant groups. A large study of 694 patients in the United States identified a strong correlation between ethnicity and genotype and found that in patients of Asian background, genotypes B and C were most common and in those of white or African American background who usually acquired hepatitis B in adulthood through sexual transmission, genotype A was most common[86].

AUSTRALIA AND THE PACIFIC

In Australia, Bell *et al*[87] showed in 2005 that in the cohort at St Vincent's Hospital, Melbourne, 8% had

Table 3 Clinical associations with hepatitis B virus genotypes

	A	B	C	D	E	F	G	H
Progression to chronicity	+++	++	+++	++				
Histological inflammation	++	++	+++	+++		+/-		
Histological fibrosis	+	+	++	++		+/-		
Association with advanced liver disease	+	++	+++	++		+/-		+
Association with HCC	+ (subgeno A1)	+	++	++		++ (subgeno F2)		+
Early HBeAg seroconversion	++	+++	+	+++		+++		++
Sustained remission after HBeAg Seroconversion	+++	+++	++	++		++		
HBsAg clearance	+++	++	+	++				+++
Response to IFN Tx	+++	++	+	+/-	+	+++	+	++
Association with PreCore mutations	-	++	+	+++	++	+++ (F1 but not F2)		
Association with BCP mutation	++		++		++			++

+/-: Possible association; - : No association; +: Slight association; ++: Moderate association; +++: Strong association. HBeAg: Hepatitis B e antigen; BCP; Basal core promotor; HCC: Hepatocellular carcinoma; IFN: Interferon.

genotype A, 29% B, 41% C and 22% D reflecting the multicultural nature of Australian Society and the patterns of migration from the Mediterranean region and more recently South East Asia. There are few studies from the Pacific Island nations however one from the Solomon Islands where Hepatitis B is hyperendemic (prevalence of 21%), found a predominance of genotypes C and D which appeared ethnicity specific[88].

GENOTYPE AND CLINICAL OUTCOMES

There are a number of studies documenting the effect of genotype on various clinical outcomes. Many of these provide comparisons of 2 prevalent genotypes in a region, *e.g.,* A *vs* D, or B *vs* C. Table 3 sets out some of what is known about the different genotypes and clinical associations. There is a paucity of information about genotype E and its associations with clinical outcome and more work is needed to further elucidate its impact on HBV related liver disease.

GENOTYPE AND RISK OF CHRONICITY

Genotype A appears to have the highest risk of progression to chronicity following acute adult acquired Hepatitis B and resolution of acute hepatitis B is often prolonged in genotype A[89]. In a cohort of Asian patients, genotype C2 was independently associated with progression to chronicity, compared to genotype B[90]. Acute infection with genotype D appears to be more commonly associated with acute liver failure than other genotypes[91].

GENOTYPE AND HBEAG/HBSAG CLEARANCE

Many Asian studies have documented the more prolonged HBeAg positive phase and delayed HBeAg seroclearance of genotype C in comparison to Genotype B[92,93] including Chu *et al*[94] who showed that HBeAg seroconversion occurs about 10 years earlier in genotype B compared to genotype C. A study of 1158 Alaskan natives which also looked at timing of seroconversion found that HBeAg seroconversion in genotype C (Subgenotype C2) patients lagged behind that of other genotypes by approximately 3 decades. Age at HBeAg seroconversion of the 75th percentile of patients was 32 years in genotype A2, 27.5 years in B6, 27.3 years in D, 24.5 in F1 but 58.1 years in C2[95]. Genotype C patients are more prone to repeated episodes of acute exacerbation with failure of HBeAg seroconversion[96] and HBeAg sero-reversion after HBeAg loss[95]. Rates of spontaneous HBsAg clearance are higher in genotype B compared to C[97]. Sustained remission following HBeAg seroconversion has been reported to be more commonly seen in genotype A than D as was HBsAg clearance[98] .

GENOTYPE AND HBV DNA LEVELS

Genotype C has been reported to have a significantly higher viral load than genotype B[99]. Viral load in genotype D has also been shown to be significantly higher than in Genotype A[100]. Genotype E is reported as being more likely to be associated with HBeAg positive disease and higher HBV DNA levels than Genotype D thus perinatal infection of infants from infected mothers is likely be an important factor in transmission for African people infected with genotype E[101].

GENOTYPE AND LIVER DISEASE

Genotype C patients are more prone to the complications of advanced fibrosis and cirrhosis[102-104] than Genotype B patients. Some small studies have shown that histological inflammation is more significant in genotype C than genotype B patients[71,105] . Genotype F, in studies of Arctic, South American and Spanish populations, also appears to be associated with worse liver disease[98,106,107]. Genotype A appears to have a

more favourable prognosis than genotype D with one small Indian study of 52 patients (46% Genotype A and 48% genotype D) showing more severe histological disease in genotype D[108] and others also attributing more severe liver disease to genotype D compared to A[109]. Genotype D is associated with HBeAg negative CHB and reports from Mediterranean countries of high rates of cirrhosis associated with HBeAg negative disease are now thought to possibly be attributable to genotype D[110]. Genotype H infection in Mexican patients is often adult acquired and thus is frequently associated with low viral loads and low risk of chronic liver disease and HCC[111]. Occult HBV infection is reported to be commonly seen in genotype H patients however this may be partly due to the suboptimal sensitivity of HBsAg assays used[112]. Furthermore the contribution of HBV genotype H to liver disease in Mexican populations is difficult to establish as alcohol, HCV coinfection and obesity are common cofactors[14].

GENOTYPE AND HCC

Genotype C has been shown to carry an increased risk for the development of HCC in the REVEAL study cohort, with an adjusted hazard ratio of 2.35[113]. In addition there is data to suggest that HCC in genotype C is associated with a higher tumour recurrence rate[114]. Genotype B on the other hand may be more likely to be associated with HCC in non cirrhotic patients and has been reported to have higher rates of solitary tumour and more satellite nodules than genotype C[115]. Genotype B has been reported to be more prevalent in patients with HCC developing at a younger age compared to age matched inactive carriers (80% *vs* 52% in those < 50 years and 90% in those < 35 years)[116]. Genotype A in Africans has also been shown to be associated with HCC and at a much younger age than in other groups. Subgenotype A1 which is the most prevalent type in sub-Saharan Africa appears to be the main factor associated with this increased risk[117]. However the contribution of aflatoxin, human immunodeficiency virus (HIV) coinfection and dietary iron overload are also factors to be considered [118]. Genotype F has also been shown to be a risk factor for HCC especially in young Alaskan natives in a case control study[119] while the rates of HCC in genotype H affected populations in Mexico are low[111].

GENOTYPE AND TREATMENT FOR HBV

Response rates to treatment with Peg IFN differ by genotype. In HBeAg positive patients treated with 52 wk of Peg IFN α-2β, HBeAg loss varied with genotype, being 47% in genotype A, 44% in Genotype B, 28% in genotype C and 25% in D[120]. A small study of Peg IFN treatment in Genotype E patients also showed poor responsiveness[121]. Limited data currently available on genotype F patients response to IFN suggests similar response to genotype A[107]. Genotype G in a small number of patients' treated with standard IFN showed poor responsiveness[122]. Based on pooled data of the 2 largest global trials of Peg IFN in HBeAg positive patients Buster *et al*[123] recommend Peg IFN be used in all genotype A patients, and in genotype B and C patients with a high alanine aminotransferase (ALT) and a low HBV DNA. In HBeAg negative CHB also, genotypes B and C have been shown to have higher response rates to Peg IFN treatment compared to genotype D[124].

Quantitative HBsAg (qHBsAg) levels are increasingly being used as predictors of response in Peg IFN therapy for CHB. In HBeAg positive disease, for patients with genotype A and D, absence of any decline in qHBsAg at week 12 has a negative predictive value (NPV) of 97-100% for poor response and in genotypes B and C, week 12 qHBsAg levels of >20000 IU/mL has a high NPV[125]. In HBeAg negative patients treated with Peg IFN, a stopping rule in genotype D based on no decline in HBsAg and < 2 $\log_{10}$ drop in HBV DNA at week 12 of therapy has also become part of recent guidelines[126] based on a very high negative predictive value for sustained response[127]. In HBeAg negative patients treated with Peg IFN, the on treatment kinetics of HBsAg has been shown to vary between genotypes. Long term virological response to Peg IFN treatment has been shown to be predicted by end of treatment qHBsAg with varying threshold levels of qHBsAg identified for different genotypes[128]. HBV genotype does not appear to influence response rates to nucleoside analogue therapy, however the patterns of drug resistant mutations that develop have been reported to be different in different genotypes[129].

VARIANT VIRUSES

HBV replicates at a high rate through the reverse transcription of an RNA intermediate and has a high spontaneous rate of error resulting in numerous mutations arising in the HBV genome[130]. Thus HBV exists as a quasispecies, ie a heterogeneous viral population composed of closely related but non identical genomes[131]. The predominant strain selected out is determined by factors such as host immune response, viral replication fitness and exogenous pressures such as antiviral therapy[132]. The most frequently occurring natural HBV variants are the precore and the basal core promotor (BCP) mutations. They result in a reduction or abolition of HBeAg production. During the early course of perinatally acquired CHB , *i.e.,* the immunotolerant phase, these mutations in the core or precore region are uncommonly seen, but they emerge during the immune clearance phase as a result of immune selective pressures[133].

HBEAG VARIANTS

Precore mutations

A point mutation at nucleotide 1896 in the HBV with

substitution of G for A results in a stop codon at this point thus preventing production of HBeAg although without affecting replication and HBcAg production[134]. The formation of this "precore" mutation is essentially precluded in certain genotypes in particular A and H and it occurs most frequently in genotype D and to a lesser extent in Genotypes B, C and E[24]. The reason for this relates to the fact that nucleotide 1896 is important in maintaining a stem loop structure (epsilon) which is necessary for encapsidation of the pregenomic RNA into the nucleocapsid. Nucleotide 1896 is opposite nucleotide 1858 in the stem loop structure and thus genotypes with a T at nucleotide position 1858 (*e.g.,* genotype D) are more likely to predispose to the development of the G1896A mutation since T-A pairing is more stable than T-G pairing[135]. Likewise genotypes with C at position 1858 (genotype A) are much less prone to development of the precore mutant virus since C-A pairing is weaker than than C-G pairing[136]. Subgenotype F2 similarly codes for C at position 1858 but F1 does not , so precore mutation may occur in some but not other genotype F CHB patients[137].

It has been suggested that stabilisation of the epsilon encapsidation signal may increase the replicative fitness of HBV which may be one reason for it being selected out[133]. Although HBV DNA levels are lower in HBeAg negative disease, this impairment in virion productivity is not thought to be related to Precore and BCP mutants but instead be the result of an independent process[138]. The HBeAg and its relationship to the host immune response is complex. It has been shown to be able to tolerize T cells[139] and cause immunomodulation of Toll-like receptor mediated signalling pathways to evade immune responses and thus is thought to contribute to viral persistence[140]. However its presence in the cytosol also acts as a target for the inflammatory response[141]. Thus it has been suggested that HBeAg may act as a tolerogen or an immunogen in different circumstances and that loss of HBeAg may be a favourable biological characteristic that renders the HBV less vulnerable to immune attack[142]. Numerous other mutations have been described in the precore region including a point mutation at G1899A (commonly seen in association with G1896A)[143]. It has been shown that viral mutation rates increase in the immune clearance phase when compared to the immune tolerant phase[144] and in fact there is significant viral sequence diversity present in the months/years leading up to HBeAg seroconversion[145]. Furthermore, the high rates of nucleotide substitution continue post seroconversion, with ongoing immune selection pressure being applied in the immune control phase although at lower viral loads[146].

Clinically, the G1896A precore mutation is a major cause of HBeAg negative chronic hepatitis and it appears to be associated with reports of fulminant hepatitis, especially in acute adult acquired HBV[147] although not in fulminant cases of CHB[148]. It was shown to be associated with a slightly lower risk of HCC than wildtype virus in a subanalysis of the REVEAL study cohort[113]. However, also recently published is a meta-analysis of 85 case control studies of 16745 patients with 5781 cases of HCC which reports that the precore mutations G1896A, G1899A as well as deletions in Pre-S region were associated with an increased risk of HCC[149].

BCP region mutations

Mutations in the core promoter region occur most commonly at nucleotides 1762 (adenine (A) to thymine (T)) and 1764 [guanine (G) to adenine (A)] and are usually found together[150, 151]. Once again this is thought to possibly confer some compensatory advantage to the virus and Tacke *et al*[152] showed that BCP mutations increased viral replication levels to above those of wildtype virus, including in strains with Lamivudine resistant mutations also present. Other mutations in the basal core promotor region have also been described, including at positions 1653, 1753-1757, 1766, 1768 and these are usually seen in addition to the A1762T and G1764A variants. Overall the association with HBeAg negativity is less strong in patients with BCP mutations than in those with precore mutations The double BCP reduces the production of HBeAg by approximately 70%[153] and this may be even further reduced in HBV variants with the additional mutations in position 1753 and 1766[153]. The BCP mutation has been found to occur more frequently in genotype C than B[78,154] and one possible reason for this may be because genotype C is more likely to have a C at nucleotide position 1858, which largely precludes formation of the precore mutation[155]. The prevalence of the BCP mutation does appear to vary between genotypes being reported at 41%, 27%, 60% and 42% in genotypes A, B, C and D[86], thus the lower prevalence in genotype A compared to C suggests that other factors apart from the nucleotide at position 1858 are also important. The BCP mutations have been implicated quite strongly in more advanced liver disease and in the development of HCC. Yuen reported an association with higher ALT levels in patients with BCP mutations compared to those with wildtype[78]. The authors also subsequently looked at 66 patients with liver biopsies, 71% of whom had the BCP mutations and found these patients had more severe necroinflammation than those without[156]. Lin *et al*[157] also showed that the BCP mutation was associated with the development of cirrhosis and HCC although this was restricted to males. Increasing prevalence of the BCP double mutation was reported by Kao *et al*[154] in patients with more significant liver disease, being 3% in inactive carriers and 64% in patients with HCC. Orito *et al*[158] also described a significant association between BCP and more advanced liver disease (OR = 4.1, 95%CI: 1.6-10.2). Recently a study by Tseng *et al*[159] of 251 spontaneous seroconverters (Genotypes B and C) showed that a higher proportion of BCP mutation (> 45%) was associated with an OR of

2.81 for the risk of cirrhosis. However, whether the BCP mutant HBV is causative or simply a reflection of more significant immune pressure and thus immune mediated inflammation and fibrosis is not known. The association of the BCP mutation with HCC has been documented by a number of groups who reported the BCP mutation was significantly associated with HCC in both genotypes B and C[154,160], in cirrhotic and non cirrhotic HCC[161,162] and by Baptista who found a high prevalence of the mutations in black African patients with HCC[163].

More recently the REVEAL study group also reported an increased risk of HCC in patients with the BCP A1762T/G1764A double mutant compared to wildtype (HR = 1.73, 95%CI: 1.13-2.67). Genotype C also had a higher hazard ratio compared to genotype B (HR = 1.76, 95%CI: 1.19-2.61) and the highest risk was amongst those with the BCP mutations, Genotype C and wildtype virus at the 1896 precore variant site (adjusted HR = 2.99, 95%CI: 1.57-5.7)[113]. There is no strong evidence that the presence or absence of the precore or BCP mutations affects the response to Interferon or nucleos(t)ide analogue treatment. However some studies have shown that the proportion of pre-core and BCP mutant virus present prior to treatment correlates with the chance of HBeAg seroconversion with an approximately 2% increase in HBeAg seroconversion rates per 1% increase of PC and BCP mutant percentages[164].

HBSAG VARIANTS

Vaccine escape mutants

Variations in the HBsAg protein can result in viral infection developing in a vaccinated subject. Anti-HBs is directed towards a highly conserved region of the surface protein (amino acids 99-160) which includes the major "a" determinant of this protein. HBsAg mutations resulting in amino acid substitutions in the region 137-147 of the surface protein can change the conformational epitope in the "a" determinant so that it is not recognized by the neutralizing anti-HBs antibodies. In particular the G145R vaccine escape mutant is known to be stable and replication competent[165,166]. The infectivity of HBsAg mutants is currently thought to be low, however another problem is their lack of detectability by serological tests[167] as was reported in a recent case of HBV with a 4 amino acid repeat insertion at position 115 in the surface protein[168]. The development of vaccine escape mutants has been thought in some parts to be related to the emergence of anti-viral drug resistant mutants because of the overlap of the polymerase gene (where nucleot(s)ide analogue associated resistant mutations occur) with the surface antigen domains recognized by anti-HBs[169]. There is evidence however that emergence of the vaccine escape mutants predates mass vaccination programs[170]. It is thought possible that immune pressure exerted on HBV by anti-HBV due to expanding vaccination programs will result in an increasing problem of vaccine escape mutants[167]. The sG145R vaccine escape mutant has also been detected in 2 of 65 patients in an Australian indigenous cohort, despite wild type polymerase gene sequences in all[171]. Graft infection with HBV following liver transplant in patients who received hepatitis B Immune globulin therapy post transplant has also been shown to be due to the development of *S* gene mutant HBV[172].

S escape mutants

Other mutations in the S protein, eg due to missense mutations in the *S* gene have also been described and are a particular concern for screening of blood donors since they result in false negatives for HBsAg serological testing[173]. The prevalence of 8 mutations associated with HBsAg diagnostic failure, including P120T, T126S, Q128H, G130N, S143L D144A and G145R was found to be approximately 1% in a study of 11,221 HBV sequences encompassing genotypes A-H[174].

A study of 4.4 million Dutch blood donations identified 23 HBsAg negative but HBV DNA positive persons and also reported the presence of multiple escape mutations in the S gene especially in Genotype D patients with occult HBV[175]. Other reasons for HBsAg negativity in the setting of HBV DNA positivity in this study were early acute HBV infection (prior to development of HBsAg), occult HBV infection, genotype G HBV with decreased HBsAg production and suppressed infection after vaccination.

Pre-S mutations

Mutations in the pre-S region, including Pre-S2 deletions, pre-S1-S2 deletions and Pre-S2 start codon mutations have been described[176,177]. Pre-S mutants have been shown to be associated with decreased synthesis and secretion of HBsAg[178]. It has been also been shown that Pre-S2 deletions are associated with more advanced liver disease[179-181] and possibly with the development of HCC especially in younger patients[162,182].

CONCLUSION

HBV genotype and viral variants have clear implications for many clinical aspects of CHB and should become more routinely utilised to help predict likely clinic course, *e.g.,* longer duration of HBeAg phase and higher risk of progression to cirrhosis in genotype C and increased HCC risk in genotypes C and F. It could be argued that formal testing of genotype should be done rather than it being assumed on the basis of ethnicity especially as populations worldwide become increasingly cosmopolitan and subtle shifts in HBV genotype prevalences are already reported (*e.g.,* in Africa and India). Easy to use nomograms derived from the Taiwanese REVEAL study's cohort for predicting HCC risk show further refinement in risk

stratification with the addition of genotype to other parameters[183]. Similarly nomograms that incorporate genotype into predictability of sustained response to Peg IFN therapy can assist with management decisions in CHB patients[123]. Thus practical tools already exist for use in clinics which are based on existing knowledge about the impact of genotype in CHB. Further research into the genotypes E, F and H are required. International agreement amongst experts on issues related to genetic and phylogenetic classification of genotypes and subgenotypes are also important for the future in this evolving area.

With regard to viral variants, knowledge of variants harboured by patients should also be increasingly incorporated into clinical decision making. Revill and Locarnini recently argued that given the evidence that the BCP mutation is an important viral biomarker of the risk of cirrhosis in genotype B and C patients, detection and quantification of BCP mutants should be performed and used as triggers for treatment in Asian CHB patients[184]. Further work on the significance of these mutations in other genotypes was also recommended.

Further elucidation of the clinical significance of other viral variants is warranted. Importantly from a population health perspective, ongoing monitoring of prevalences of vaccine escape and *S* escape mutants is necessary and further research into vaccines that remain efficacious against mutant forms of HBV will be needed.

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REVIEW

Spontaneous bacterial peritonitis: The clinical challenge of a leaky gut and a cirrhotic liver

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Abstract

Spontaneous bacterial peritonitis (SBP) is a frequent, life-threatening bacterial infection in patients with liver cirrhosis and ascites. Portal hypertension leads to increased bacterial translocation from the intestine. Failure to eliminate invading pathogens due to immune defects associated with advanced liver disease on the background of genetic predisposition may result in SBP. The efficacy of antibiotic treatment and prophylaxis has declined due to the spread of multi-resistant bacteria. Patients with nosocomial SBP and with prior antibiotic treatment are at a particularly high risk for infection with resistant bacteria. Therefore, it is important to adapt empirical treatment to these risk factors and to the local resistance profile. Rifaximin, an oral, non-absorbable antibiotic, has been proposed to prevent SBP, but may be useful only in a subset of patients. Since novel antibiotic classes are lacking, we have to develop prophylactic strategies which do not induce bacterial resistance. Farnesoid X receptor agonists may be a candidate, but so far, clinical studies are not available. New diagnostic tests which can be carried out quickly at the patient's site and provide additional prognostic information would be helpful. Furthermore, we need tools to predict antibiotic resistance in order to tailor first-line antibiotic treatment of spontaneous bacterial peritonitis to the individual patient and to reduce mortality.

Key words: Ascites; Cirrhosis; Farnesoid X receptor; Liver; Nucleotide-binding oligomerization domain containing 2; Rifaximin; Prophylaxis; Spontaneous bacterial peritonitis; Toll-like receptor 2

Core tip: Spontaneous bacterial peritonitis (SBP) is a frequent infection in patients with liver cirrhosis which is associated with a poor prognosis. Portal hypertension leads to translocation of intestinal bacteria which cannot be eliminated due to immune defects caused by liver cirrhosis and genetic predisposition. Empirical antibiotic treatment has become less effective because of wide-spread antibiotic resistance. This review summarises key features of SBP and points out how diagnosis, treatment and prophylaxis may be improved in the future in order to reduce mortality.

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INTRODUCTION

Patients in advanced stages of liver cirrhosis tend to develop bacterial peritonitis without evident source of infection, a form of infection which has been termed spontaneous bacterial peritonitis (SBP) in 1963[1]. Next to urinary tract infection, SBP is the most frequent infection in patients with advanced liver cirrhosis[2]. While it develops in up to 3.5% of patients that are treated as outpatients[3], its prevalence is as high as 12% in hospitalized patients[2,4]. In patients at high risk, SBP incidence can be reduced by prophylactic antibiotic treatment[5-7]. However, efforts to decrease the high mortality associated with SBP, ranging between 16% and 52%, had to face disappointing limitations[2,8,9] Concerning antibiotic treatment and prophylaxis, the rise of bacterial resistance to antibiotics commonly used in patients with liver cirrhosis has reduced the therapeutical options[10]. In addition, attempts to decrease the prevalence of the indispensable underlying condition of SBP, liver cirrhosis, by modern antiviral treatment of viral hepatitis B and C, will probably be counterbalanced by the rising number of patients with non-alcoholic fatty liver disease[11]. Furthermore, SBP is recognised as an important marker of liver disease progression which might be the decisive watershed in the management of advanced liver disease[12]. It can be conceived as the clinically evident manifestation of bacterial translocation from the intestine, linking intestinal microbiome, genetic and acquired immune defects to the development of infection. Thus, SBP stays not only at the centre of liver disease pathophysiology, but also remains a challenge in clinical management. Neither reduction of the burden of liver disease nor development of new antibiotics to overcome bacterial resistance will occur in near future. Therefore, the challenge is to define subgroups of patients for optimal therapy in order to decrease failure of empirical therapy and exert low selection pressure on bacteria.

LEAKY GUT

The usual bacteria causing SBP in patients without prior antibiotic treatment or frequent hospitalisations are enteric bacteria, mostly *Escherichia coli* (*E. coli*)[13,14]. Upper gastrointestinal bleeding is the only major risk factor with sudden onset[15]. Usually, an external source of infection cannot be identified[16]. Taken together, these facts suggest that SBP is an endogenous infection, in general caused by transmigration of enteric bacteria to the ascites[17].

Apart from these clinical observations, experimental data also support this hypothesis. Bacterial translocation of enteric bacteria to mesenterial lymph nodes was not only observed in animal models[18,19], but also in patients with liver cirrhosis, in whom the prevalence of bacterial translocation increased with liver disease severity assessed by the Child-Pugh-Score[20]. In addition, indirect signs of bacterial translocation, such as elevated levels of lipopolysaccharide binding protein (LBP)[21] or bacterial DNA[22] are frequently found in patients with liver cirrhosis.

Nevertheless, bacteria from other sources are also found in ascites. Pyrosequencing of ascitic DNA for viable bacteria revealed that a substantial amount of non-enteric bacteria have access to the peritoneal cavity[23]. In patients, SBP may be caused by bacteria not known from the intestine: examples like SBP by Pasteurella multocida after a scratch of a pet dog[24] or in a pet holder[25] and SBP by bacillus cereus[26] indicate that any kind of bacteremia in cirrhotic patients might end up in ascites infection. In addition, a recent study in Chinese patients suggested that the intestinal microbiome of patients with liver cirrhosis, in contrast to healthy controls, might contain bacteria which normally reside in the oral cavity[27]. Therefore, it is difficult to distinguish the source of infection by identifying the causative microorganism. It is not known to which extent different routes of infection contribute to the development of SBP.

In general, intestinal bacterial translocation is conceived as a key feature of liver cirrhosis[17]. However, measuring bacterial translocation directly is not feasible, so surrogate parameters like lipopolysaccharide (LPS) - a component of the wall of Gram-negative bacteria - bacterial DNA or LPS binding protein (LBP) are used[28]. In animal models, elevated levels of LPS or LBP can be induced by liver damage[29,30]. Markers of bacterial translocation have been linked to all major complications of liver cirrhosis, including ascites formation[21], severe portal hypertension[31], variceal bleeding[32], hepatorenal syndrome, SBP[33] and hepatic encephalopathy[34]. Three factors are considered as key mechanisms to increase bacterial translocation in patients with liver cirrhosis: changes in the amount and composition of the intestinal microbiome[35], a decreased barrier function of the intestine[36] and impaired host responses to translocating bacteria[37].

In healthy subjects, the small bowel contains a relatively small number of bacteria[38]. By contrast, in patients with liver cirrhosis, bacterial overgrowth in the small bowel occurs[39,40] . With the advances in microbiome research, the composition of intestinal bacteria in patients with liver cirrhosis can now be assessed in more detail. Significant differences compared to healthy subjects have been found[27]. In addition, it is not only the bacterial species present in the intestine that may lead to complications of liver cirrhosis[41], but also the products of bacterial metabolism. In line with this, intake of rifaximin

improves cognition along with altering metabolites from intestinal bacteria, but does not influence the composition of the intestinal microbiome[42]. An intriguing question is in how far the intestinal microbiome is only the consequence of liver disease or - once pathologically changed - contributes to the development of more severe disease[35].

It is important to note that the virulence of bacterial strains concerning onset and course of infection differs considerably. *E. coli* strains causing SBP display higher motility than *E. coli* causing urinary or biliary tract infections[43]. In addition, SBP by encapsulated *E. coli* is associated with more complications[44] and in the special case of the K1 antigen with lower survival[45].

A decreased barrier function of the intestine in advanced liver disease has been found in animal models[46-48] and humans[31,49,50]. Recently, the farnesoid X receptor (FXR), a nuclear receptor for bile acids[51], has emerged as an important molecule for maintaining the intestinal barrier. Bacterial translocation from the intestine is increased in FXR knock-out and in bile-duct ligated mice[52]. Synthetical FXR agonists block bacterial translocation in the latter[52] and decrease portal hypertension in animals models of cirrhosis[53]. In addition, a FXR polymorphism which leads to a reduced translation of FXR target genes is associated with the occurrence of SBP[54]. So far, it is not known if synthetic FXR agonists may reduce bacterial translocation in humans.

SBP is associated with polymorphisms in pattern recognition receptors, for example the nucleotide-binding oligomerization domain containing 2 (NOD2) gene[55,56]. The same NOD2 polymorphisms predispose for Crohn's disease[57], which is also characterised by a leaky gut. Unfortunately, the mechanism by which these polymorphisms lead to increased bacterial translocation is still debated. Nevertheless, this joint association provides a clear hint for a shared mechanism and underlines the involvement of the innate immune system in bacterial translocation.

CIRRHOTIC LIVER

Portal hypertension is a hallmark of advanced liver cirrhosis. Decreasing portal hypertension reduces bacterial translocation[31]. However, data on a possibly protective role of non-selective beta blockers, which reduce portal pressure, concerning the occurrence of SBP in patients with liver cirrhosis are contradictory[12,58]. Another treatment for portal hypertension is the placement of a transjugular intrahepatic portosystemic shunt (TIPS)[59]. A meta-analysis on TIPS for refractory ascites found no signficantly decreased incidence of SPB in patients with TIPS[60], but studies focussing directly on this issue are missing.

Apart from portal hypertension, cirrhosis leads to the development of various immune defects and might unmask minor genetic immune defects. The importance of genetic predisposition is stressed by the high recurrence rate of SBP after a first episode if no antibiotic prophylaxis is given. In addition to polymorphisms in the *NOD2*[55,56] gene, which have not only been linked to an impaired intestinal barrier but also to altered innate immune responses[57], polymorphisms in the toll-like receptor 2 (*TLR2*) gene[61] and the monocyte chemotactic protein 1 (*MCP1*) gene[62,63] have been associated with the occurrence of SBP. TLR2 and NOD2 are pattern recognition receptors that sense bacterial components and trigger immune responses[64]. Patients carrying both a NOD2 and a TLR2 risk variant have a particularly high susceptibility for SBP[61]. Overall, patients with liver cirrhosis and ascites carrying a NOD2 risk variant display a higher mortality than patient with wild-type alleles[55,56]. MCP1 is a chemokine attracting immune cells, in particular monocytes, to the site of infection[65]. Monocytes from patients with the G allele at position -2518 produce more MCP1 than monocytes from patients with the A allele at this position[66], so that patients with the A allele are probably more prone to SBP because of a deficit to raise adequate levels of MCP1. Taken together, these genetic studies point at an eminent role of the innate immune system in the development of SBP. Determination of these polymorphisms has no diagnostic impact, because not all patients carrying these mutations will develop SBP, probably due to the presence of so far unknown protective genetic variations and competing risk factors, e.g. death from variceal bleeding or hepatocellular carcinoma. In addition, the presence of these polymorphisms does not predict the onset of SBP - while some patients will develop SBP at first decompensation, other patients receive several large-volume paracentesis till SBP occurs.

Synthesis of proteins by a cirrhotic liver is reduced and fluid accumulates, leading to lower ascites protein concentration, which has been described as one of the major risk factors for SBP[6,7]. In addition, defects in neutrophil[67], monocyte[68], T cell[69] and dendritic cell[70] function have been shown in patients with liver cirrhosis. It is probable that these immune defects impair the normal clearance of translocated bacteria, leading to a state of permanent immune activation and inflammation[21]. The most common causes of liver cirrhosis, viral hepatitis and alcoholic abuse, differ by the mechanisms of liver damage. However, studies demonstrating differences in immune function of ascites cells between these two etiologies are rare. One study found that ascites macrophages are more pro-inflammatory in alcoholic liver disease than in liver cirrhosis induced by hepatitis C virus[71]. Nevertheless, the scarcity of such studies rather seems to indicate that alterations in the immune system concerning the susceptibility to bacterial infections in chronic liver disease are determined mainly by liver failure in general, while the cause of liver disease is secondary.

Although many aspects of bacterial translocation

Table 1 Important risk factors for spontaneous bacterial peritonitis

Variceal bleeding[15]
Previous SBP[6]
Genetic polymorphisms in the *NOD2*[55,56], *TLR2*[61], *MCP1*[62,63] and *FXR*[54] gene
Low ascites protein content (below 1-1.5 g/dL)[7]
Advanced liver disease[116]
Intake of proton pump inhibitors[96,97]

SBP: Spontaneous bacterial peritonitis; NOD2: Nucleotide-binding oligomerization domain containing 2; TLR2: Toll like receptor 2; MCP1: Monocyte chemotactic protein 1; FXR: Farnesoid X receptor.

are known, it is still not fully understood how and when bacterial translocation finally leads to SBP. Important risk factors for SBP are listed in Table 1.

DIAGNOSIS OF SBP

This limitation in our understanding led to simplified diagnostic criteria, which are easy to use in clinical practice, but may not reflect differences in disease. Diagnosis of SBP is made according to international guidelines[6,7] in patients with liver cirrhosis if the ascites polymorphonuclear (PMN) cell count exceeds 250 cells/μL and other forms of peritonitis have been excluded. Among others, differential diagnosis comprises malignant ascites, bowel perforation, intraabdominal abscess formation, pancreatitis and peritonitis due to special bacteria like mycobacterium tuberculosis or chlamydia. Hints for secondary bacterial peritonitis due to bowel perforation are polymicrobial culture growth in combination with two of the following findings in the ascites: a total protein above 1 g/dL, lactate dehydrogenase above the normal for serum and glucose levels below 50 mg/dL[7].

A PMN count of 250 cells/μL has been chosen because it constitutes a sensitive diagnostic marker[16]. Growth of bacteria in the ascites culture does not establish the diagnosis of SBP, since bacteria are detected only in about 40% of SBP cases[6,9]. Conversely, detectable bacteria in ascites samples with a PMN count below 250 cells/μL lead only in 38% to SBP, because most patients eliminate the bacteria without therapeutic intervention[72]. Attempts to improve the sensitivity of microbiological ascites analysis had limited success. Overall, detection of bacteria in the ascites by PCR-based methods failed to improve test accuracy[73-76]. A pilot study using in-situ hybridisation in ascites leukocytes detected bacteria in 10/11 SBP cases, but this study is limited by the small sample size and by the fact that species identification was not possible[77]. However, even if a molecular method could prove superior to traditional culture methods regarding detection rate, a problem of increasing importance is rapid detection of resistance to antibiotics[78], since failure of first-line treatment due to increasing rates of bacterial resistance is associated with poor prognosis[79]. However, reliable determination of resistance profiles can so far only be done by phenotypical tests after conventional culture.

One of the advantages of the current diagnostic definition of SBP is its simplicity. However, a differential leukocyte count of the ascites can be obtained only in some clinical settings. Therefore, alternative tests that can be performed easily, rapidly and reliably are needed. The most advanced form of these tests is a urinary dipstick that is calibrated especially to ascites[80]. Calprotectin, a protein secreted by neutrophils, is another candidate for a bedside test[81].

TREATMENT OF SBP

Antibiotic therapy for 5 d with third generation cephalosporines is the established treatment for SBP[6,7]. Randomised trials concerning the antibiotic treatment of SBP are summarised in Table 2. In addition to antibiotics, substitution of albumin to prevent occurrence of hepatorenal syndrome is recommended, in particular for patients that present with total bilirubin > 4 mg/dL or creatinine > 1 mg/dL or urea nitrogen > 30 mg/dL[7]. Treatment with albumin reduces the incidence of renal failure and death[82]. However, the rise in bacterial resistance has reduced the efficacy of third generation cephalosporines and quinolones, especially in nosocomial infections[78]. In addition, enterococci, which are per se resistant to cephalosporines, have become more frequent as a source of SBP[83]. Failure of first line treatment is associated with worse survival[84]. Therefore, it would be necessary to replace cephalosporines with a more effective empiric therapy. The regional variability of antibacterial resistance limits a general approach. Considering isolates from culture-positive SBP, only combinations of modern broad spectrum antibiotics like carbapenems and glykopeptides are considered as reliably effective first line therapy in all patients[78,85]. Renal toxicity, costs and concerns about induction of even more multi-resistant microorganisms are drawbacks of such a treatment. First results of a randomised trial comparing ceftazidime *vs* meropenem + daptomycin (NCT01455246) presented at the congress of the American Association for the Study of Liver Diseases 2014 (poster 574)[86] indicate a benefit for the combination therapy.

Therefore, it seems more adequate to identify risk factors for resistance to standard treatment in order to select patients who profit from broader antibiotic treatment. Known risk factors are nosocomial infection, previous antibiotic prophylaxis with norfloxacin, use of beta-lactams during the past 12 wk and a history of infection by multi-resistant bacteria[10]. For patients with these risk factors, treatment adapted to the local resistance profiles is recommended. However, therapy should be started immediately after diagnosis of SBP, and most clinicians might not know the local resistance profiles. A more general recommendation is

Table 2 Randomised controlled trials concerning antibiotic treatment of spontaneous bacterial peritonitis

Ref.	No. of patients	Study arms	Resolution of infection	*P*	Comment
Felisart *et al*[117]	73	Ampicillin + tobramycin *vs* cefotaxime	56% *vs* 85%	< 0.02	Also patients without SBP included
Rimola *et al*[118]	143	Cefotaxime 8 g/24 h *vs* 4 g/24 h	77% *vs* 79%	NS	
Navasa *et al*[119]	123	Ofloxacin *po vs* cefotaxime *iv*	84% *vs* 85%	NS	Only patients with uncomplicated SBP included
Ricart *et al*[120]	48	Amoxicillin-clavulanic acid *vs* cefotaxime	88% *vs* 83%	NS	
Terg *et al*[121]	80	Ciprofloxacin only *iv vs* 2 d *iv* then *po*	76 *vs* 78%	NS	
Piano *et al*[86] (NCT01455246) (preliminary results presented at the AASLD 2014, Abstract 574)	32	Daptomycin + meropenem *vs* ceftazidime	87% *vs* 25%	< 0.001	Only patients with nosocomial SBP included

NS: Not significant; SBP: Spontaneous bacterial peritonitis.

to give piperacillin/tazobactam or - in regions with high prevalence of multi-resistant bacteria - carbapenems in combination with glykopeptides[78]. In addition, a second paracentesis after 48 h of treatment should be performed[6]. Based on the results from a first study, a decrease of less than 25% of PMN indicates treatment failure and should prompt a change in treatment[6]. Recognizing treatment failure as early as possible is essential to reduce mortality. Thus, studies to define more and better parameters of treatment response are needed. Of course, rapid microbiological analysis and communication of the results to the clinician is another important factor to guide therapy. However, it is not only response to antibiotic treatment that reduces mortality, but also prevention of renal failure, which might be the most important prognostic factor[8,87]. Albumin substitution to prevent renal failure in the context of SBP was already discussed above.

In summary, the challenges of SBP therapy are various given the rise in resistant bacteria. New classes of antibiotics need to be developed. More knowledge about distinguishing patients who can be treated with standard antibiotics from those who need special treatment is required. Last but not least, failure of first line treatment must be detected as early and as reliably as possible. Still, effective prophylaxis of SBP might alleviate all these problems.

PROPHYLAXIS OF SBP

Primary and secondary prophylaxis of SBP has been established based on some of the known risk factors for SBP: gastrointestinal bleeding, previous SBP and low ascites protein content[6,7]. Primary prophylaxis of SBP is recommended in all patients with gastrointestinal bleeding and mostly done with cephalosporines[78,88]. In this context, antibiotic prophylaxis has been reported to reduce SBP incidence about 70%[89]. Low ascites protein content has been identified early on as risk factor for SBP[90], which has been explained by a low complement activity[91]. A randomised controlled trial[92] in 68 patients with low ascites protein and advanced liver failure or impaired renal function showed that prophylaxis with norfloxacin significantly reduced the occurrence of SBP and improved 3-mo survival, so that primary antibiotic prophylaxis for such patients should be considered according to current guidelines[6,7]. So far, no study has investigated if the rise in resistant bacteria counterbalances the benefit of primary prophylaxis in these patients.

Secondary prophylaxis of SBP with quinolones is widely recommended[6,7] based on the result of a clinical trial[93] and data from studies including patients with and without prior SBP[5,94]. However, an increase of infections with quinolone - resistant bacteria has been reported after the introduction of secondary prophylaxis into clinical practice[10,95]. Again, data from randomised trials to evaluate the efficacy of secondary prophylaxis in the context of a high prevalence of antibiotic resistance are missing. Naturally, long term prophylaxis has to be carried out with oral antibiotics, so that not only parenteral, but also oral new antibiotic classes are needed. Randomised studies on primary and secondary antibiotic prophylaxis of SBP are summarized in Table 3.

Most risk factors for SBP cannot be modified easily. However, use of acid suppressive therapy, in particular with proton pump inhibitors, has been shown to increase the risk for SBP[96,97]. Therefore, acid suppressive therapy should be prescribed only if a clear indication exists, which is not often the case[84]. Interestingly, this harmful side-effect of proton pump inhibitors seems to be caused rather by impaired oxidative burst of granulocytes and monocytes[98] than by inducing small bowel bacterial overgrowth[99]. Probiotics can reduce bacterial translocation and the associated inflammatory changes in animal models of liver cirrhosis[100,101]. However, clinical trials did not show a significant reduction of SBP incidence under treatment with probiotics[102,103].

A new approach for SBP prophylaxis is to consider non-absorbable antibiotics that might reduce the intestinal bacterial load without systemic side effects[16]. The main candidate is rifaximin[104], which prevents

Table 3 Randomised controlled trials concerning antibiotic prophylaxis of spontaneous bacterial peritonitis

Ref.	No. of patients	Study arms	Kind of prophylaxis	Occurence of SBP	*P*
Ginés *et al*[93]	80	Norfloxacin *vs* placebo	Secondary	12% *vs* 35%	0.014
Soriano *et al*[122]	63	Norfloxacin *vs* control	Primary/secondary	0% *vs* 23%	< 0.05
Singh *et al*[123]	60	Trimethoprim-sulfamethoxazole *vs* control	Primary/secondary	3% *vs* 27%	0.025
Grangé *et al*[124]	107	Norfloxacin *vs* placebo	Primary	0% *vs* 9%	NS
Rolachon *et al*[125]	60	Ciprofloxacin *vs* placebo	Primary/secondary	4% *vs* 22%	< 0.05
Novella *et al*[126]	109	Norfloxacin permanently *vs* only during hospitalisation	Primary	2% *vs* 17%	< 0.01
Fernández *et al*[92]	68	Norfloxacin *vs* placebo	Primary	7% *vs* 61%	< 0.001
Terg *et al*[127]	100	Ciprofloxacin *vs* placebo	Primary	4% *vs* 14%	0.076

NS: Not significant; SBP: Spontaneous bacterial peritonitis.

hepatic encephalopathy[105,106] and is widely used in patients with liver cirrhosis. In addition, it belongs to a class of antibiotics which is normally not used in therapy of SBP and was originally reported to induce no bacterial resistance[107]. A small study reported that patients who responded to rifaximin treatment by reduction of hepatic venous pressure gradient displayed a significant reduced rate of complications from liver cirrhosis including SBP over 5 years of follow-up[108]. Another retrospective study comprising 404 patients with liver cirrhosis and ascites requiring paracentesis described a significant reduction of SBP by rifaximin. However, patients with prior SBP or SBP occurring in the course of gastrointestinal bleeding had been excluded[109]. In addition, a prospective observational study of 152 patients with advanced liver cirrhosis found a reduction of SBP incidence only by quinolones, but not by rifaximin[110]. The different results of these studies may be explained by variations in the risk for SBP and severity of liver disease, suggesting that rifaximin might be effective only in the subgroup of patients who have relatively low risk for SBP and less severe liver disease. In summary, rifaximin cannot be recommended for SBP prophylaxis until prospective, randomised studies are available.

An ongoing clinical trial investigates if primary antibiotic prophylaxis with quinolones is beneficial in patients with a genetically determined high risk (EudraCT number 2013-001626-26).

Nevertheless, all antibiotics, including rifaximin[111-113], will lead to the emergence of bacterial resistance. Therefore, strategies avoiding the use of antibiotics might be more promising on the long term. Potential candidates are FXR agonists, since reduced FXR function is associated with increased bacterial translocation[52,54]. FXR agonist have already been tested for non-alcoholic fatty liver disease and primary biliary cirrhosis and show a good safety profile[114,115]. Thus, this new class of drugs may become a novel tool to decrease bacterial translocation in the future.

CONCLUSION

SBP occurs frequently in patients with liver cirrhosis, because liver disease leads to increased rates of bacterial translocation from the gut, but is also associated with a compromised immune system. Mortality of SBP has remained high and bacterial resistance to antibiotics threatens to increase mortality even more in the future. The challenge is to improve treatment efficacy by understanding the pathophysiology of SBP in more detail, by tailoring the therapy to the needs of the individual patient and by identifying new approaches for prophylaxis.

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REVIEW

Non-alcoholic fatty liver disease and psoriasis: So far, so near

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Abstract

Psoriasis is a chronic inflammatory immune-mediated skin diseases which is frequently associated to comorbidities. Non-alcoholic fatty liver disease (NAFLD) is defined as an excessive accumulation of triglycerides in hepatocytes and includes a wide spectrum of liver conditions ranging from relatively benign steatosis to non-alcoholic steatohepatitis with fatty infiltration and lobular inflammation and to cirrhosis and end-stage liver disease. Actually, psoriasis is considered a systemic diseases associated to comorbidities, as metabolic syndrome and NAFLD is seen the hepatic manifestation of the metabolic syndrome. The possible link between psoriasis, obesity and metabolic syndrome, which are known risk factors for NAFLD has been recently documented focusing in the crucial role of the adipose tissue in the development of the inflammatory background sharing by the above entities. According to recent data, patients with psoriasis show a greater prevalence of NAFLD and metabolic syndrome than the general population. Moreover, patients with NAFLD and psoriasis are at higher risk of severe liver fibrosis than those with NAFLD and without psoriasis. The link between these pathological conditions appears to be a chronic low-grade inflammatory status. The aim of this review is to focus on the multiple aspects linking NAFLD and psoriasis, only apparently far diseases.

Key words: Psoriasis; Non-alcoholic fatty liver disease; Adipose tissue; Adipocytokines; Biologic therapies; Non-biologic therapies

Core tip: The review focuses on the multiple physio-pathogenetic aspects of the possible link between psoriasis and non-alcoholic fatty liver disease (NAFLD) emphasizing the most recent scientific data. The importance of the multidisciplinary approach to patients affected by psoriasis is underlined and the therapeutic options to treat concomitant psoriasis and NAFLD is discussed evaluating the risk benefit of both biologic and non-biologic therapies.

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INTRODUCTION

Psoriasis is an immune-mediated, chronic, and inflam-

matory disease with an estimated prevalence of 2%-3% in the worldwide[1].

Recent evidence have shown that psoriasis is not only the disease of the skin surface but a complex entity with multi-systemic involvement and with a deep influence on patients' quality of life, morbidity and mortality[2].

In particular, psoriasis is frequently associated to the so-called "psoriatic comorbidities", including artropathy, uveitis, inflammatory bowel diseases and a cluster of medical conditions known as metabolic syndrome[3] (Figure 1).

According to the National Cholesterol Education Program Adult Treatment Panel Ⅲ (NCEP ATPⅢ), a patient is affected by metabolic syndrome, if he/she shows at least three of these following criteria: Abdominal obesity, defined as waist circumference ⩾ 102 cm in men and ⩾ 88 cm in women; Triglycerides plasma levels ⩾ 150 mg/dL; high-density lipoproteins (HDL) cholesterol plasma levels not more than 40 mg/dL in men and 50 mg/dL in women; Blood pressure more than 130 mmHg (systolic) and 85 mmHg (diastolic); Fasting plasma glucose levels more than 100 mg/dL[4].

Non-alcoholic fatty liver disease (NAFLD) is defined as the hepatic excessive accumulation of triglycerides in patients without a history of potus[5,6].

NAFLD include a wide variety of liver conditions ranging from relatively benign steatosis, consisting in fatty infiltration, to non-alcoholic steato-hepatitis (NASH) with fatty infiltration and lobular inflammation and to cirrhosis and hepatocellular carcinoma[7,8].

NAFLD is currently the most common chronic liver disease in Western countries with a prevalence of 10%-25% and, it is now seen as the most frequent liver disorder, in particular in obese people[9]. Actually, NAFLD is strictly linked to the MetS being its hepatic manifestation[10]. It is known that metabolic syndrome is associated either to psoriasis and to NAFLD, thus it could be reasonable that both entities could be present in an individual simultaneously[11]. This review provides an overview on the most recent findings on the possible link between NAFLD and psoriasis, evaluating the common underlying physio-pathogenic process and the role of systemic drugs on their management.

NAFLD: THE MULTIPLE HITS HYPOTHESIS

The liver plays a central role in lipid metabolism, importing serum free fatty acids (FFA), storing and exporting lipids and lipoproteins. Although the NAFLD pathophysiology has not been completely elucidated, the so called "multiple-hit hypothesis" describes it as a complex, two-step liver injury[12].

The first hit is characterized by hepatic triglyceride accumulation contributing to steatosis; therefore, steatotic liver appears to be more vulnerable to the

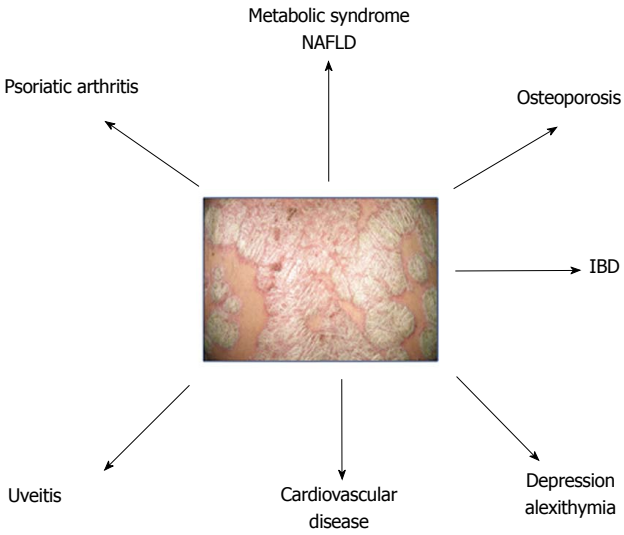

Figure 1 Comorbidities in psoriasis. NAFLD: Non-alcoholic fatty liver disease. IBD: Inflammatory bowel diseases.

"second hit" of adipokine-induced liver injury, oxidative and endoplasmic reticulum stresses, mitochondrial dysfunction, and hepatic apoptosis, which subsequently promote the transition from simple steatosis to steatohepatitis[13-16] (Figure 2). Insulin resistance (IR) appears to exert a central role in both the first and second hits[17]. Insulin is an anabolic hormone that regulates glucose metabolism, gene expression, energy homeostasis and enzymatic functions[18]. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the Ras-mitogen activated protein kinase (MAPK) pathway are the two most important pathway that are involved in insulin-mediated functions[17,18].

In particular, the inhibition of gluconeogenesis and the uptake of glucose is linked to PI3K-AKT, while cell proliferation and differentiation depend on the interaction between MAPK and PI3K-AKT.

Liver, adipose tissue and skeletal muscle are the most important insulin-target tissues[17,18]. In the liver, insulin regulates the glucose metabolism, while in the adipose tissue it reduces the hormone sensitive lipase activity with the subsequent inhibition of the free fatty acid efflux out of adipocytes[17,18].

A subject is considered insulin resistant when his or her insulin mediated glucose uptake by muscle and adipose tissue is impaired. As a compensatory mechanism, beta cells in the pancreas start to secrete increased amounts of insulin to maintain normoglycemia, leading to hyper-insulemia[19].

In the adipose tissue, insulin allows free fatty esterification and triglyceride fat storage. When insulin resistance develops, free fatty acids are inappropriately shifted to non-adipose tissues, as the liver. Moreover, the hepatic lipogenesis and the activation of pro-fibrotic cytokines are mediated by hyperinsulinemia[20,21].

Insulin resistance and hyperinsulinemia increase the excretion of triglycerides by the liver, resulting in elevated serum levels of triglycerides[20,21].

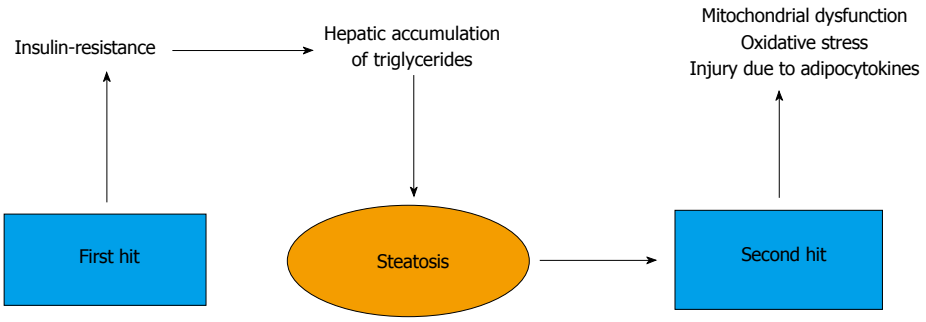

Figure 2 Non-alcoholic fatty liver disease and the two hits hypothesis.

In summary, different mechanisms are implicated in the hepatic triglyceride storage, as the increased triglyceride or FFA synthesis, the reduced FFA transport from the hepatocytes, an excessive transport of FFA to the liver or an abnormal dietary intake of FFA[22-24].

Thus, this lipids' (tryglicerides and FFAs) accumulation in the liver leads to lipotoxicity and consequently to the mitochondrial disfunction and the oxidative stress, which represent the further liver damage[17].

Many studies demonstrated a direct link between NAFLD and cardiovascular disease: inflammation, oxidative stress, insulin resistance, ectopic adipose tissue distribution, dyslipidemia, endothelial dysfunction, and adipocytokines are considered the common and shared pathogenetic processes[21,22].

PSORIASIS: A SYSTEMIC DISEASE

Epidermal hyperproliferation, abnormal keratinocyte differentiation, angiogenesis and activated $CD4^+$ and $CD8^+$ T-cell infiltrates in the dermis and epidermis are the most common detectable histologic features in psoriasis[25].

The pathogenesis of psoriasis is characterized by the involvement of both innate and adaptive immunity. In the beginning of the inflammatory process, NK cells play an important role releasing pro-inflammatory cytokines; subsequently the Th1-Th17 interaction is crucial in the amplification of the flogosis[26].

Clinically, psoriasis is characterized by sharply demarcated erythematous plaques covered by silvery-white scales preferentially on the elbows, knees, scalp, umbilicus and lumbar area[27,28].

Psoriasis and cardiovascular risk

Approximately 80% of psoriatic patients have limited disease, involving < 10% body surface area, but approximately 20% have more extensive skin involvement. Although psoriasis is rarely life-threatening, it exerts an important impact on patients' quality of life, similar to that caused by diabetes, cancer or heart disease[29-31].

The underlying low and persistent inflammatory status with increased levels of pro-inflammatory cytokines, such as TNF-α and IL-6, seems to be responsible of the metabolic dysregulation in psoriasis[32].

Cardiovascular risk factors, such as diabetes, hypertension, dyslipidemia and obesity, are more prevalent in patients affected by psoriasis; moreover it has been suggested that the chronic inflammatory nature of psoriasis is also a contributing and potentially an independent risk factor for the development of cardiovascular disease[33].

Recent evidence have demonstrated that psoriasis may represent an independent risk factor for cardiovascular morbidity and mortality: the cardiovascular risk is increased of about almost three times in patients affected by moderate to severe psoriasis, particularly in young people. For this reason, patients with severe psoriasis appear to have a reduced life expectancy of about 6-year[34].

Furthermore, recent data have demonstrated that NAFLD may be linked to increased risk of cardiovascular events independently from conventional risk factors[35,36].

Many evidences have shown that psoriatic patients have altered lipid metabolism with high levels of plasma cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, very low-density lipoprotein cholesterol, and decreased HDL cholesterol and antioxidant capacity. This dyslipidemic profile could precede the development of psoriasis[37].

In 1994, Offidani *et al*[38] evaluated the lipid profile in children affected by psoriasis, demonstrating higher levels of plasma total cholesterol and no significant changes of plasma triglycerides[38]. Moreover, high percentage content of total cholesterol and of the cholesterol/protein ratio in LDL and in HDL was found with an alteration of their fluidity.

Raised levels of LDL and decreased levels of HDL cholesterol are responsible of coronary artery disease and of mortality for cardiovascular disease[39].

Psoriasis and hypertension

Hypertension shows an higher prevalence in patients affected by psoriasis compared with controls, thus a link between hypertension and psoriasis has been postulated[37].

The increased production of angiotensinogen by adipose tissue, subsequently converted to angiotensin

Ⅱ through angiotensin converting enzyme (ACE) could represent the central causative element of the hypertension in psoriatic patients[40]. It has been demonstrated an increase of ACE serum levels in psoriatic patients[37,40]. Angiotensin Ⅱ is responsible for kidney salt retention and, acting as vasoconstrictor, for vascular tone regulation; moreover, it stimulates T-cell proliferation promoting inflammation and atherosclerosis[40].

The association between hypertension and psoriasis may also be attributed to the increased oxidative stress in psoriatic patients: greater levels of reactive oxygen species can damage endothelium-dependent vasodilation[40].

Furthermore, endothelin-1 could be involved in hypertension pathogenesis of psoriatic patients. Endothelin-1 is produced by keratinocytes and it promotes blood vessels vasoconstriction with consequently blood pressure increase. In lesional skin and in serum of psoriatic patients, endothelin-1 expression appears to be altered correlating to psoriasis disease severity[40].

Psoriasis and prothrombotic state

Metabolic syndrome and psoriasis have been associated to a pro-inflammatory and/or pro-thrombotic state probably related to elevated serum levels of PAI-1, fibrinogen and reactive C-protein (RCP). IL-6 induces RCP increase and it has been shown to be predictive of future cardiovascular disease (CVD) in healthy subjects. Moreover, the risk of in patients with either diabetes or MetS is significantly increased in the presence of elevated RCP levels[37].

McDonald *et al*[41] have shown that the global risk of arterial and venous diseases appeared to be 2.2 times higher in psoriatic patients compared with control patients affected by different skin diseases[41]. Another study conducted in a large cohort of psoriatic patients have underlined that the risk of CV mortality was 50% higher compared to the general population[42].

These data were confirmed by Lin *et al*[43], who detected that patients affected by psoriatic arthritis (PsA) had an increased prevalence of metabolic syndrome with significantly greater carotid intima-media thickness compared to patients with psoriasis only. Furthermore, greatest CIMT measurements were detectable in PsA patients with metabolic syndrome compared to PsA patients without metabolic syndrome and psoriasis patients with or without metabolic syndrome[43].

PSORIASIS AND NAFLD: EPIDEMIOLOGY

Recent studies focused on the possible link between psoriasis, obesity and metabolic syndrome, which are known risk factors for NAFLD[44].

It is known that, after the age of 40 years, the psoriatic patients have a higher prevalence of metabolic syndrome and an increased risk for the each components of MetS than controls[11].

Moreover, the association between psoriasis and MetS is directly correlated to the severity of psoriasis and it is independent from the presence of obesity in psoriasis[45-47]. Considering that metabolic syndrome is detected both in NAFLD and psoriasis, it is likely that these two pathological entities can coexist in the same patient[11]. Lonardo *et al*[48] in 2001 firstly documented three cases of concomitant psoriasis and NASH, confirmed by liver biopsy. All patients were obese or overweight and showed MetS components. In an Italian prospective observational study, the prevalence NAFLD in psoriatic patients was significantly increased, compared to general Italian population[49,50].

Furthermore, NAFLD was unrelated to the severity of the skin disease, but logistic regression showed that patients with psoriasis and NAFLD showed a higher risk of psoriatic arthritis. This aspect could reflect the actions of pro-inflammatory cytokines in both diseases. Moreover, psoriatic patients with NAFLD had also significantly higher AST/ALT ratio and higher non-invasive fibrosis scores compared with controls with NAFLD not associated with psoriasis. AST/ALT ratio has proven to be an independent predictive factor for liver fibrosis in patients with NAFLD[25].

Consequently, the risk of severe liver fibrosis is higher in patients affected by both NAFLD and psoriasis than patients with only NAFLD. These two parameters are considered independent predictors of liver fibrosis in NAFLD patients[49,50].

These data were confirmed by Gisondi *et al*[51] in a case-control study, who assessed the frequency and characteristics of NAFLD in patients with chronic plaque psoriasis *vs* healthy controls. Among 130 psoriatic patients, up to nearly half (47% *vs* 28%of controls) resulted affected by NAFLD, which was strongly related to psoriasis severity according to Psoriasis Area Severity Index (PASI) score. Moreover, patients with psoriasis and NAFLD showed metabolic syndrome and higher serum C-reactive protein[51].

Van der Voort *et al*[52] in 2013 conducted a large prospective population-based cohort study in subjects up to 55 years. Among 2292 participants, 118 (5.1%) were affected by psoriasis and the prevalence of NAFLD was 46.2% in psoriatic patients compared with 33.3% of participants without psoriasis. Thus, after adjustment for alcohol consumption, smoking status, presence of MetS components and alanine aminotransferase, psoriasis remained a significant predictor of NAFLD[52].

PSORIASIS AND NAFLD: THE PATHOGENIC LINK

The pathogenesis of both NAFLD and psoriasis seems to be multifactorial and complex and the precise link between these two entities has not completely elucidated. It could be speculated that a low, chronic and persistent inflammatory status may be the

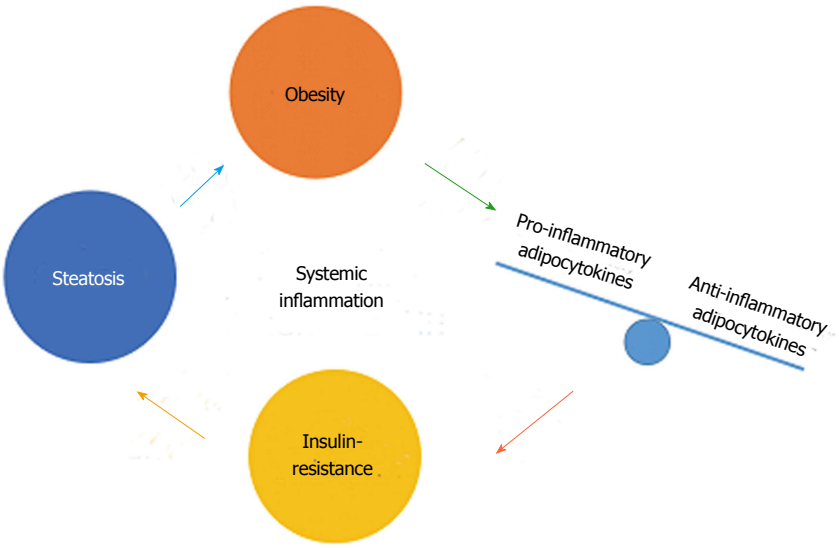

Figure 3 The vicious circle.

"primum movens" linking NAFLD and psoriasis.

It is known that psoriasis and obesity are strictly associated: obesity seems to predispose to psoriasis and psoriasis seems to increase the risk of obesity. A recent meta-analysis of epidemiological studies was evaluating the associations between psoriasis and obesity have evidenced that an 1.46 or and 2.23 or for obesity among patients with mild psoriasis and severe psoriasis respectively. One incidence study found that psoriasis patients have a HR of 1.18 for new-onset obesity. Thus, psoriatic patients showed a higher prevalence and incidence of obesity directly correlated to the severity of psoriasis itself[6,53,54]. It is known that the increasing prevalence of NAFLD parallels the rise of obesity and its complications[55]. Thus, psoriasis and NAFLD could be linked by obesity itself, which may contribute to the development of further MetS components and comorbidities[55].

As psoriasis and NAFLD, obesity is considered a persistent and low-grade inflammatory process[4,56].

The adipose tissue accumulation seems to lead to adipocyte hypertrophy and hyperplasia with a sort of local ischemia; subsequently an inflammatory process and the release of pro-inflammatory chemokines start, attracting macrophages which amplify and spread the inflammatory process in neighboring adipocytes[57,58] (Figure 3).

Subcutaneous and central fat (omental and inta-abdominal) are the two most important part of the adipose tissue; the central one, also called visceral adipose tissue (VAT), is considered more metabolically active than the subcutaneous fat. A higher risk of developing insulin resistance and of MetS components is detected in patients affected by central obesity than patients with excess of subcutaneous fat[59-61].

The energy storage, the endocrine role and the partecipation in the immune system are three important actions of the VAT[62].

Thus, excess adipose tissue results in an unbalance between pro- and anti-inflammatory cytokines and the increased inflammatory stimuli is responsible for the starting of the persistent low-grade inflammation[4,58,59].

Adipocytokines are bioactive molecules able to modulate appetite-energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure and lipid metabolism by autocrine, paracrine and endocrine way. Furthermore, they play a crucial role in the pathogenesis of metabolic syndrome. Among adipocytokines, TNF-α, IL-6, leptin, visfatin, resistin appear to exert a pro-inflammatory effect, whereas adiponectin has anti-inflammatory properties[58,59,62].

The pathogenesis of both psoriasis and NAFLD is strictly dependent on the above cytokines[63].

Adipocytes and stromo-vascular cells are responsible for the secretion of TNF-α; adipose tissue TNF-α is not secreted in systemic circulation and acts both in autocrine and paracrine way. In adipose tissue, TNF-α mRNA correlates with body mass index, percentage of body fat and hyper-insulinemia; moreover, weight loss decreases TNF-α levels[63].

TNF-alpha interfere with insulin action reducing the auto-phosphorylation of tyrosine residues of insulin receptor and phosphorylation of insulin receptor substrate 1 (IRS-1), thus contributing to the first hit of NAFLD[37,64]. In addition, the production of adiponectin is inhibited by TNF-α[65].

Finally, a positive correlation between TNF-α and BMI had been shown, and a higher serum levels was reported in patients with NAFLD[66,67].

TNF-α plays a crucial role in the pathogenesis of psoriasis: in fact, psoriatic patients show elevated serum levels of TNF-α which positively correlate with PASI[68]. Moreover, TNF-α levels and plasma levels of adiponectin are negatively correlated[69-71].

In psoriasis, TNF-α increases keratinocyte proliferation, pro-inflammatory cytokines' production, expression of vascular endothelial cell adhesion molecules, angiogenesis. In obese adipose tissues, the release TNF-α directly participate in the macrophage recruitment and lipolysis creating and perpetuating a

Table 1 Role of adipocytokines in non-alcoholic fatty liver disease and psoriasis

	Adipocytokines	Psoriasis	NAFLD
Anti-inflammatory	Adiponectin	↓ Promotes anti-inflammatory cytokines	↓ Increase insulin-sensitivity
Pro-inflammatory	IL-6	↑ Keratinocyte proliferation	↑ Contributes to insulin-resistance
	TNF-α	↑ (1) Keratinocyte proliferation; (2) Angiogenesis; and (3) Promotes expression of adhesion molecules; and (4) Increase pro-inflammatory cytokines	↑ Contributes to insulin-resistance; Increase hepatic fibrogenesis
	Leptin	↑ (1) Keratinocyte proliferation; (2) Promotes Th1 responses; and (3) Angiogenesis	↑ (1) Leptin-resistance; and (2) Contributes to hepatic fibrogenesis
	Resistin	↑ Increase pro-inflammatory cytokines	↑ (1) Contributes to insulin-resistance; and (2) Controversial data on NAFLD
	Visfatin	↑	Not altered in early stage; Protection toward liver injury (?); Negatively correlated to TNF-α
	Ghrelin	↑	↑

NAFLD: Non-alcoholic fatty liver disease.

vicious circle[72] (Table 1).

Adiponectin is an anti-inflammatory adipocytokine secreted by adipocytes acting as an insulin-sensitizing hormone. Moreover, adiponectin seems to down-regulate the synthesis of TNF-α in fat tissue and in heart muscle cells, to inhibit the production of IL-8, vascular adhesion molecule-1, and reactive oxygen species in endothelial cells and to stimulate the synthesis of IL-10[73]. Data on literature have demonstrated a negative correlation between adiponectin serum levels and body mass index (BMI)[73-78]. Obesity, type 2 diabetes, coronary disease, hypertension and non-alcoholic fatty liver disease in obese patients are associated to low plasma and serum levels of adiponectin[73,77,78].

Moreover, it has been shown that serum adiponectin values were lower in patients affected by both psoriasis and NAFLD than inpatients affected by psoriasis without hepatic involvement. This aspect could be linked to the adiponectin-mediated suppression of type 1 T helper cell-cytokines preventing psoriasis-prone individuals from developing disease until obesity and other factors antagonize its effects[79,80] (Table 1). Among pro-inflammatory adipocytokines, IL-6, leptin and resistin seem to be involved in the pathogenesis of both hepatic steatosis and psoriasis[81].

Leptin regulates the appetite and body weight: scientific evidence have underlined higher leptin levels in obese patients with a sort of leptin resistance, just as in type 2 diabetes, where insulin resistance is observed[81-83].

Furthermore, leptin resistance could promote the development of NAFLD by reducing intracellular lipid levels in skeletal muscle, liver and pancreatic beta cells. In patients affected by psoriasis, leptin serum levels are higher and psoriasis itself represents an independent risk factor for hyperleptinemia. In psoriasis, leptin seems to enhance the synthesis of Th1 cytokines, to reduce the synthesis of Th2 and to mediate proliferative and anti-apoptotic processes in T-cells[84].

Furthermore, there is a positive correlation between leptin serum levels and BMI[85,86] (Table 1). Resistin is a dimeric protein able to induce the synthesis of TNF-α and IL-12 and to increase blood glucose and insulin concentrations. It has been shown that psoriatic patients have elevated levels of resistin that is positively correlated with PASI index[85,87]. As resistin is a pro-inflammatory cytokine, it could be hypothisezed that resistin might be involved in the pathogenesis of MetS in psoriatic patients[85,88]. Actually, it has not been completely elucidated the precise role of resistin in obesity, NAFLD and insulin resistance and/or diabetes[63] (Table 1).

Visfatin is a pro-inflammatory and insulin-mimetic adipocytokine contributing to glucose and lipid metabolism. The role of visfatin in NAFLD is debated. Recent study detected that although plasma visfatin levels are not altered in the first stages of NAFLD, it is inversely associated with TNF-alpha, suggesting its possible protective role against liver damage in this widespread disease. Data on the possible role of visfatin on psoriasis are insufficient[89] (Table 1).

Ghrelin has been suggested to be involved in metabolic syndrome, and obesity, type 2 diabetes and hypertension are associated to decrease levels of ghrelin. In NAFLD, it has been demonstrated that an imbalance in adiponectin, leptin, and ghrelin seems to be associated with more severe hepatic disease[90]. Moreover, serum ghrelin concentration is correlated with a low risk of developing NAFLD[91].

In patients with psoriasis, serum level of ghrelin

was higher, although not statistically significant, than those of the control group, with a strong negative correlation with PASI score[92] (Table 1).

IL-6 is an inflammatory cytokine involved both in psoriasis and NAFLD. It regulates the migration of T cells into the epidermis, the growth and differentiation of dermal and epidermal cells[68].

Obesity, inadequate glucose tolerance, and resistance to insulin are positively correlated with IL-6 serum levels; body weight reduction is associated with decreased IL-6 concentration[93,94].

In psoriatic patients, serum levels of IL-6 are significantly increased with a positive correlation with PASI score[85].

Moreover, a negative correlation between IL-6 and adiponectin plasmatic levels in obese patients has been observed; obese patients with psoriasis show a statistically significant increase of IL-6 compared to control group[87,95-97] (Table 1).

The role of IL-17 in pathogenesis of both psoriasis and NAFLD has been recently elucidated. Although the precise role has not completely clarified, elevated levels of IL-17 have been reported in obese patients and type 2 diabetes[98].

T cells of adipose tissue can produce IL-17, and adipogenesis and glucose metabolism are regulated by IL-17. In NAFLD, Th17 and IL-17 seem to promote the evolution from simple steatosis to steatohepatitis. Finally, in psoriasis IL-17 induces IL-6 expression in keratinocytes; moreover severity of psoriasis is positively correlated with elevated serum levels of IL-17[98].

However, in addition to the emerging role of adipose tissue in pathogenesis of psoriasis and NAFLD, it is also possible that NAFLD itself might contribute to the psoriasis severity by releasing of inflammatory mediators from inflamed liver, as C-reactive protein, reactive oxygen species, IL-6 and other pro-inflammatory cytokines. These inflammatory mediators are remarkably higher in patients with NAFLD than in those without[99,100].

CONVENTIONAL PSORIATIC TREATMENTS AND LIVER FUNCTION IMPLICATIONS

Successful treatment is imperative in order to improve signs and symptoms of psoriasis, and to improve physical or psychological distress. According to the European consensus mild psoriasis is defined as BSA < 10 and PASI < 10 and DLQI < 10 and moderate-to-severe as BSA > 10 or PASI > 10 and DLQI > 10. There is an agreement that mild psoriasis should preferentially be treated with topical therapy and, in case of inadequate response, UV-light should be added. In case of moderate-to-severe psoriasis systemic therapy should be initiated[101-104].

Methotrexate (MTX), cyclosporine A (CsA) and retinoids are common and efficacious systemic agent used for the treatment of moderate to severe psoriasis, but their long-term use is hindered by safety concerns and, in particular, by the risk of hepatotoxicity. A recent retrospective review conducted on 710 patients with moderate to severe psoriasis treated with MTX have shown that a high proportion (57.6%) of patients on MTX had deranged transaminases[105].

MTX-mediated liver toxicity does not show specific histological aspects being similar to those of NASH. Moreover, metabolic syndrome and NASH are associated with several risk factors for methotrexate-mediated liver damage, as obesity, diabetes, and hyperlipidemia. Thus, the use of MTX in psoriatic patients should be carefully monitored for the possible worsening of a pre-existing steatohepatitis[24]. Thus, in MTX users, FibroTest can accurately predict the presence of liver fibrosis and the Fibroscan significantly predict the absence of significant liver fibrosis[106]. CsA is a potent immunosuppressor used for organ transplantations and various autoimmune disorders; one of the most detectable side effects is hepatotoxicity[107]. Acitretin is a synthetic retinoid which could induce liver toxicity by impairing mitochondrial phosphorylation efficiency without affecting the membrane potential[108]. Increased serum hepatic enzyme levels have been observed in approximately 25% of patients treated with acitretin but no clinically significant biopsy-proven hepatotoxicity was found after two years intermittent acitretin therapy[24].

ERA OF BIOLOGICS AND LIVER IMPLICATIONS

In recent years, the so-called biological therapies or biologic respond modifiers have led to a revolution in the treatment of moderate to severe psoriasis. Currently approved biological products for psoriasis treatment fall into two main classes: cytokine modulators and biologics targeting T cells[109].

These treatments include fusion of proteins and monoclonal antibodies that target the T cells or specific inflammatory cytokines[110].

TNF-α inhibitors link the TNF-α blocking its activity and reducing the interactions between immune cells and keratinocytes. Currently, three anti-TNF-alpha are been approved for the treatment of psoriasis: Infliximab, Adalimumab and Etanercept[110]. In literature, 20 cases of autoimmune hepatitis triggered by anti- TNF-α therapy have been reported to date. The median time and the number of doses of anti-TNF-α drugs to the onset of liver damage were 2 mo and 3 times, respectively. After the onset of liver damage, anti-TNF-α therapy was discontinued in all cases and six were treated with corticosteroid, with or without azathioprine. All cases had good response to the therapies, and the liver damage was resolved within approximately 3 mo in five cases.

However, it is difficult to distinguish a drug-induced autoimmune hepatitis (drug induced AIH) from a de novo autoimmune hepatitis (AIH) because the clinical, biochemical, serological, and histological patterns may be overlapping. Furthermore, patients treated with biological agents may have various forms of simultaneous autoimmune disease and ANAs before treatment. Moreover, about 3% of AIH cases had psoriasis as a concurrent autoimmune disease[110].

Despite the pathogenesis of AIH triggered by anti-TNF-α therapy remains unclarified, some hypotheses had been postulated: TNF-α itself could contribute to the development of AIH and/or it could be linked to reactive metabolites of the anti-TNF-α drugs which are recognized by the immune system as neoantigens[111,112].

Ustekinumab is a human monoclonal antibody approved for the treatment of psoriasis binding the p40 subunit of IL-12 and of IL-23, preventing their interaction with the cell surface IL-12Rβ1 receptor and subsequently inhibiting IL-12- and IL-23-mediated cell signaling, activation, and cytokine production. No data are reported about the role of Ustekinumab on liver function[113,114].

PSORIASIS, METS AND NAFLD: TOWARD COMMON THERAPEUTIC STRATEGIES?

Psoriasis is now perceived as a systemic disorder with skin manifestation and associated comorbidities responsible for increased morbidity and mortality. Thus, a multidisciplinary approach in the prompt diagnosis of psoriasis-related comorbidities and in their managements appear useful to obtain an appropriate tight control of psoriasis from an early stage[53,101].

Given that TNF-α shows a pivotal role in psoriasis and related comorbidities, there could be a rationale in the use of TNF-α inhibitors in their treatment[11].

The effect on psoriatic therapies on adipocytokines

Data on literature show conflicting results about the role of psoriatic treatments on adipocytokines' levels. Ozdemir *et al*[92] have demonstrated that, after cyclosporine treatment, a significant increase was seen in the serum level of adiponectin and resistin, not correlated to disease parameters[92].

About the possible biologics' action on adipocytokines' levels, Shibata *et al*[115] demonstrated an increased in adiponectin and IL-6 level after infliximab treatment. We have recently demonstrated that Adalimumab and Etanercept could be able to rebalance, but not normalize, adipocytokines' levels, mainly related to a reduction of pro-inflammatory ones rather than an increase of adiponectin[62].

The effect of biologic response modifiers on blood lipids

About lipid profiles, total cholesterol, triglycerides are not significantly modified by Infliximab[116,117]. Conversely, another study have shown that Infliximab modifies plasma lipid and lipoprotein levels in patients with rheumatoid and psoriatic arthritis resulting in a more atherogenic profile and significantly increasing triglyceride levels and lowering HDL cholesterol[118].

A retrospective study reviewing the medical records of 45 patients affected by psoriasis treated with Etanercept for 24 wk showed no statistically significant modifications on the lipid profile[119,120].

Moreover, Puig *et al*[121] emphasized that Etanercept treatment may provide some potentially favorable modulation of insulin sensitivity, HDL-C, Apo A1 and Apo B:Apo A1 ratio[121]. Finally, an *in vitro* study focused that the use of Etanercept can lead to a reduction in lipid peroxidation and an improvement in HDL antioxidant and anti-inflammatory properties[122].

Biologic response modifiers and insulin resistance

Few data are available about the role of TNF-α inhibitors on insulin resistance. A study conducted by Marra *et al*[123] have evidenced that the use of Etanercept, in patients affected by psoriasis, can improve insulin sensitivity, by a sort of modulation of the inflammatory background. Similarly, an improvement in insulin sensitivity has been obtained 120 minutes after the Infliximab infusion to up to one year[41,123].

Anedoctical cases of psoriasis with diabetes developing unpredictable hypo- or hyperglycemia has been reported after treatment with TNF-α inhibitors; this aspect resolved after the drug discontinuation[41].

Biologic response modifiers and body mass index

Significant weight gain of about 2 kg in 1 year and the increase of BMI has been reported in patients affected by psoriasis and psoriatic arthritis after long-term use of TNF-alpha inhibitors[41,71].

Gisondi *et al*[124] demonstrated that BMI and body weight were significantly increased in patients treated for 7 mo with infliximab compared with those treated with Ustekinumab.

Biologic response modifiers and NAFLD

There are few data on the role of biological response modifiers on NAFLD. The only study on this topic was a retrospective case-control study in patients with psoriasis, metabolic syndrome, and NAFLD, conducted by Campanati *et al*[25]. The authors found that the risk of developing hepatic fibrosis, shown by change in the AST/ALT ratio, was significantly correlated with insulin sensitivity assessed with the HOMA index and the QUICKI. However, the correlation coefficients between the above parameters were less than 0.1: this could be explained by the fact that the development of hepatic fibrosis in NAFLD depends on multiple factors not only by insulin resistance. Moreover, Etanercept attenuated insulin resistance, patients gained significant weight with increases in the waist-hip-ratio and BMI during

treatment, and these changes could themselves represent risk factors for the development of hepatic fibrosis in NAFLD[25].

CONCLUSION

Despite the relationship between NAFLD and psoriasis needs to be further investigated, data on literature show that psoriatic patients show an increased prevalence of NAFLD.

Moreover, NAFLD and psoriasis seem to be related to an increased cardiovascular risk, probably linked to the common low and persistent inflammatory state.

No specific pharmacological treatments should be suggested to these patients, as lifestyle modification and dietary recommendations, can play an important role in the treatment of metabolic complications of psoriasis[125].

In fact, investigations have assessed the effect of weight-loss on psoriasis and conclusively found that the severity of psoriasis can be significantly improved. The most convincing data are derived from bariatric surgery where the majority of patients show a significant improvement of psoriasis severity[101]. However, there is no data yet available that demonstrates a lasting effect of weight loss measures on psoriasis severity/activity after the intervention. It could be underline that a significant weight loss can lead to significant improvements in circulating levels of leptin, and adiponectin with a possible action and inflammatory state[126].

Improvement of insulin resistance and insulin sensitivity and a significant reduction in systemic inflammation could be obtained by the use of TNF-α inhibitors[118-124]. Thus, given that biological therapies present the potentiality to reduce the TNF-α-related metabolic comorbidities in psoriatic patients, further prospective studies are needed on this issue. Finally, evaluating the coexistence of NAFLD in psoriatic patients, dermatologists should consider methotrexate and other conventional therapies for psoriasis as possible triggers of liver disease in this population.

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REVIEW

Hepatitis C virus syndrome: A constellation of organ- and non-organ specific autoimmune disorders, B-cell non-Hodgkin's lymphoma, and cancer

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Abstract

The clinical course of chronic hepatitis C virus (HCV) infection is characterized by possible development of both liver and extrahepatic disorders. The tropism of HCV for the lymphoid tissue is responsible for several immune-mediated disorders; a poly-oligoclonal B-lymphocyte expansion, commonly observed in a high proportion of patients with HCV infection, are responsible for the production of different autoantibodies and immune-complexes, such as mixed cryoglobulins. These serological alterations may characterize a variety of autoimmune or neoplastic diseases. Cryoglobulinemic vasculitis due to small-vessel deposition of circulating mixed cryoglobulins is the prototype of HCV-driven immune-mediated and lymphoproliferative disorders; interestingly, in some cases the disease may evolve to frank malignant lymphoma. In addition, HCV shows an oncogenic potential as suggested by several clinico-epidemiological and laboratory studies; in addition to hepatocellular carcinoma that represents the most frequent HCV-related malignancy, a causative role of HCV has been largely demonstrated in a significant percentage of patients with isolated B-cells non-Hodgkin's lymphomas. The same virus may be also involved in the pathogenesis of papillary thyroid cancer, a rare neoplastic condition that may complicate HCV-related thyroid involvement. Patients with HCV infection are frequently asymptomatic or may develop only hepatic alteration, while a limited but clinically relevant number can develop one or more autoimmune and/or neoplastic disorders. Given the large variability of their prevalence among patients' populations from different countries, it is possible to hypothesize a potential role of other co-factors, *i.e.*, genetic and/or environmental, in the pathogenesis of HCV-related extra-hepatic diseases.

Key words: Hepatitis C virus; Mixed cryoglobulinemia; Thyroid; Diabetes; Lymphoma

Core tip: The proposed definition of hepatitis C virus (HCV) syndrome encompasses the multiform complex of clinico-pathological conditions potentially correlated to chronic HCV infection. The natural history of HCV syndrome is the result of multifactorial and multistep pathogenetic process, which usually proceeds from mild, often isolated manifestations, to systemic immune-mediated disorders, and less frequently to overt malignancies. Here we analyze the clinical, epidemiological, and pathogenetic aspects of this multifaceted condition, including the updated results of the world literature.

Ferri C, Sebastiani M, Giuggioli D, Colaci M, Fallahi P, Piluso A, Antonelli A, Zignego AL. Hepatitis C virus syndrome: A constellation of organ- and non-organ specific autoimmune disorders, B-cell non-Hodgkin's lymphoma, and cancer. *World J Hepatol* 2015; 7(3): 327-343 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i3/327.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i3.327

INTRODUCTION

Hepatitis C virus (HCV) has been isolated in 1989; it represents the main agent of the so-called nonA/nonB chronic hepatitis[1]; soon after the HCV discovery, several clinico-epidemiological, pathological, and laboratory studies definitely evidenced the role of this agent in the symptom complex associated to mixed cryoglobulinemia (MCs)[2-4]. Clinically, MCs is systemic autoimmune disorder, also termed cryoglobulinemic vasculitis (CV), characterized by cutaneous and visceral organ involvement, including chronic hepatitis present in a relevant number of patients[4-7]. The presence of liver involvement, rarely found in systemic vasculitis, suggested a role of hepatotropic viruses in CV since the seventies[4,6,7]; this hypothesis was ultimately confirmed by the high rate of HCV ongoing infection in large CV patients' series[2-4,8]. On the other hands, MCs mimics some immune-mediated disorders and malignancies, especially B-cell non-Hodgkin's lymphoma (B-NHL); this peculiar clinical feature suggested a possible role of HCV also in other immune-mediated and neoplastic conditions during the past two decades[4,6,7]. Besides liver involvement, a growing number of clinico-laboratory studies progressively evidenced a possible pathogenetic role of chronic HCV infection in various extrahepatic manifestations, among which the MCs represents the true prototype[4,6,7].

In this complex scenario, the demonstration of HCV lymphotropism represented a decisive advance in the knowledge of the pathogenesis of HCV-associated disorders[9,10]. The spectrum of these conditions includes both hepatic, organ-specific and systemic autoimmune disorders, and malignancies[4,6,7]; therefore we previously proposed the term "HCV syndrome" referring to this constellation of virus-driven clinical conditions[11]. The different clinical phenotypes of HCV-syndrome can be the results of genetic/environmental co-factors as suggested by the heterogeneous geographical prevalence of single HCV-associated manifestations[4,6,7].

The present review focuses on the state of the art of this multifaceted condition with particular emphasis to extra-hepatic manifestations.

PATHOGENESIS OF HCV SYNDROME

Numerous studies regarding the biological aspects of HCV and the interactions between viral genome with the immune system of the infected subjects may explain the complex clinical spectrum of HCV-associated disorders[4,6,7,9-16]. Soon after HCV identification, the hepato- and lymphotropism of this virus has been clearly demonstrated[9,10]; in particular, the HCV infection of lymphoid tissue may represent a decisive step in the development of virus-driven autoimmune-lymphoproliferative diseases[4,11]. Epidemiological studies firstly suggested a possible pathogenetic role of HCV in MCs, a systemic disease sustained by indolent B-cell expansion[4-7]; moreover, cryoglobulinemia may be detected in a relevant percentage of individuals with HCV infection, frequently associated with circulating autoantibodies and/or mixed cryoglobulinemia, the hallmark of overt MCs[4-7,17].

A positive, single-stranded RNA characterizes the HCV; given the absence of a DNA intermediate in the viral replication, HCV RNA sequences cannot incorporate into the genome of infected individuals; consequently, it may represent a chronic stimulus to the immune system, which through a multistep process may lead to clonal B-lymphocytes expansion[4,12-15]. In particular, a relevant number of patients with HCV infection show t(14;18) translocation, which in turn may lead to Bcl-2 proto-oncogene activation; more frequently HCV-associated MCs with mono-oligoclonal type Ⅱ mixed cryoglobulins[4,6,7,18] (Figure 1). In addition, viral envelope E2 protein able to bind the molecule CD81, which is widely expressed on cell membrane of both hepatocytes and B-cells, might be decisive for HCV-associated autoimmune phenomena[4,6,7,19]. The interaction of HCV-E2 with CD81, part of cell-surface protein complex CD81-CD21-CD19-Leu 13 on the B-lymphocytes, may be able to reduce the threshold for the activation of B-cells by bridging complement recognition CD21-mediated and antigen-specific recognition (4, 6, 7, 13, 14, 19). The HCV-E2 and CD81 interaction in antigen-reactive B-lymphocytes may amplify the VDJ rearrangement frequency[13,14]; the t(14;18) translocation represents one of possible genetic aberration with consequent activation of bcl-2 proto-oncogene detectable in HCV-infected individuals[18]. The Bcl-2 over expression may prevent apoptosis and consequently may extend the survival B-lymphocytes[13,14,18]. The expansion of B-cells may lead to autoantibody production, including

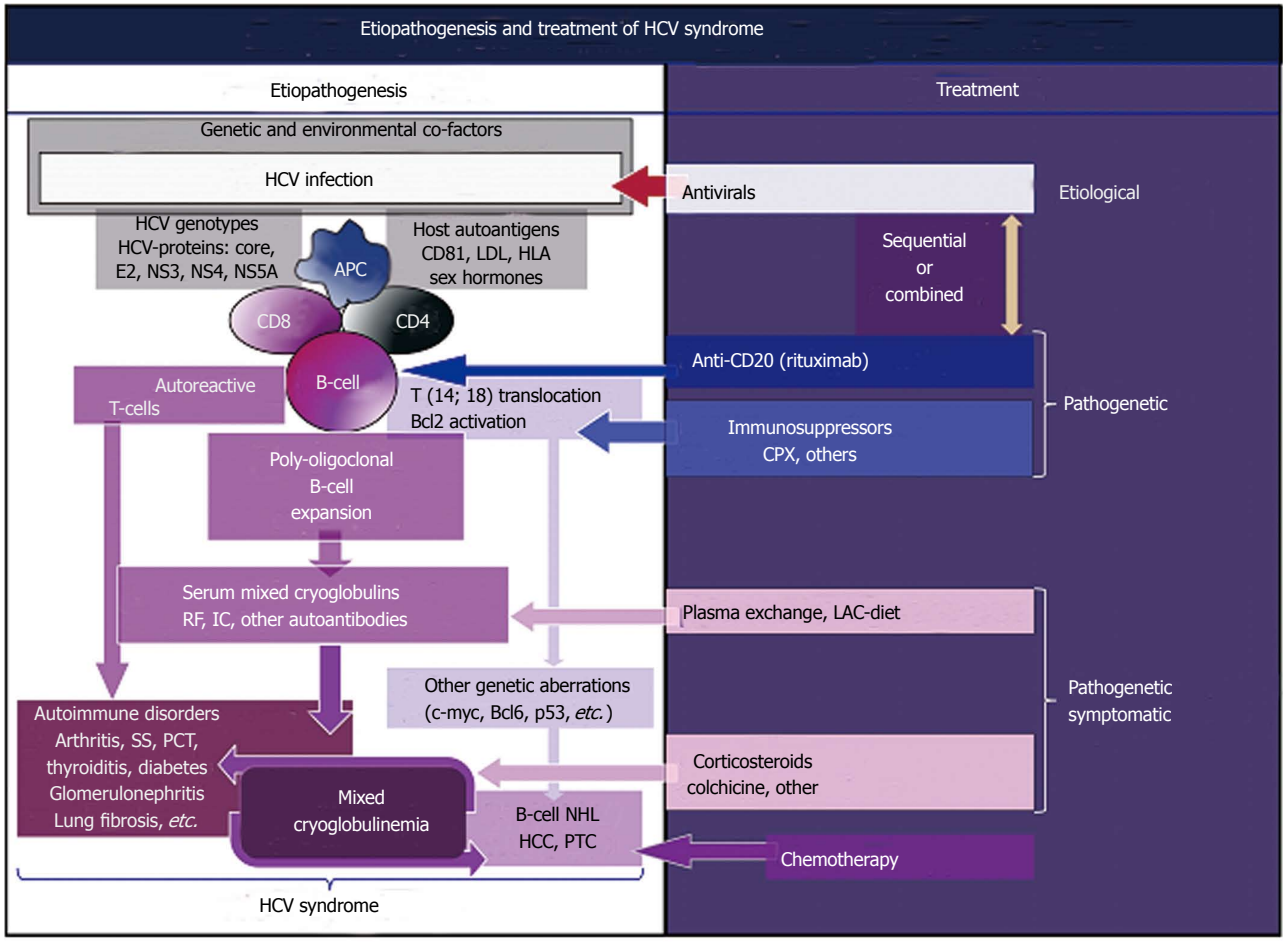

Figure 1 Etiopathogenetic cascade of hepatitis C virus syndrome, including both hepatic and extra-hepatic disorders, is a multifactorial and multistep process: The remote events include hepatitis C virus infection, predisposing genetic factors and, possibly, unknown environmental/toxic triggers (Left). The HCV-driven immune-system alterations with prominent "benign" lymphoproliferation, from one side, and oncogenic alterations, from another side, may be the result of different pathogenetic mechanisms not mutually exclusive, through a multifactorial and multistep process: Viral antigens (core, envelope E2, NS3, NS4, NS5A proteins) may exert a chronic stimulus on the host immune system, the high-affinity binding between HCV-E2 and CD81 and consequent t(14;18) translocation with bcl-2 proto-oncogene activation, a cross-reaction between particular HCV antigens and host autoantigens, *i.e.*, a molecular mimicry mechanism, and a direct infection of B-lymphocytes by HCV responsible for neoplastic cell transformation. Predisposing host factors may include particular HLA alleles, and both metabolic and hormonal conditions. The main consequence is a "benign" B-cell proliferation with production of various autoantibodies, among which RF and cryo- and non-cryoprecipitable IC. These serological alterations may be correlated with different organ- and non-organ-specific autoimmune disorders, including the systemic manifestations of MCs, or cryoglobulinemic vasculitis. Moreover, the activation of Bcl2 proto-oncogene, responsible for prolonged B cell survival, may be a predisposing condition to other genetic aberrations (c-myc, Bcl6, and p53 activation), which may lead to frank B cell lymphomas (B-NHL) and other malignancies (HCC: Hepatocellular carcinoma; PTC: Papillary thyroid cancer). The appearance of malignant neoplasias can be seen in a small but significant percentage of patients, usually as a late complication. Both immunological and neoplastic disorders show a clinico-serological and pathological overlap. Often, autoimmune organ-specific manifestations may evolve to systemic conditions, such as mixed cryoglobulinemia syndrome, and less frequently to overt malignancies. Conversely, it is not uncommon that patients with malignancies develop one or more autoimmune manifestations. In this scenario, MCs is at the crossing road between autoimmune and neoplastic disorders; Right: There are not comprehensive therapeutical guidelines for the HCV syndrome because of the complexity of its pathogenetic and clinico-prognostic characteristics; we can adopt in part the therapeutical strategy used for MCs, which often encompasses the different clinical variants of HCV syndrome. This therapeutical approach is essentially based on three main levels of intervention: the etiological treatment by means of antiviral drugs directed at HCV eradication, the pathogenetic therapies with immunomodulating/antineoplastic drugs, and the pathogenetic/symptomatic therapies such as corticosteroids and plasma exchange (see also text). HCV: Hepatitis C virus; IC: Immune complexes; SS: Sicca syndrome; PCT: Porphyria cutanea tarda; RF: Rheumatoid factor; MCs: Mixed cryoglobulinemia syndrome; CPX: Cyclophosphamide.

the anti-IgG rheumatoid factor constitutive of IgG-IgM immune-complexes, including mixed cryoglobulins[4,6,7,20].

While specific autoantibodies are typically found in patients with single-organ or systemic autoimmune disorders, cryoprecipitable and non-cryoprecipitable IgG-IgM immune-complexes are the serological hallmarks of MCs[4,6,7,20]. Moreover, a molecular mimicry mechanism that may involve specific HCV proteins and host autoantigens might produce B-cell activation with increased production of autoantibodies[4,6,7,20] (Figure 1). While the prolonged survival of B-lymphocytes can lead to additional genetic aberrations, such as c-myc, Bcl6, and p53 responsible to the development of malignant B-NHL in predisposed individuals[4,6,7,12-14,20] (Figure 1). The HCV oncogenic potential has been clearly identified in hepatocellular carcinoma and in a relevant number of patients with B-NHL[4,6,7,12-14,20-22] or thyroid cancer[23,24]. Comparably to the association between *Helicobacter pylori* infection and MALT

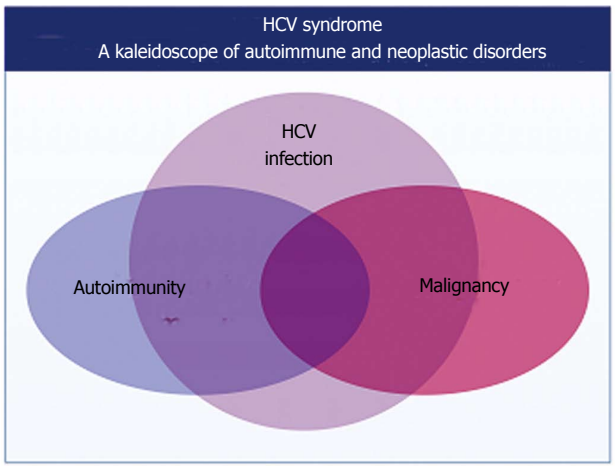

Figure 2 Patients with chronic hepatitis C virus infection may develop a complex of both hepatic and extra-hepatic disorders. Hepatitis C virus (HCV) syndrome represents an important example of coexistence of autoimmune and neoplastic conditions in humans; moreover, it can be one of the most useful models of study of the complex interactions between autoimmunity and oncogenesis[4] (Figure 2).

lymphoma of the stomach, the same pathogenetic mechanism of chronic antigen stimulation producing malignant lymphoproliferation may be hypothesized for HCV-driven lymphomagenesis[4,6,7,12-14] (Figure 1). This important topic, in particular the possible role of HCV infection in B-NHL, was underlined in a recent review focusing on the proposed epidemiologic/virological guidelines to support a causative role for a given virus in human cancerogenesis[14].

The HCV-driven lymphomagenesis may be the result of various oncogenetic mechanisms that may be not mutually exclusive, through a multifactorial and multistep process[4,6,7,12-14]: chronic external stimulation by HCV antigens of B-cell receptors (CD19, CD21, CD81, B-cell receptors), in particular the high-affinity binding of HCV-E2 and CD81 may lead to bcl-2 activation; HCV replication in B-lymphocytes with oncogenic potential through viral proteins; the direct infection of B-lymphocytes by HCV may produce permanent genetic B-cell damage, the so-called mutator B-cell phenotype due to "hit and run" mechanism of cellular transformation. The potential role of viral penetration and replication in B-lymphocytes remains to be definitely elucidated; however, some studies supported the oncogenic role of HCV genome or viral proteins in B-lymphocytes[12-16].

More recently, a role of the B-cell-activating factor (BAFF or BLyS) in the pathogenesis of HCV-related lymphomagenesis has been suggested. BAFF is a specific cytokine of B-lymphocytes, which is essential for the development and survival of B-lymphocytes. A higher serum levels of BAFF are detectable in patients with HCV infection if compared to healthy controls, and more frequently in HCV-positive subjects developing lymphoproliferative disorders[15,16]; but the exact mechanisms of this enhanced concentration remain still to be deeply clarified. The evaluation of polymorphic variants of *BAFF* gene promoter suggested a possible explanation; namely, a particular allelic variant (-871T), possibly correlated with the *BAFF* gene increased transcriptional activity, was significantly more frequently found in HCV-positive subjects with than in those without MCs. As regards HCV-related NHL, increased levels of circulating osteopontin were associated with HCV-positive B-cell lymphoma. Moreover, the highest levels of osteoponin were observed among HCV-positive individuals with associated MC type Ⅱ regardless the presence of B-NHL[15,16].

On the whole, HCV-infected patients represent one of the most useful models of study as regards the complex interactions between autoimmunity and oncogenesis in humans[4] (Figure 2).

CLINICAL MANIFESTATIONS OF HCV SYNDROME

The HCV biological activities, *i.e.,* the hepato- and lymphotropism, are responsible for HCV syndrome, a mosaic of hepatic as well as organ-specific and systemic diseases[11] (Figures 1 and 3).

Several clinico-epidemiological studies published in the world literature demonstrated that the prevalence of HCV-related autoimmune/neoplastic disorders shows a manifest geographical heterogeneity[4,6,7,25]. This finding does not perfectly coincide with the varying prevalence of HCV infection in different parts of the world; therefore, we can hypothesize that HCV alone is not sufficient to produce the wide spectrum of diseases that may complicate the natural course of HCV infection. It is supposable that specific HCV genotypes, host genetic and/or environmental factors should cooperate in the pathogenesis of HCV syndrome[4,6,7,26,27]; even if the actual relevance of these putative co-factors should be fully elucidated.

Moreover, contrasting data as regards the prevalence of different HCV-associated disorders has been also reported among clinical studies on HCV-infected patients' series from the same country, depending on specific specialization and/or investigative approach (sample sizes and choice of patients and/or control subjects) of different referring centers (Figure 3).

It is well known that the majority of individuals with HCV infection are asymptomatic, often for long-time periods, without consequences for their quality of life as well as the overall outcome; while a limited but relevant number of subjects may develop hepatic as well as extrahepatic diseases, often as late complication (Figure 1). The prolonged clinical follow-up of large series of HCV-positive patients suggests that the natural course of HCV infection may proceed through a multistage pathological process, usually from mild-moderate to clinically severe conditions, such as the MCs or malignant neoplasias[4,6,7] (Figures 1 and 3). However, it is possible that the slow progression of HCV syndrome in some individuals may

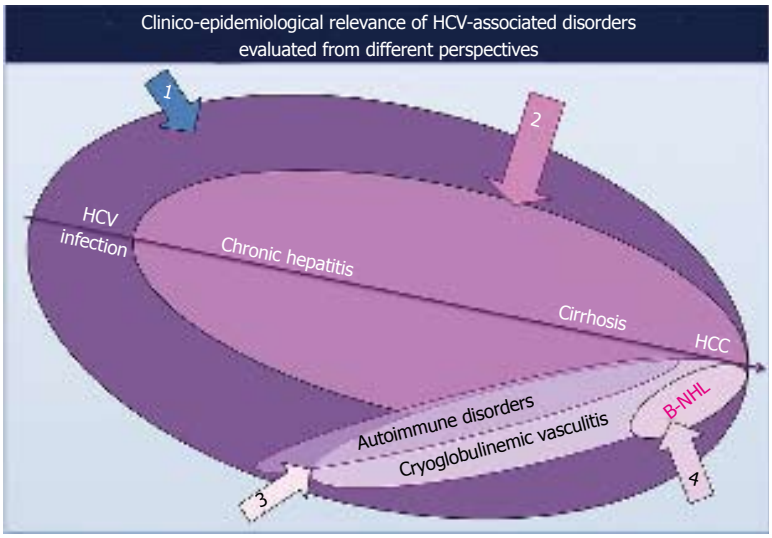

Figure 3 Schematic representation of the constellation of hepatitis C virus -associated disorders, varying from the great number of individuals with asymptomatic hepatitis C virus infection to patients with one or more harmful manifestations. Epidemiological studies demonstrated a great geographical heterogeneity among different HCV-associated disorders, as well as with important discrepancies with regards to their prevalence reported in clinical studies from the same country; this latter discrepancy may be dependent on specific specializations and/or variable methodological approaches (sample sizes, choice of patients and/or control subjects) of investigators from different referring centers. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 1 Hepatitis C virus syndrome: strength of association between hepatitis C virus infection and different diseases

Strong association[1]	Significant association[2]	Possible association[3]	Anecdotal association[4]
Chonic hepatitis	B-cell NHL	Sicca syndrome/SS	PM/DM
Cirrhosis	Monoclonal gammopthies	Polyarthritis	PAN
HCC	Porphyria cutanea tarda	Pruritus	Behçet's syndrome
Mixed cryoglobulinemia s./	Glomerulonephritis	Osteosclerosis	Chronic urticaria
Cryoglobulinemic vasculitis	Autoimmune thyroiditis	Fibromyalgia	Psorias
	Papillary thyroid cancer	Peripheral neuropathy	Mooren corneal ulcer
	Diabetes m. type 2	Lung alveolitis	
		Autoimmune hepatitis	
		Cardiovascular inv.	
		Lichen planus	

[1]The HCV is the etiological agent or it is detectable in the majority of patients; [2]HCV is detectable in a significant proportion of pts compared to general population, with heterogeneous geographical distribution, its potential role is supported by pathogenetic studies; [3]A role of HCV infection has been suggested by cohort studies or [4]by some anecdotal observations. HCC: Hepatocellular carcinoma; SS: Sjögren's syndrome; PM/DM: Polymyositis/dermatomyositis; PAN: Panarteritis nodosa; HCV: Hepatitis C virus.

be abruptly complicated by one of the most severe complications[4,28].

The symptom complex of HCV syndrome includes numerous autoimmune diseases and malignant neoplasias[11]. The statistical and clinical relevance of HCV association with different disorders is markedly variable; in this light, HCV-driven conditions can be classified in four groups according to the strength of association[4,11] (Table 1): level 1: the HCV is the etiological agent or it is present in a large percentage of patients; level 2: HCV is detectable in a statistically significant number of patients when compared to the general population, at least in some geographical areas, often demonstrated by clinical and laboratory investigations; level 3: a causative role of HCV has been suggested by some cohort studies or level 4: by isolated, anecdotal observations without definite pathogenetic link. The following paragraphs focuses on the main clinical and laboratory features of extra-hepatic manifestations of HCV syndrome.

Autoimmune diseases

Extrahepatic manifestations of HCV syndrome are immune-mediated diseases[11]; MCs represents the most common condition, it represents a crossover between "benign" organ-specific and systemic autoimmune diseases, from one side, and malignant neoplastic disorders, from the other side[4,11] (Figure 1).

MCs, cryoglobulinemic vasculitis: MCs is commonly classified among small-vessel systemic vasculitides; thus, the terms MCs and CV should be referred to the same clinico-serological and pathological disorder[4-7,20]. The first one underlines the presence of the serological hallmark, *i.e.*, mixed cryoglobulins, which characterize the disease, along with the "benign" B-cell lymphoproliferation, and the classical symptoms, namely purpura, weakness, and arthralgias[4-7,20]; while the term CV focuses on the pathological hallmark, *i.e.*, the leucocytoclastic vasculitis, affecting small-medium sized vessels (Figure 4). Vascular damage is the consequence of the deposition of circulating cryo- and non-cryoprecipitable immune-complexes and complement; they produce both skin and internal organ damage[4-7,20] (Figures 1 and 4; Table 2 with MCs symptoms). CV represents a crossroads among immune-mediated disorders and malignancies (Figure 1); it is not rare to observe in a single patient during

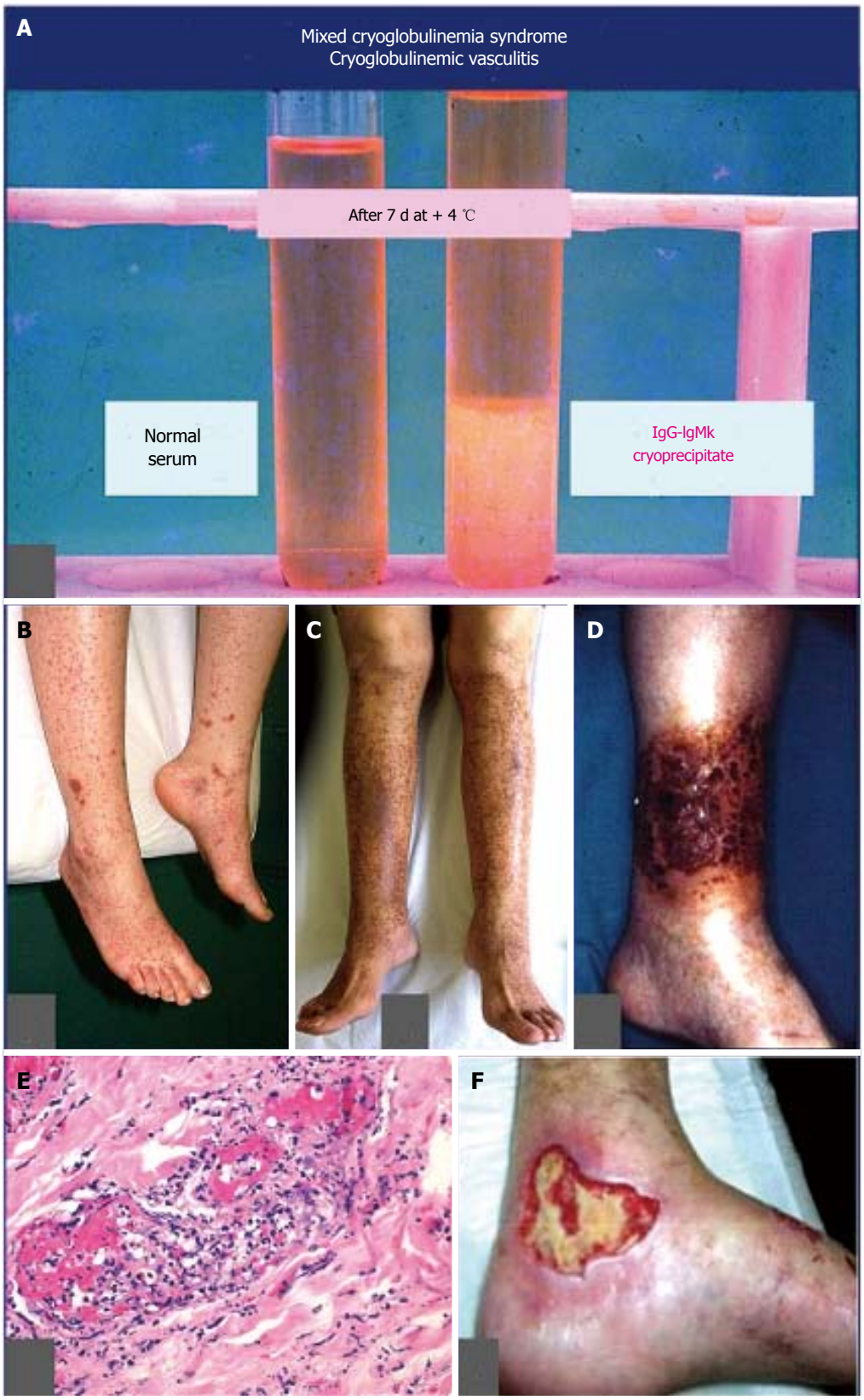

Figure 4 The main serological, clinical, and pathological hallmarks of mixed cryoglobulinemia syndrome, or cryoglobulinemic vasculitis: A: On the right serum cryoprecipitate (evaluated after 7 d storage at 4 ℃) composed by polyclonal IgG (autoantigen) and monoclonal IgMk (autoantibody) immune-complexes, compared to normal serum sample; B: Recent onset, palpable purpuric lesions of the lower limbs; C: Sock-like ochraceous hyperpigmentation of the legs and feet, consequence of repeated episodes of purpura; D: Severe, necrotizing vasculitic skin lesion of the leg; E: Typical histological pattern of cutaneous leukocytoclastic vasculitis involving the small vessels and characterized by diffuse fibrinoid necrosis and disintegrated neutrophil permeation of the vessel walls; F: Wide non-healing skin ulcer, often resistant to treatment.

Table 2 Clinico-epidemiological and laboratory features of mixed cryoglobulinemia[1]

Epidemiological features	
Mean age at disease onset ± SD, yr (range)	55 ± 12 (29-72)
Mean disease duration ± SD, yr (range)	12 ± 10 (1-40)
Female/male ratio	3
Clinical features	
Purpura	98
Weakness	98
Arthralgias	91
Arthritis (non-erosive)	8
Raynaud's phenomenon	32
Sicca syndrome	51
Peripheral neuropathy	81
Renal involvement[2]	31
Liver involvement	73
B cell non-Hodgkin's lymphoma	11
Hepatocellular carcinoma	3
Serological and virological features	
Mean cryocrit (SD)	4.4% (12)
Type Ⅱ/type Ⅲ mixed cryoglobulins	2/1
Mean C3 (SD) (*nv* 60-130 mg/dL)	93 (30)
Mean C4 (SD) (*nv* 20-55 mg/dL)	10 (12)
Anti-nuclear antibodies	30
Anti-mitochondrial antibodies	9
Anti-smooth muscle antibodies	18
Anti-extractable nuclear antigen antibodies	8
Anti-HCV antibodies ± HCV RNA	92
Anti-HBV antibodies	32
HBsAg	1

[1]Data are referred to 100 consecutive, unselected Italian patients with mixed cryoglobulinemia syndrome (MCs) evaluated at the end of follow-up; [2]Membranoproliferative glomerulonephritis type I. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

the natural course of the disease the entire spectrum of symptoms, from mild manifestations to overt vasculitic syndrome, and finally to most severe complications such as hepatic/renal failure, and/or cancer[4,28]. Compared to other systemic vasculitides, CV shows two distinctive symptoms, namely B-cell NHL and chronic hepatitis. While B-cell NHL affects a minority of patients (Table 2), generally as late complication, liver involvement is detectable in almost 3/4 of individuals[4-7,20]. The clinical course of chronic hepatitis is generally mild to moderate, and often asymptomatic for long time period; it may be complicated by cirrhosis and in some cases by hepatocellular carcinoma[4,28]. Renal involvement, usually a type Ⅰ membranopro liferative glomerulonephritis, represents one of the most severe organ damage of CV[4,28,29]. Moreover, the rare, diffuse vasculitis is a life-threatening complication prognostically similar to classical systemic vasculitides[4,28]. Laboratory investigations are characterized by largely variable amounts of serum cryoglobulins and low complement with typically marked C4 reduction and normal C3; nonetheless, levels of cryocrit and complement are frequently not correlated with the severity/activity of CV[4]. Typically, B-lymphocyte expansion represents the substrate of CV, which in a number of patients may be complicated by overt lymphoma, usually a late disease manifestation[4,28,30]. The large majority of patients with CV show a chronic HCV infection; this association is particularly frequent in particular geographical areas, mainly Southern Europe[4]; generally in those countries where the presence of other HCV-associated extra-hepatic disorders are rather observed[4,25]. Conversely, other classical systemic vasculitides are significantly more frequent in other countries of Northern Europe and Northern America where HCV-associated CV is less frequently observed[4,31].

Other systemic rheumatic diseases: CV is characterized by clinical polymorphism; therefore it is not rare to observe a clinical overlap between CV and other rheumatological disorders, mainly Sjögren's syndrome or rheumatoid arthritis[4,28,31-35] (Table 1, Figure 1). Chronic arthritis, generally oligoarthritis, can develop during the natural course of HCV infection; the joint involvement commonly appears as non-erosive, less aggressive arthritis. HCV-associated MCs patients may develop mild oligoarthritis, on the contrary moderate-severe polyarthritis, comparable to classical rheumatoid arthritis, may be sporadically observed in HCV-infected patients treated with alpha-interferon[4,31,32]. On the other hand, given the relatively high prevalence of these conditions, we can observe a pure association of HCV infection with frank rheumatoid arthritis; the same association can be occasionally observed with primary Sjögren's syndrome. While the possible involvement of HCV in the pathogenesis of Sjögren's syndrome is still a controversial topic[4,31-35]. Differential diagnosis between CV and primary Sjögren's syndrome may be very difficult in individual cases such patients with sicca syndrome, serum mixed cryoglobulins, HCV infection, and anti-RoSSA/LaSSB antibodies; these latter generally at low serum concentration[4,31-35]. This peculiar symptom complex that may satisfy classification criteria of both CV and primary Sjögren's syndrome seems to identify a worse clinical variant, more frequently complicated by malignant lymphoma[31-35]. In these instances, it is preferable to consider these individuals as having Sjögren's/MCs overlapping disease that should be treated according to individual clinical manifestations[31,32].

The presence in the clinical practice of overlapping MC/Sjögren's suggests that HCV may trigger complex immunological alterations that, in genetically predisposed subjects, may cause various clinical phenotypes mimicking some well-known disorders, such as rheumatoid arthritis, Sjögren's syndrome, and dermato-polymyositis[4,31,32] (Figure 3, Table 1).

Actually, the clinical value of other rheumatic disorders possibly triggered by HCV observed in limited patients' series or anecdotal case reports deserve further investigations[4,31,32] (Table 1).

Endocrine disorders: Thyroiditis, diabetes type 2, and male gonadal dysfunction can be included among

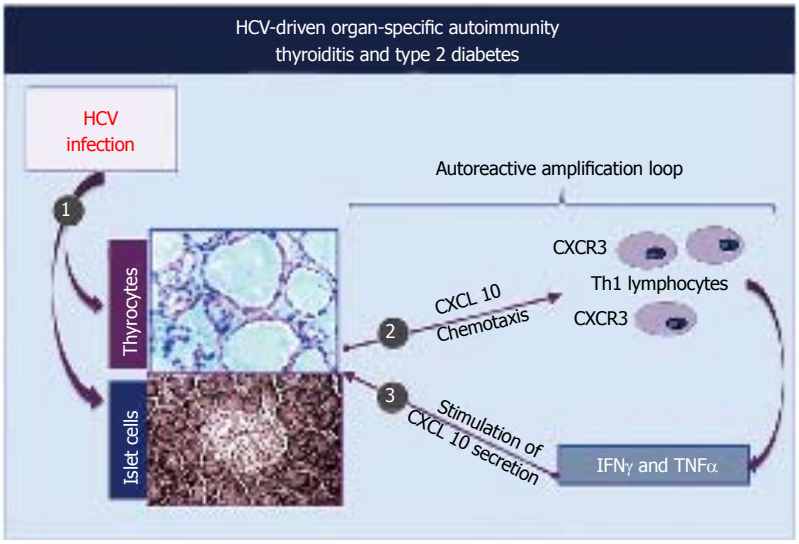

Figure 5 Immune-mediated thyroid involvement and diabetes type 2 can be observed in a significant percentage of patients with hepatitis C virus infection. The figure summarizes the possible etiopathogenetic mechanisms involved in these two endocrine disorders. HCV thyroid infection may act by upregulating CXCL10 gene expression and secretion in thyrocytes (and pancreatic b-cells); CXCL10 may promote the recruitment of Th1 lymphocytes, which secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα). In turn, these cytokines may induce CXCL10 secretion by thyrocytes (and pancreatic b-cells), thus perpetuating the immune cascade. The consequence may be the appearance of thyroid autoimmune disorders and/or diabetes type 2 in genetically predisposed subjects. HCV: Hepatitis C virus.

the most frequent endocrine alterations that may complicate HCV-positive individuals with and without MCs[4,31,36,37].

The same geographic heterogeneity that characterizes different disorders complicating HCV infection is reported for the prevalence of serum levels of anti-thyroid antibodies, which markedly varied from 2% to 48% of individuals in several cohort studies[36,38].

A possible explanation of this heterogeneity may be related to the variable contribution of environmental factors and host genetic predisposition among different patients' populations, for example the iodine intake or the presence of other infectious factors[11,36,38].

In addition, hypothyroidism can be found in 2%-9% of subjects with HCV infection, generally as subclinical finding[36]; while various thyroid disorders and anti-thyroid antibodies are generally more frequently detected in hepatitis type C compared to hepatitis B or D[36]. In fact, the prevalence of these findings has been evaluated in a wide patients' series with type C hepatitis compared to general population groups from geographical areas with variable iodine intake and subjects with hepatitis type B[36]. Hypothyroidism and autoimmune thyroiditis were found in patients with type C hepatitis in a statistically higher percentage if compared to control subjects; while comparable percentages of hyperthyroidism were detected. Comparable findings have been reported in another study evaluating thyroid alterations in HCV-associated MCs[36-38].

Overall, thyroid involvement can be considered as one of possible manifestations of MCs as well as of HCV syndrome[4,31,36-38].

Thyroid involvement should be periodically evaluated in HCV-infected patients to early diagnose the thyroid dysfunction and the possible malignant complications[36].

Diabetes type 2 can be considered another relevant endocrine disorder of the HCV syndrome, generally not correlated with presence/severity of hepatic involvement[36]. Preliminary, clinico-epidemiological studies reported an increased prevalence of diabetes type 2 in HCV-infected non-cirrhotic subjects compared with chronic hepatitis of different origin, a finding not confirmed in a subsequent report[36]. Successively, a case control study evaluated 564 Italian HCV-infected non-cirrhotic patients compared with 302 control individuals without history of drug or alcohol addiction, or positive for viral hepatitis serological markers, and 82 HBV-infected non-cirrhotic subjects[36]. Diabetes type 2 was significantly more frequent in HCV-infected non-cirrhotic patients compared to controls (12.6% *vs* 4.9% and 7%, respectively; $P = 0.008$). Of interest, prevalence of diabetes type 2 in HBV-infected non-cirrhotic subjects (7%) resulted within the age-adjusted range of prevalence rates for the Italian general population (4%)[36].

The clinical phenotypes of subjects with hepatitis and diabetes type 2 were quite different: individuals with non-cirrhotic HCV-positive diabetes type 2 were slightly older, with higher levels of triglycerides, blood pressure, and BMI, but lower concentrations of cholesterol HDL[36].

In addition, HCV-positive, non-cirrhotic subjects with diabetes type 2 had significantly lower BMI compared to control subjects with diabetes type 2 alone and significantly higher BMI ($P < 0.05$) compared to non-cirrhotic, non-diabetic HCV-infected subjects. Classical diabetes type 2 is characterized by the "metabolic syndrome" phenotype, *i.e.,* overweight, older age, higher arterial pressure, and dyslipidemia. Conversely, non-cirrhotic, non-diabetic HCV-infected subjects resulted lean and with low levels of cholesterol LDL. These latter have been correlated with the hypobetalipoproteinemia induced by the binding competition of HCV with hepatocyte LDL receptors[36].

Patients with HCV chronic infection manifested a peculiar clinical variant different from the usual form of diabetes type 2. The classification of HCV-positive patients as diabetes type 2 is quite traditional; with the new pathogenetic information the boundaries between diabetes type 1, latent autoimmune diabetes,

and diabetes type 2 are progressively weakening. In patients with HCV-associated MCs complicated by diabetes, an immune-mediated mechanism has been postulated for the endocrine disorder; a comparable autoimmune pathogenesis might be suggested also for diabetes observed in HCV-infected patients without MCs. This supposition is reinforced by the increasing observation of autoimmune alterations in patients with diabetes type 2[36,39-47]. HCV-related MCs patients showed abnormal serum levels of sex hormones[48]. Interestingly, erectile dysfunction was anecdotally observed in subjects with type C during antiviral treatment with interferon-alpha[49]. The putative relationship between HCV infection and gonadal dysfunction has been evaluated in 207 HCV-infected males (102 with MCs) in comparison with 207 age- and sex-matched individuals, selected randomly from 2010 subjects of Italian general population previously evaluated for the presence of erectile dysfunction[50]. The study adopted some important exclusion criteria, namely patients' age over 55 years, recent treatment with interferon-alpha, presence of cardio-vascular and psychiatric disorders, diabetes, hypothyroidism, and renal failure.

HCV-positive patients showed a higher prevalence of erectile dysfunction compared to controls ($P < 0.001$). In addition, abnormally low testosterone plasma levels were detected in HCV-infected individuals with complicating erectile dysfunction. Of interest, erectile dysfunction as well as low serum testosterone was independent of the severity of hepatic damage. The alterations of sex hormones along with the frequent peripheral neuropathy might explain the erectile dysfunction[4,31,32].

The above-mentioned findings are in keeping with the hypothesis of a possible role of patient's hormonal status in immune mediated conditions triggered by HCV infection; we could hypothesize that low androgen levels may reduce endogenous depressive activity and consequently may amplify the proliferation of autoreactive B-lymphocytes triggered by HCV infection[4,31,32]. With regards the pathogenetic mechanisms responsible for HCV-related thyroid disorders, possibly HCV infection of thyrocytes may act by upregulating gene expression and secretion of CXCL10 (as previously shown in human hepatocytes) by recruiting Th1 lymphocytes, which secrete IFNγ and tumor necrosis factor alpha (TNF-α)[40,41].

In turn, these cytokines may induce the secretion of CXCL10 by thyrocytes, with consequent perpetuation of the immune cascade[36] (Figure 5).

The consequence may be the appearance of thyroid autoimmune diseases in subjects genetically predisposed. This pathogenetic hypothesis has been confirmed by a recent study that evaluated the serum levels of CXCL10 in patients with HCV-positive MCs, with and without autoimmune thyroid involvement[36,42], and also studying the association of thyroiditis with other autoimmune disorders[43-45].

Chronic immune-mediated inflammatory thyroid lesions may be responsible for the papillary thyroid cancer found in a significant number of HCV-infected individuals compared to controls[23,24,46].

Analogous pathogenetic mechanisms can be involved as consequence of HCV infection of pancreatic β-cells responsible for the up-regulation of *CXCL10* gene expression and secretion[36] (Figure 5). The recruited Th1 lymphocytes, which secrete IFNγ and TNFα, amplify the secretion of CXCL10 by B-lymphocytes. The final result is the appearance of B-cell dysfunction, with the probable contribution of genetic predisposition. Both thyroid function abnormalities and diabetes type 2 should be considered among the manifestations of HCV syndrome[4,31,32,36].

It is opportune to evaluate these patients at regular intervals for the above endocrine disorders in order to identify subjects needing treatment and to early diagnose possible thyroid malignancies[47].

Porphyria cutanea tarda: Porphyria cutanea tarda (PCT) constitutes the most common clinical variant of porphyria; the disease is characterized by low activity of uroporphyrinogen decarboxylase (URO-D), the enzyme involved in the heme synthesis[51-55]. The URO-D deficiency is necessary but not sufficient for the clinical development of PCT, therefore possible pathogenetic co-factors have been proposed, including hepatotropic virus infection; this hypothesis was also suggested by the frequent chronic liver involvement in patients with PCT. Thus, the possible role of HCV has been investigated worldwide; numerous reports showed a broad range of prevalence of HCV infection in patients with PCT[51-55]. The HCV-associated PCT is particularly intriguing with regards the pathogenetic implications[52]. A direct role of HCV can be excluded considering the absence of alteration in porphyrine metabolism in HCV-positive patients without PCT[53]; while it is supposable that a cross-reactivity between host and HCV antigens and/or metabolic factors such as altered genes connected with iron metabolism[52] may contribute to a genetically-driven reactivity that may be relevant in individuals with PCT.

Renal involvement: Renal involvement represents a frequent complication of HCV-related MCs, which may seriously affect the overall outcome of these patients[4,28,29,31,32]. Glomerulonephritis is the result of immune complex glomerular deposition, mainly cryoglobulins. The actual pathogenetic relevance of HCV in the etiopathogenesis of glomerulonephritis remains not completely established; the detection of viral sequences in immune-complexes suggested an indirect role of HCV in the glomerular inflammation[4,28,29,31,32].

Moreover, a role of HCV has been suggested for tubulo-interstitial and glomerular renal damage in both transplanted and native kidney[56,57]. HCV-related glomerular injury may include different pathological patterns: mainly membranoproliferative glomeru-

lonephritis (MPGN) in the presence/absence of cryoglobulinemia, while membranous nephropathy, rapidly progressive GN, immunotactoid and fibrillary GN, exudative-proliferative GN were less frequently observed[4,29]. Classical cryoglobulinemic type I MPGN is found in a significant proportion of HCV-associated MCs, while "primary" or clinically isolated MPGN is detected in less than one third of HCV-associated MPGN[56]. The latter variant has been observed mainly in Japan and United States[29,56,58]. Nevertheless, the actual prevalence of MPGN in HCV-positive patients without serum cryoglobulinemia is difficult to evaluate; possibly this condition may constitute a mild or subclinical variant of MCs, probably due to methodological difficulties in detecting circulating cryoglobulins[4]. It is not rare that MPGN is one of presenting symptoms of HCV-associated MCs, while the overt syndrome can appear as late manifestation of HCV infection[4,28]. Therefore, HCV-positive patients with apparently isolated GN should undergo to careful clinico-serological assessment in order to exclude possible hepatic and extrahepatic disorders, especially the MCs.

Miscellanea: Miscellanea of immune-mediated disorders may complicate HCV infection (Table 1); the association of lichen planus, in particular oral lesions, with HCV has been reported with a variable geographic prevalence[59,60]. Moreover, a wide number of mucocutaneous manifestations, generally as acute episode or chronic manifestation of well-known cutaneous diseases, are observed with variable prevalence in HCV-infected patients, often as limited series or anecdotal case reports[4,59,60] (Table 1). These symptoms may be the expressions of immune-mediated skin damage, triggered by viral proteins and possibly amplified by interferon-alpha treatment[4]. Peripheral nerve involvement is a frequent HCV-related manifestation, more often in patients with CV[4,61]; while involvement of central nervous system (CNS) is rarely observed. Vascular symptoms that may involve CNS may represent late manifestations of HCV syndrome, generally as comorbidities in subjects with particularly severe extra-hepatic symptoms treated with long-term corticosteroids. HCV-infected individuals may develop cardiovascular complications, which are not constantly confirmed by different studies[4,62,63]. Another controversial feature of HCV syndrome is the putative role of this virus in autoimmune hepatitis[4,31,32,64,65]. Circulating mixed cryoglobulins and some extrahepatic manifestations such as sicca syndrome, arthritis, and thyroiditis, can be observed in patients with autoimmune hepatitis; vice versa, HCV-infected patients may show serum non organ-specific autoantibodies[11,65]. Often, antigenic specificity of autoantibodies detectable in HCV-positive individuals presents only titer differences when compared to those found in "primary" autoimmune hepatitis[4,11]. Possibly, the heterogeneity of geographical distribution of HCV-associated autoimmune hepatitis[25] can be correlated to the variable cooperation of different causative agents, including HCV; therefore, this virus might be responsible for a distinct subset of AIH in infected individuals from specific geographical areas.

Neoplastic disorders

Following the discovery of HCV the oncogenic role of this virus has been established by several clinico-epidemiological and laboratory studies; besides hepatocellular carcinoma, HCV chronic infection may represent the trigger factor of papillary thyroid cancer and B-cell NHL.

Hepatocellular carcinoma: Hepatocellular carcinoma (HCC) is a primary hepatic cancer and occurs commonly as late complication of chronic hepatitis and cirrhosis[21,22]. Over the past two decades, the incidence of this malignancy is steeply increasing, analogously to the HCV prevalence worldwide[21,22]. In HCV-infected individuals HCC represents the most frequent malignancy[21,22]. The development and progression of HCC is a multistep process; a chronic insult, *i.e.,* HCV, HBV, and alcohol, induces liver injury through oxygen species production, cellular DNA damage, endoplasmic reticulum stress, and necrosis of damaged hepatocytes. In this context, chronic inflammation and oxidative DNA damage favor the accumulation of mutations and epigenetic aberrations in hepatocytes or liver stem cells, which in turn may foster the development of dysplastic nodules and their malignant transformation to overt HCC[21,22]. This harmful complication can appear in the context of isolated type C hepatitis, as well as in patients with extrahepatic HCV-related manifestations[4,21,22]; even if the prevalence of HCC seems to be lower in HCV-associated MCs compared to the entire population of HCV-infected patients[4,28]. This intriguing topic needs to be confirmed by wider clinico-epidemiological investigations. Tentatively, it is possible that in patients with HCV-related extrahepatic diseases the rather frequent involvement of particular HCV genotypes such as the 2a/2b[4,26] and/or genetic background, as well as a different clinical course of the disease, including specific treatments such as immunomodulating drugs, may counteract the risk for developing this tumor.

Papillary thyroid cancer: Papillary thyroid cancer represents a rare neoplasia that may be associated to chronic HCV infection[23,24]. A significantly increased prevalence of thyroid cancer in type C hepatitis as well as in HCV-related MCs compared with controls was first noticed in 1999[23,46]; it was subsequently confirmed by a case control study reporting an increased prevalence of HCV infection in individuals with papillary thyroid cancer undergoing surgery[24]. In addition, high prevalence of papillary thyroid cancer in subjects with a past history of blood transfusions and/or liver disease seems to indirectly support the role of HCV in this malignancy[36]. However, a review of the

literature shows discordant results, possibly owing to important epidemiological and methodological bias[36,66]. A recent study on large cohort of HCV-positive patients seem to confirm the increased prevalence of papillary thyroid cancer by excluding some possible biases such as iodine intake, gender and/or patients' age[36]. In our studies, thyroid autoimmune alterations were more commonly found in patients developing thyroid papillary cancer irrespective of whether they had type C hepatitis alone or HCV-related MCs[23,24,46]. This observation suggests that immune-mediated thyroid alterations *per se* may be a predisposing condition for this malignancy. Although a possible causative role of HCV infection in papillary thyroid cancer is suggested by the above clinico-epidemiological studies, this association needs to be verified by further investigations.

B-cell NHL: Over the last two decades a putative role of HCV in B-lymphocyte lymphomagenesis has been progressively investigated considering the following observations: the lymphotropism of HCV was definitely demonstrated in individuals chronically infected, including those with HCV-related MCs[9,10]; in the same time, HCV revealed as the major etiological factor of MCs, a "benign" autoimmune-lymphoproliferative diseases that may be complicated by frank B-cell lymphomas[4,6,7,28,30,67,68]. Thus, the logical supposition was that the same virus might be also involved in the etiopathogenesis of 'idiopathic' B-cell NHL. In 1994, unexpected high rate of HCV ongoing infection in Italian series of patients with 'idiopathic' B-NHL was first reported[69]. Since this initial report numerous clinico-epidemiological and laboratory studies on different patients populations and in animal models, plainly established the causative role of this virus in a significantly higher percentage of patients with B-NHL compared to controls[69-103]. Once more, this association showed a geographical heterogeneity similarly to that observed for other HCV-associated diseases, especially the MCs; in fact, the association between HCV and B-cell lymphomas was not confirmed by other epidemiological studies[104-119]. This particular virus-related malignant lymphoproliferation may presents two major clinical variants: B-NHL as neoplastic manifestation of HCV-positive MCs, more often as late disease manifestation, or B-NHL complicating HCV infection without relevant extrahepatic manifestations[4,6,7,31,67,68]. The B-NHL of cryoglobulinemic patients can vary from extranodal, nodal or splenic marginal-zone lymphoma to diffuse large B-NHL or, less frequently, lymphoplasmacytic lymphoma/immunocytoma (LPL/Ic) and B-cell chronic lymphocytic leukemia (B-CLL)[4,120]. Lymphomas may be correlated to the expansion of peripheral B-lymphocytes and to lymphoid cell infiltrates frequently detectable in bone marrow and liver of patients with MCs[4,120]. These infiltrates containing lymphoid elements closely comparable to those characterizing B-CLL and LPL/Ic have been classified as "early lymphomas"[4,120]. However, different from overt malignant lymphomas, they usually remain stable for a long time and are followed by frank lymphatic malignancy in about 10% of individuals[4,120]. According to the above clinico-pathological features the term "monotypic lymphoproliferative disorder of undetermined significance (MLDUS)" has been proposed[4,120]. Interestingly, MLDUS observed in HCV-positive type II MC shows a significantly high incidence in those countries also characterized by frequent association between "idiopathic" B-NHL and HCV infection, as well as by rather high prevalence of genotype 2a/2c[4,120].

With regard to primary B-NHL a number of epidemiological studies (Table 3) confirmed the association of these malignancies with HCV in a significant proportion of patients; HCV-associated B-NHL showed the same geographical heterogeneity observed for other HCV-related extrahepatic disorders[69-119]. This epidemiological feature may reflect the multifactorial etiopathogenesis, including both genetic background and environmental cofactors, of this heterogeneous group of lymphatic malignancies[12-15]. Moreover, some discordant data among studies from the same country might be the consequence of recruitment bias (choice of patients and/or control subjects, sample sizes) at different referring centers as aforementioned (Figure 3). Whether an increased risk for all B-cell NHL is associated with HCV infection or only particular subtypes remains still open question[98]. Apart from the above epidemiological aspects, HCV can be included among other well known lymphotropic viruses, namely human T lymphotropic virus type I, Epstein Barr virus, human herpesvirus 8, and human immunodeficiency virus, responsible for a significant proportion of B-cell NHL[121]. Of interest, some meta-analysis studies of epidemiological investigations confirmed the strongly positive association between HCV infection and increased risk of B-NHL[90,98,122,123].

Finally, in HCV-infected patients with splenic marginal zone or indolent B-cell NHL, combined treatment with interferon-alpha and ribavirin may lead to HCV clearance and concomitant regression of lymphomas[124-126]. These observations clearly presuppose that the virus is the causative agent in at least some NHL subsets by directly driving the B-cell lymphoproliferation.

TREATMENT OF HCV SYNDROME

Considering the complexity of HCV syndrome because of its variable composition of clinical symptoms with specific pathogenetic, clinical, and prognostic characteristics, it is impossible to draw comprehensive therapeutical guidelines. In clinical practice, it can be useful to look at the therapeutical strategy developed for patients with MCs, which takes into account the different clinical variants of HCV syndrome[4,28,31,32]. This strategy is essentially based on three main levels

Table 3 Association between hepatitis C virus infection and B-cell non-Hodgkin's lymphoma: Epidemiological studies

Significant association[1]			No association[1]		
Ref.	Country	Prevalence[2]	Ref.	Country	Prevalence[2]
Ferri *et al*[69]	Italy	34% (17/50)	Brind *et al*[104]	United Kingdom	0% (0/63)
Luppi *et al*[70]	Italy	42% (29/69)	Hanley *et al*[105]	United Kingdom	0% (0/72)
Mazzaro *et al*[71]	Italy	28% (56/199)	McColl *et al*[106]	Scotland	0% (0/110)
Musolino *et al*[72]	Italy	20.8% (5/24)	Thalen *et al*[107]	The Netherlands	0% (0/115)
Silvestri *et al*[73]	Italy	8.9% (42/470)	Ellenrieder *et al*[108]	Germany	4.3% (3/69)
De Rosa *et al*[74]	Italy	22.4 (59/263)	Timoraglu *et al*[109]	Turkey	0% (0/48)
Zuckerman *et al*[75]	Mexico and United States	22% (26/120)	Collier *et al*[110]	Canada	0% (0/101)
Catassi *et al*[76]	Italy	11.2% (16/143)	Shariff *et al*[111]	Canada	2.3% (2/88)
Kashyap *et al*[77]	United States	11.5% (36/312)	Udomsakdi-Auewarakul *et al*[112]	Thailand	2.3% (3/130)
Luppi *et al*[78]	Italy	22.3% (35/157)	Hausfater *et al*[113]	France	1.83% (3/164)
Cucuianu *et al*[79]	Romania	29.5% (20/68)	Isikdogan *et al*[114]	Turkey	0% (0/119)
Vallisa *et al*[80]	Italy	37.1% (65/175)	Giannoulis *et al*[115]	Greece	1.9% (2/108)
Paydas *et al*[81]	Turkey	11.4% (26/228)	Sonmez *et al*[116]	Turkey	2.8% (3/109)
Harakati *et al*[82]	Saudi Arabia	21.4% (12/56)	Okan *et al*[117]	Turkey	3.1% (8/258)
Mizorogi *et al*[83]	Japan	17% (17/100)	Park *et al*[118]	South Korea	2.1% (5/235)
Zucca *et al*[84]	Switzerland	9.4% (17/180)	Varma *et al*[119]	India	1.75% (1/57)
[3]Pioltelli *et al*[85]	Italy	16% (48/300)			
Sanchez Ruiz *et al*[86]	Spain	11.7% (9/77)			
Chindamo *et al*[87]	Brazil	8.2% (9/109)			
De Renzo *et al*[88]	Italy	17.3% (39/227)			
Imai *et al*[89]	Japan	13.4% (21/156)			
Gisbert *et al*[90]	Spain	7% (7/99)			
Mele *et al*[91]	Italy	17.5% (70/400)			
Yenice *et al*[92]	Turkey	7.1% (6/84)			
Iwata *et al*[93]	Japan	11% (16/145)			
Gisbert *et al*[94]	Spain	5.8% (5/86)			
Talamini *et al*[95]	Italy	19.6% (44/225)			
Cowgill *et al*[96]	Egypt	42% (95/227)			
Engels *et al*[97]	United States	3.9% (32/813)			
de Sanjose *et al*[98]	Spain	3.9% (172/4784)			
Spinelli *et al*[99]	Canada	2.4% (19/795)			
Chuang *et al*[100]	Taiwan	11% (31/346)			
Libra *et al*[101]	Italy	19.7% (539/2736)			
Kang *et al*[102]	South Korea	2.8% (76/3932)			
Nosotti *et al*[103]	Italy	9.2% (19/207)			
Range		2.4%-42%			0%-4.3%

[1]Compared to controls; [2]HCV-RNA and/or anti-HCV; [3]The prevalence of HCV infection in B-NHL was statistically significant compared to controls, but not confirmed by odds ratio method to estimate the relative risk. HCV: Hepatitis C virus.

of intervention (Figure 1): the etiological treatment by means of antiviral drugs directed at HCV eradication, the pathogenetic therapies with immunomodulating-antineoplastic drugs, and the pathogenetic/symptomatic therapies such as corticosteroids and plasmapheresis[4,28,31,32]. These three different therapeutic lines are not mutually exclusive; they are usefully employed in HCV-positive MCs, considering the composition and severity of clinical features observed in the single patient[4,28,31,32] (Figure 1). The etiological treatment, alone or in combination with immunosuppressors, may lead to HCV eradication and MCs remission[31,127-134]; as above mentioned the beneficial effect of HCV eradication is also observed in patients with B-cell NHL, as isolated condition or complicating the MCs[124-126]. In theory, antivirals should be regarded as the gold standard treatment in patients with overt HCV-associated clinical symptoms, considering the whole individual patient condition and the potential side effects or contraindications to these therapies[4,127-131]. The preemptive use of the novel direct antiviral drugs in HCV-infected individuals even in the absence of relevant clinical manifestations is a very critical issue, considering the necessary cost-benefit analysis.

On the other hand, clinico-biological parameters predictive of possible recovery of immune-system alterations after HCV eradication are still lacking. Combined pathogenetic and symptomatic therapies may be able to improve a single clinical manifestation of HCV syndrome; the clinical usefulness of these treatments has been largely reported in cryoglobulinemic patients, particularly for patients treated with anti-CD20 monoclonal antibody therapy[130,132-134]. In all cases, etiological, pathogenetic, and symptomatic treatments, in sequence or in combination, should be tailored on the single patient after a careful clinical evaluation[4,31,131-134]. Finally, long-term clinical monitoring of HCV-infected patients, including those with mild or asymptomatic clinical variants, is mandatory for a timely diagnosis and treatment of the

most severe complications.

CONCLUSION

The hepato- and lymphotropism are the distinctive biological features of HCV responsible for a wide symptom complex including both hepatic and systemic autoimmune and neoplastic diseases. The strength of association largely varies among potentially HCV-driven disorders, a well as for a specific disease among patients' series from different countries. Besides liver involvement, HCV represents the etiological agent of the majority patients with MCs, and may be implicated in a significant proportion of other autoimmune disorders and B-NHL.

A putative HCV-associated disease *per se* may constitute a clinical syndrome, characterized by a spectrum of clinico-serological variants; these latter can be regarded as the resulting phenotypes of a multifactorial and multistep process secondary to a variable combination of genetic, environmental, and infectious factors. In this context, HCV can be considered as one of possible causative agents producing distinct autoimmune or neoplastic disease subsets. Considering the frequent clinical overlap and the presence of multiple serum autoantibodies, it is frequent very difficult to make the differential diagnosis among "idiopathic" and HCV-associate autoimmune disorders.

The HCV syndrome is a multifaceted condition that encompasses the complex of HCV-related disorders. The syndrome may be considered as a continuum; this hypothesis is frequently suggested by the clinical history of individual patients that may develop most of HCV-driven immunological/neoplastic disorders over their clinical follow-up.

Future investigations should better define the boundaries of HCV syndrome, along with the actual etiopathogenetic role of this virus in different disorders and the involved, often unknown, co-factors, the effects of HCV eradication, and the correct therapeutic strategies for different HCV-related clinical symptoms. Finally, considering the ongoing variations of the epidemiology of HCV infection and other possible co-factors, as well as the effects of the gradual improvement of therapeutical armamentarium, it is supposable that the spectrum of HCV syndrome, *i.e.*, prevalence, clinical characteristics, and prognosis of different symptoms, might change over the time. The timely description of this evolving framework should be another intriguing issue of clinical investigations in the next future.

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REVIEW

Risk of hepatitis B virus reactivation in rheumatoid arthritis patients undergoing biologic treatment: Extending perspective from old to newer drugs

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Abstract

Hepatitis B virus (HBV) reactivation in rheumatoid arthritis (RA) patients undergoing biological therapy is not infrequent. This condition can occur in patients with chronic hepatitis B as well as in patients with resolved HBV infection. Current recommendations are mainly focused on prevention and management strategies of viral reactivation under tumor necrosis factor-α inhibitors or chimeric monoclonal antibody rituximab. In recent years, growing data concerning HBV reactivation in RA patients treated with newer biological drugs like tocilizumab and abatacept have cumulated. In this review, epidemiology, pathogenesis and natural history of HBV infection have been revised first, mainly focusing on the role that specific therapeutic targets of current biotechnological drugs play in HBV pathobiology; finally we have summarized current evidences from scientific literature, including either observational studies and case reports as well, concerning HBV reactivation under different classes of biological drugs in RA patients. Taking all these evidences into account, some practical guidelines for screening, vaccination, prophylaxis and treatment of HBV reactivation have been proposed.

Key words: Rheumatoid arthritis; Hepatitis B virus; Biologics; Anti-TNF; Rituximab; Tocilizumab; Abatacept

Core tip: Hepatitis B virus (HBV) infection represents a major issue in patients with rheumatoid arthritis (RA) undergoing biological disease-modifying anti-rheumatic drugs (bDMARDs). While several observational studies and trials deal with the risk of HBV reactivation under anti-TNF agents, there is limited experience with newer drugs as tocilizumab (TCZ) and abatacept (ABA). In this paper, literature concerning the risk of HBV reactivation in RA patients undergoing different classes of bDMARDs, including ABA and TCZ, has been revised. Finally, some evidence-based practical suggestions for the management of this condition are proposed.

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INTRODUCTION

Over the last decade, the introduction of biologic drugs for the treatment of rheumatoid arthritis (RA) led to significant improvements in clinical and radiographic disease outcomes. From available scientific evidences, low disease activity or even clinical remission resulted more frequently achievable with biologic and conventional synthetic disease-modifying antirheumatic drugs in combination (bDMARDs and csDMARDs, respectively) rather than with therapeutic regimens including csDMARDs only[1]. According to the current recommendations for RA management[2-6], bDMARDs may be initiated in patients who have failed to respond to at least one csDMARD, including methotrexate (if not contraindicated). Table 1 summarizes the principal bDMARDs currently licensed for RA treatment. The first generation of biological drugs targeting specific effector molecules in RA pathogenesis is represented by tumor necrosis factor alpha (TNF-α) inhibitors (TNFI). Five agents of such class of bDMARDs are currently approved for RA: three monoclonal antibodies [infliximab (IFX), adalimumab (ADA), and golimumab (GOL)], one modified antibody Fab fragment [certolizumab pegol (CZP)] and one soluble receptor [etanercept (ETN)]. Moreover, B-cell targeted therapy with rituximab (RTX), a chimeric monoclonal antibody against CD20, a B-lymphocyte antigen that specifically depletes mature B-cells, is another well-established therapeutic option for RA treatment. Recently other therapeutic agents targeting molecules involved in RA pathogenesis have been approved: tocilizumab (TCZ), a humanized monoclonal antibody against interleukin 6 (IL-6) receptor which selectively blocks IL-6 biological effects, and abatacept (ABA), a soluble CTLA4-Fc fusion protein, that, by mimicking the cytotoxic T-lymphocyte antigen 4 (CTLA4), a negative regulator of T-cell costimulation, mainly prevents the activation of the T-cell compartment. Either anti-TNF, anti-IL-6 or costimulation blockade agents can be prescribed as first-line biologic therapies, while RTX is usually indicated as a second-line treatment option, that means for patients who failed and/or are intolerant to a first bDMARD, even if under certain circumstances, it can be considered as a first-line drug[4]. The combination therapy of a biologic agent with methotrexate (or another equivalent csDMARD such as leflunomide[7,8]) is generally more effective than monotherapy, as shown for TNFI[9], RTX[10] and TCZ[11]. Nevertheless, bDMARD monotherapy might be considered in case of contraindications for csDMARDs[12]. Biologic therapy is more effective than csDMARDs in achieving clinical response (measured by validated composite indexes) and in preventing radiographic damage as well. For instance, randomised controlled trials on TNFI show a remission rate of 50% *vs* 28% with ETN + methotrexate over methotrexate alone respectively[13]; similarly, ADA + methotrexate resulted in a remission rate of 34%, against 17% for methotrexate alone[14]. Furthermore, most evidences support the superiority of bDMARDs over csDMARDs in preventing structural and functional deterioration, even for long-term periods[14-17]. Compared to TNFI, newer bDMARDs, like TCZ and ABA, showed similar efficacy profiles, even if slight differences have been reported in specific contexts[18,19]. Despite their recognised therapeutic effects in most treated patients, biologic therapy with TNFI might result inadequate in approximately 30% of cases, thus carrying on the risk of further joint damage[20]. After TNFI failure, no particular bDMARD seems to be better than the others as second line choice[18-21].

All these drugs carry a potential for serious infective complications, especially involving the reactivation of latent infections with intracellular pathogens. This is particularly the case for latent tuberculosis, or latent infection with viruses such as Herpes Simplex Virus, hepatitis C and hepatitis B virus (HBV). The prevalence of HBV infection in patients affected by rheumatic diseases is similar to the one of the general population. In the last decade, increasing attention has been paid to the possibility of HBV reactivation in RA patients undergoing treatment with bDMARDs. Growing evidences, coming first from oncology, then from gastroenterology and rheumatology fields, suggest that HBV reactivation can occur not only in hepatitis B surface antigen (HBsAg) carriers, but also in HBsAg negative individuals presenting liver HBV-DNA with detectable or undetectable serum HBV-DNA [occult HBV infection (OBI)], during or eventually after immunosuppression. Among the available bDMARDs approved for RA, the risk of HBV reactivation under TNFI has been well-described in several observational studies. Moreover, the evidences about the well-established risk of HBV reactivation in hematological patients treated with RTX, have been replicated in several reports on RA patients. In contrast, for this topic, observational data for newer bDMARDs with different targets like ABA and TCZ are still lacking, with only a few reports published. Consequently the existing practical guidelines for the prevention and management of viral reactivation are mostly targeted to the "older" rather than to the newer bDMARDs.

In this paper, literature concerning the risk of HBV reactivation in RA patients undergoing different classes of the most commonly prescribed bDMARDs has been revised. Finally, some evidence-based practical suggestions for the prevention and the treatment of this condition are proposed.

Table 1 Four classes of immunotherapies licensed to treat rheumatoid arthritis

Class	Mode of action	Drug	Mechanism of action	Route of administration	Therapeutic regimen	Half-life	Major approved indications (FDA)
TNF inhibitors	TNF-α neutralization	Adalimumab (fully human monoclonal IgG1 antibody)	Binding to TNF	SC	40 mg every 2 wk	13 d	RA, JIA, PsA, AS, CD, UC, PP, non radiographic axial SpA
		Golimumab (fully human monoclonal IgG1 antibody)	Binding to soluble and membrane bound TNF	SC	50 mg every 4 wk	13 d	RA, PsA, AS, UC
		Certolizumab (pegilated FabI fragment of a human monoclonal antibody)	Binding to TNF	SC	200 mg every 2 wk or 400 mg monthly	14 d	RA, CD, PsA, AS, non-radiographic axial SpA
		Etanercept (p75 TNF receptor - IgG1 Fc fusion protein	Works as a decoy receptor. It binds to soluble TNF, blocking the binding to its receptor	SC	50 mg weekly or 25 mg twice a week	3-6 d	RA, PsA, AS, poliarticular JIA, PP
		Infliximab (chimeric mouse-human monoclonal IgG1 antibody)	Binding to soluble and membrane bound TNF	IV	3 mg/kg (up to 7.5 mg/kg if not effective) every 8 wk after loading at 0, 2 and 6 wk	9 d	RA, PsA, AS, CD, pediatric CD, UC, pediatric UC, PP
B-cell depletion	B-cell lysis	Rituximab (chimeric mouse-human monoclonal IgG1 antibody)	Binding to CD20 and depletion of $CD20^+$ B cells	IV	Two infusions of 1000 mg 2 wk apart. This can be repeated every 6 mo if symptoms return. Infusions are preceded by IV methylprednisolone to reduce the incidence of infusion reactions	18 d (range 5-76)	BNHL, CLL, RA, GPA, MPA
T-cell inhibition	T-cell costimulation blockade	Abatacept (extracellular domain of CTLA4-IgG1 Fc recumbinant human fusion protein)	Binding to CD80/ CD86, blocking T-cell co-stimulation	IV	500-750-1000 mg infusions (for body weight < 60, between 60 and 100 or > 100 kg) every 4 wk following three loading infusions at 0, 2 and 4 wk	13 d (range 8-25)	RA, poliarticular JIA
				SC	125 mg weekly		
IL-6 inhibition	IL-6 receptor blockade	Tocilizumab (humanized monoclonal IgG1 antibody)	Binding to soluble and membrane bound IL-6 receptor	IV	8 mg/kg every 4 wk	10-13 d	RA, poliarticular JIA, sistemic JIA

FDA: Food and Drug Administration; CTLA-4: Cytotoxic T-lymphocyte associated-antigen 4; IL-6: Interleukin 6; IV: Intraveneous injection; PEG: Polyethylene glycol; SC: Subcutaneous; TNF: Tumour necrosis factor; JIA: Juvenile Idiopathic Arthritis; PsA: Psoriatic arthritis; AS: Ankylosing spondylitis; SpA: Spondiloarthritis; CD: Chron's disease; UC: Ulcerative colitis; PP: Plaque psoriasis; BNHL: B-cell non-Hodgkin lymphoma; CLL: Chronic lymphocytic leukemia; GPA: Granulomatosis with polyangiitis (Wegener's disease); MPA: Microscopic polyangiitis.

HBV: EPIDEMIOLOGY AND NATURAL HISTORY

Chronic infection with HBV is still a significant global health problem: about 2 billion people worldwide have been infected with HBV and approximately 5% of the world population is affected with chronic HBV infection, which is the leading cause of HBV-related complications such as chronic hepatitis, HBV-related cirrhosis, and hepatocellular carcinoma (HCC), accounting for 500000 to 700000 deaths per year[22]. The geographic distribution of HBV infection can be described as follows: 88% of global population lives in areas of intermediate ($HBsAg^+$ prevalence 2%-7%) or high endemicity (> 7%) corresponding to African and East-Asian territories where most infections occur from vertical transmission; whereas the remaining 12% lives in low endemicity areas ($HBsAg^+$ prevalence < 2%), roughly corresponding to North Europe and United States, where HBV infection usually primarily occurs in adulthood[23]. In western countries the incidence of HBV infection has been furtherly diminished by widespread vaccination programs since the 1980s[24]. HBV is a partially double-stranded DNA

Table 2 Nomenclatures and definitions used in hepatitis B virus infection

Markers	Chronic inactive carrier	HBeAg positive CHB	HBeAg negative CHB	"Resolved hepatitis B"
HBsAg	+	+	+	-
HBeAg	-	+	-	-
Anti-HBe	+	-	+/-	+/-
Anti-HBs	+	-	+/-	+/-
Anti-HBc	+	+/-	+	+/-
ALT	-	+/-	+	-
Serum HBV-DNA	Undetectable/< 2000 IU/mL	Persistent/intermittent ↑ (> 20000 IU/mL)	Persistent/intermittent ↑ (> 2000 UI/mL)	Detectable or undetectable
Liver injury	+/-	Moderate/severe CHB	Minimal to severe CHB	-
Necroinflammation	No (> 90%)	Yes (> 90%)	No (> 90%)	No

CHB: Chronic hepatitis B; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; anti-HBe: Antibodies to hepatitis B e antigen; anti-HBs: Antibodies to hepatitis B surface antigen; anti-HBc: Antibodies to hepatitis B core antigen; ALT: Alanine aminotransferase.

virus transmitted by percutaneous or permucosal exposure to infected body fluids and perinatally from mother to infant. The virus genome is able to convert into a covalently closed circular form (cccDNA) that can persist lifelong into the nuclei of infected cells, leading to the likelihood of viral reactivation during or after immunosuppression[25].

Natural history of HBV infection is a dynamic process and runs throughout 4 distinct phases[26].

The immune tolerant phase is characterized by positive serum hepatitis B e antigen (HBeAg), serum HBV-DNA >10^5 copies/mL, and normal aminotransferase levels. In this phase cellular response is absent, with consequent minimal liver inflammation. After vertical infection this phase may last > 40 years (possibly leading to HBV genome integration, with decreased likelihood of viral eradication), while it is usually short-term in adults.

The HBeAg-positive immunoactive phase is characterized by a weak cellular response against infected hepatocytes, expressed by elevated aminotransferase levels (hepatitis), HBV DNA levels > 10^5 or between 10^4-10^5 copies/mL, and histological evidence of liver inflammation. As serum viral load falls, *HBe seroconversion* (from HBeAg to anti-HBe) can occur at a rate of 4%-10% per year.

Most people after HBeAg seroconversion enter the so-called HBV inactive phase, associated with a more effective cytotoxic T-cell response leading to normalization of aminotransferases and undetectable or < 2000 UI/mL serum viral load (inactive carrier state). During this phase, which may last lifelong, liver inflammation improves along with a lower risk of serious complications. Nevertheless, an estimated 20% of patients will reactivate, possibly leading to HBV-related complications: this condition, known as *HBeAg-positive* or HBeAg-negative chronic hepatitis B (CHB), is defined as HBsAg serum positivity for > 6 mo, along with serum HBV-DNA between 2000 and 20000 IU/mL or > 20000 IU/mL as well as elevated aminotransferases[26,27].

A proportion of patients develops HBsAg clearance at a rate of 0.5%-0.8% per year[28] (resolution phase). Generally in these patients only anti-hepatitis B core antigen (anti-HBc) antibodies are detectable, because protective anti-HBs antibodies can be lost in time. The concept of occult HBV infection (OBI, defined with detectable liver HBV-DNA with serum undetectable or < 200 IU/mL HBV-DNA in $HBsAg^-$ individuals) has been recently introduced to describe the underlying risk of HBV reactivation under immunosuppressive therapy[29-31]. In this phase the risk of development of cirrhosis is minimal, whereas the risk of HCC is reduced but still significant[32]. Definitions of HBV status in virological, biochemical and serological terms are summarized in Table 2.

CURRENT MANAGEMENT OF CHRONIC HEPATITIS B

Antiviral therapy is generally recommended for CHB patients who have HBV DNA levels > 2000 IU/mL, serum aminotransferases above the upper limit of normal (ULN) and moderate to severe active liver necroinflammation and/or at least moderate fibrosis[33]. Patients with alanine aminotransferase (ALT) > 2 times ULN and serum HBV-DNA > 20000 IU/mL may start treatment even without liver biopsy. The ideal endpoints of antiviral therapies are long-term suppression of viral replication, sustained HBeAg seroconversion for $HBeAg^+$ individuals, and HBsAg clearance. Long-term viral suppression can now be achieved in > 95% cases with oral nucleic acid analogues (NAs), although HBsAg loss remains a hard to achieve target (< 10%). Drugs available for the treatment of CHB include interferon-α (IFN), pegylated-INF-α2a (PEG-IFN) and six NAs, that can be classified into nucleosides (lamivudine, telbivudine, emtricitabine, entecavir) and nucleotides (adefovir and tenofovir) analogues. While IFN-based regimens, despite a good efficacy profile, lead to frequent side effects and contraindications[33,34]; oral NAs show a better safety profile. Though inexpensive, the first generation agents (such as lamivudine) are associated with a high rate of viral resistance (about 70% after 5 years), which limit their use in clinical practice. Entecavir and tenofovir are

potent HBV inhibitors with a high barrier to resistance, and they are currently recommended as first-line monotherapies. These agents have to be given either indefinitely (HBeAg$^-$ CHB) or for 12 mo following HBeAg seroconversion in HBeAg$^+$ CHB[35].

HBV REACTIVATION: DEFINITION AND RISK FACTORS

HBV reactivation in patients with chronic inactive/resolved HBV infection undergoing immunosuppressive treatment is defined as the combination of two findings: a > 1 log10 IU/mL increase in serum HBV-DNA level or the detection of previously undetectable HBV-DNA; and serum ALT elevation > 2-3 times the ULN[36,37]. The increase in liver function tests (hepatitis) usually follows viral reactivation[36]. Reactivation clinically ranges from a subclinical and transient event to severe hepatitis; in a limited proportion of patients acute liver failure and death have been reported, with a higher risk for patients affected by CHB and advanced histological injury[36].

The host serological status and HBV-DNA levels are major risk factors for HBV reactivation: HBsAg carriers are at greater risk, and the risk increases with HBV-DNA load[36,37]. For HBsAg negative patients, the risk of reactivation is considerably lower in individuals with both anti-HBc and anti-HBs antibodies[38]. The risk may be higher in the event of concomitant methotrexate therapy[38]. A variety of immunosuppressive drugs may lead to HBV reactivation: corticosteroids, cytotoxic agents, cs- and bDMARDs. In most cases reactivation occurs after treatment withdrawal, as a result of the restored immunity[39]. The liver damage due to HBV reactivation is a two-stage process: initially during intense immunosuppression an enhanced viral replication occurs as reflected by increased serum and liver HBV-DNA; then, during the subsequent immune restoration after treatment withdrawal a rapid immune-mediated destruction of HBV-infected hepatocytes overcomes, clinically manifested with hepatitis, hepatic failure and even death. In a recent retrospective study of 35 cases of HBV reactivation in patients undergoing immunosuppressive treatment for immune-mediated inflammatory disease, reactivation appeared a median of 35 wk (2-397) after treatment start, with earlier occurrence in RTX-treated and in HBsAg$^+$/HBV-DNA$^+$ patients[38]. However, earlier appearance was not associated with an increased clinical severity. Factors significantly associated with HBV-related serious complications (death and/or fulminant hepatitis) were identified in Asian ethnicity, delay from immunosuppressive therapy initiation to HBV reactivation diagnosis, HBV-DNA load and aminotransferases at HBV diagnosis. The use of specific TNFI/antiviral molecules did not differ between patient who experienced or not serious events, but patients with a poor outcome tended to receive IFX, ADA or 1st-generations NAs as compared with those with a favorable outcome (P = 0.07 and P = 0.03)[38].

ROLE OF PRO-INFLAMMATORY CYTOKINES IN HBV INFECTION

RA is a chronic inflammatory disease affecting 1% of adult population that predominantly involves the synovial membrane of diarthrodial joints. The disease is characterized by the activation of resident synovial macrophages in association with a massive synovial infiltration of lymphocytes and neutrophils, and by the local development of an inflammatory milieu, which in turn promotes the proliferation of synoviocytes and fibroblasts, and neoangiogenesis: all this leads to the production of an aberrant, hyperplastic tissue, the so-called rheumatoid pannus, and to the differentiation and activation of chondrocytes and osteoclasts and subsequent cartilage and bone destruction. Apart from macrophages and other innate immunity "effector" cell types (dendritic cells, neutrophils, synoviocytes, osteoblasts, osteoclasts, and chondrocytes), three major pathways of RA immunity response have become recognized as pivotal: B-cells, T-cells, and a wide range of inflammatory cytokines that, acting as an intricate and redundant network both systemically and locally, shift the balance towards a proinflammatory state[40]. Synovitis is ultimately driven by a number of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1. TNF-α is overexpressed in the synovial fluid of patients with RA. Moreover, TNF-α transgenic mice spontaneously develop arthritis[41]. IL-6, a typical cytokine featuring redundancy and pleiotropic activity, also plays a key role in the development of RA[42], promoting an imbalance between Th17 and regulatory T (Treg) cells and the production of autoantibodies, such as rheumatoid factor (RF) and anticitrullinated peptide antibodies (ACPA). IL-6 also promotes synovial inflammation and cartilage and bone destruction and exerts systemic effects leading to cardiovascular, psychological, and skeletal disorders.

RA was historically considered a T-cell driven disease, although adaptive humoral immunity also plays a pivotal role in its pathogenesis, as shown by the production of RF and ACPA, that are considered to be the serological hallmarks of RA. It has long been recognised that activated CD4 and CD8$^+$ T-cell subsets, B-cells, plasmablasts and plasma cells are abundant in rheumatoid synovial tissue. A substantial proportion of patients show ectopic germinal centres that may support B cell maturation and class switching and thereby promote autoantibody production[43].

HBV infection causes acute and chronic necroinflammatory hepatitis. The pathogenesis of HBV infection, similarly to that of RA, is still largely unknown. Massive hepatic injury occurring during CHB seems to be immune-mediated and depends on HBV-specific cytotoxic T-cells[44]; moreover, an efficient

control of HBV infection requires the synergic actions of both innate and adaptive immunity[45].

Innate immunity induces in HBV infected cells the production of type I interferons and several proinflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-10, some of which are reported to suppress viral replication and/or to exert non-cytolytic viral clearance. In HBV infected patients TNF-α is produced by intrahepatic leukocytes in a HBc-dependent fashion[46]. Several reports show intrahepatic and serum TNF-α elevation in patients with acute or chronic hepatitis B, suggesting a potential role in inhibiting viral replication[47]. The persistence of HBV infection may be associated with $CD8^+$ T-cell loss of the ability to secrete enough TNF-α to kill infected hepatocytes (the so-called "exhausted phenotype")[48]. Genetic polymorphisms leading to lower TNF-α expression associate with an increased risk of HBV chronicization[49]. It has been recently demonstrated that in TNF-α knockout mice and in ETN-treated mice HBV infection persists, with subsequent increase in HBV-specific $CD8^+$ T-cells, serum and liver HBV-DNA, and antigen expression. Thus in this animal model TNFI may be able to suppress HBc-dependent viral clearance effects[49]. However, in patients with CHB this cytokine may contribute to liver injury[50]. Other cytokines, like IL-1, might be involved in the natural history of HBV infection but data concerning their real role and meaning are still most elusive[51,52].

Cellular immunity is critical for the outcome of HBV infection, too: HBV-specific T-cells are involved in the control of viral infection, whilst non-specific NK cells infiltrate the liver leading to hepatocellular injury[53]. In humans, IL-6 in combination with TGF-β and IL-1β drive naive $CD4^+$ T-cell to differentiate into Th17 cells in a HBc-dependent fashion[54]. Th17 cells can produce multiple cytokines that trigger the recruitment and activation of neutrophils leading to massive tissue inflammation[55]. Recent reports showed that in CHB patients antigen non-specific Th17 response is increased and that the peripheral Th17 frequency is associated with the degree of liver damage[56,57]. A recent study revealed that the increased Th17 response in CHB patients correlates with the enhanced IL-6 receptor (IL-6R) expression on $CD4^+$ T-cells, suggesting IL-6R as a potential novel target for CHB immunotherapy[58]. In addition, several studies demonstrated that IL-6 facilitates *in vivo* and *in vitro* HBV infection[59] and might be able to predict the development of HCC in HBV infected patients[60]. However, conclusive data are still lacking and the immunosuppressive action of the anti-IL-6R antibody TCZ might actually play a detrimental role.

CTLA-4, an inhibitory receptor expressed by activated T-cells that acts as a negative regulator of T-cell responses seems to be involved in HBV infection: studies in humans suggest an influence of CTLA4 polymorphisms in HBV clearance, HBV-related carcinogenesis and HBV reactivation after immunosuppression, but the underlying mechanisms are still unclear[61-63].

Recent reports suggest that humoral immunity plays an important role in the immune response to HBV. HBcAg is able to directly activate B-cells to produce specific antibodies in the absence of regulatory T-cells[64]. In patients with $HBeAg^-$ CHB, $CD20^+$ B-cells infiltrate the liver and correlate with histologic necroinflammation and fibrosis[65]. However, immunosuppression and B-cell suppression are associated with viral reactivation. B-cells are thus involved in liver inflammation in HBV infected patients, but whether their influence on disease progression is harmful or beneficial is still unknown.

SAFETY OF BMARDS IN PATIENTS WITH COMORBID HBV INFECTION

While in the last decade an amount of data has been cumulated regarding the effect of TNFI and RTX on RA patients with CHB or even OBI; the likelihood of HBV reactivation in patients undergoing newer bDMARDs TCZ and ABA has been more recently described. Available evidences stratified by bDMARDs about this topic will be exposed in the following sections.

ANTI-TNF AGENTS

It has been 11 years since the first reports of HBV reactivation after TNF-α blockade have been reported[66,67]; since then, a cumulative evidence from case reports and small retrospective or prospective studies has been collected[68-81]. TNFI-induced immunosuppression is able to decrease HBV antigen presentation: the abrupt increase in viral presentation at treatment discontinuation induces an acute cytotoxic response, which can cause extremely severe hepatitis. Table 3 summarizes the observational studies of HBV reactivation in RA patients undergoing TNFI. A distinction has to be made between CHB patients ($HBsAg^+$) and patients with resolved infection ($HBsAg^-$/anti-HBc^+).

Patients with chronic HBV infection ($HBsAg^+$)

In a 2011 review of 87 published cases of TNFI-treated CHB patients, Perez-Alvarez et al reported an HBV reactivation rate of 38%[76], similar to that observed under chemotherapy (50%)[36]. Half of the patients had received IFX, 33% ETN and 17% ADA. In most cases (about 75%), viral reactivation was accompanied by an increase in aminotransferase levels. Among patients with severe reactivation, 5 developed liver failure and 4 died from HBV-related complications. Interestingly, only 25% of the patients who developed HBV reactivation had received antiviral prophylaxis, compared to 62% of those who didn't experience viral reactivation. Apart from potential publication bias, these data indicate an increased risk of HBV

Table 3 Hepatitis virus B reactivation in rheumatoid arthritis patients receiving tumor necrosis factor-α inhibitors: studies in patients with markers of chronic or remote hepatitis virus B infection

Ref.	Study design	Target population	No. of patients	Treatment	Antiviral Prophylaxis	HBsAg$^+$	Anti-HBc$^+$ and anti-HBs$^-$	Anti-HBc$^+$ and anti-HBs$^+$	HBV-DNA$^+$	Anti-HBs$^+$/ anti-HBc$^-$	Reactivation
Carroll *et al*[69]	Case series	Rheumatic pts or pts with IBD with CHB	13	11 treated with IFX, 2 treated with ETN	Lamivudine	13 pts (100%)	-	-	-	-	7 cases with IFX, 2 cases with ETN
Charpin *et al*[70]	Prospective	RA (12)/SpA (9) pts with resolved HBV infection	21	4 treated with IFX, 14 with ETN, 3 with ADA	0 pts	0 pts	0 pts 100% (with 3 pts < 100 UI/mL)		0 pts	-	Mean decrease in anti-HBs titre 8%; no cases of HBV reactivation
Chung *et al*[71]	Retrospective	RA (41), SpA (60), JIA (2) pts	103	TNFα inhibitors	0 pts	8 pts	-	-	0 pts	-	1/8 HBsAg$^+$ (12.5%) after 6 wk of IFX
Caporali *et al*[72]	Prospective	RA (59), SpA (8) pts with resolved HBV infection	67	25 treated with IFX, 23 with ETN, 19 with ADA	0 pts	0 pts	46 pts	28 pts	0 pts	-	No cases of HBV reactivation
Vassillopoulos *et al*[73]	Prospective	RA (66), SpA (64), other (1) patients with actual/remote HBV infection or vaccinated for HBV	131	43 treated with IFX, 64 with ETN, 62 with ADA	14 pts (100% of CHB group): 11 with lamivudine, 2 with entecavir, 1 with telbivudine	14 pts	19 pts		0 pts among CHB group	19 pts (vaccinated)	No cases of HBV reactivation in pts with resolved HBV infection. In vaccinated pts, slight decrease in anti-HBs titres (median 163 > 105 IU/mL, $P = 0.01$). Among CHB pts, 1 (7%) treated with Lamivudine + ETN developed HBV reactivation due to a resistant mutant strain
Mori *et al*[74]	Prospective	RA pts with actual/remote HBV infection	239	9 treated with IFX, 18 with ETN, 2 with ADA, 5 with TCZ, 28 with csDMARDs	2 (100% of HBsAg$^+$ pts) with entecavir	2 pts	60 pts		0 pts	-	2 cases of HBV reactivation in anti-HBc$^+$ pts (3.3%), 1 with csDMARDs and 1 with ADA
Tamori *et al*[75]	Prospective	RA pts with positive anti-HBc	50	22 treated with IFX, 20 with ETN, 2 with ADA	Entecavir	5 pts	45 pts		-	-	2/5 (40%) cases of HBV reactivation among HBsAg$^+$ pts; 1/45 (2%) cases of HBV reactivation among HBcAb$^+$/HBsAg$^-$ pts, not under TNFI
Pérez-Alvarez *et al*[76]	Systematic review	TNFI-treated pts	257	Anti-TNF (not specified)	Not specified	89 pts	168 pts		-	-	HBV reactivation in 35 (39%) pts among HBsAg+ group, fatal in 4 cases. Lower risk if pre-emptive NAs (23% *vs* 62%, $P = 0.003$). Higher risk with IFX *vs* ETN. Nine cases (5%) of HBV reactivation in HBcAb$^+$/HBsAg$^-$ pts, fatal in 1 pt
Lan *et al*[77]	Prospective	RA anti-HBc$^+$ pts	88	40 pts treated with ETN, 48 with ADA	10 HBsAg$^+$ pts treated with lamivudine	18 pts	12 pts	58 pts	0 pts	22 pts (vaccinated)	Among HBsAg+ pts, no cases of HBV reactivation if pre-emptive NAs; mean decrease in HBV-DNA = 153 IU/mL ($P < 0.001$); 5/8 cases of reactivation without antivirals. 1 case of HBV reactivation in the HBsAg-/anti-HBs- group
Lee *et al*[78]	Systematic review	HBsAg$^+$ rheumatic disease-positive pts	122	14 pts treated with IFX, 56 with ETN and 25 with ADA	48 pts (drug not specified)	122 pts	-	-	Not specified	-	15 cases (12.3%) of HBV reactivation, including 1 SpA patient treated with pre-emptive lamivudine
Lee *et al*[79]	Systematic Review	HBsAg$^-$/anti-HBc$^+$ rheumatic disease-positive (RA 327, SpA 121) pts	468	100 pts treated with IFX, 269 with ETN and 95 with ADA	Not specified	0 pts	Not specified	Not specified	Not specified	-	8 cases (1.7%) of HBV reactivation, 7 with ETN and 1 with ADA; satisfactory clinical outcomes with antiviral therapy

Droz *et al*[38]	Retrospective	Pts with immune-mediated inflammatory diseases (RA 14, CTD 7, vasculitis 5, other 9) developing HBV reactivation	35	7 pts treated with TNF-α inhibitors (not specified), 4 with RTX, 1 with ABA, 1 with TCZ, the others with steroids and/or other immunosuppressants	5 pts (drug not specified)	23 pts	12 pts		Not specified	-	Reactivation occurred a median of 35 wk after therapy start. 88.6% were asymptomatic; 25.7% had severe hepatitis. Management were NAs in 91.4% cases and decrease/withdrawal of immunosuppressants in 45.7%. Pooling these data with literature, earlier reactivation for RTX and HBsAg/HBV-DNA$^+$ pts
Ye *et al*[80]	Prospective	Inflammatory arthritis pts (50 RA, 37 SpA)	87	TNFα inhibitors: 56 treated with IFX, 31 with ETN)	4 pts among CHB group , 9 pts among inactive carriers (not specified)	37 (6 HBV-DNA+, 31 HBV-DNA-) pts	50 pts		Not specified	-	2 cases of HBV reactivation among CHB pts not receiving pre-emptive NAs, none in those receiving it. Among inactive HBsAg carriers, 6 cases of reactivation in pts who didn't receive NAs, none in those who did. No cases in HBsAg$^-$ pts
Nakamura *et al*[81]	Retrospective	RA	57	48 treated with TNFα inhibitors (including 9 receiving also TCZ); 7 with TCZ alone and 2 with TCZ and ABA	0 pts	0 pts	11 pts	38 pts	0 pts	8 pts (not vaccinated)	HBV-DNA detected in 3 pts (5.3%), 2 receiving TCZ and 1 receiving ETN, with serum HBV-DNA < 2,1 log copies/mL, and subsequent undetectable HBV-DNA within months

Pts: Patients; CHB: Chronic hepatitis B; IFX: Infliximab; ETN: Etanercept; IBD: Inflammatory bowel disease; SpA: Spondyloenthesoarthropaties (psoriatic arthritis included); csDMARDs: Conventiona Synthetic disease-modifying antirheumatic drugs; JIA: Juvenile idiopathic arthritis; TCZ: Tocilizumab; NAs: Nuclet(s)ide analogues; CTD: Connettive tissue disease; RTX: Rituximab; ABA: Abatacept. Adapted from Lunel-Fabiani *et al*[82] *Joint Bone Spine* 2014.

reactivation in TNFI-treated CHB patients, particularly without proper antiviral prophylaxis. In a 2010 prospective study 14 HBsAg$^+$/anti-HBc$^+$/anti-HBs$^-$ patients, 8 classified as inactive HBsAg carriers and 6 suffering from active CHB, were treated with combination therapy with oral NAs and TNFI: antiviral prophylaxis could prevent viral reactivation in > 90% of cases[73]. Only one active CHB patient developed HBV reactivation (7%) after 3-year prophylactic lamivudine: viral sequencing indicated a resistant mutant strain, as frequently happens during long-term lamivudine administration. Lee *et al*[78] have recently reported 15 HBV reactivation cases (12.3%) in a systematic review of 122 HBsAg$^+$ rheumatic patients undergoing TNFI or csDMARDs. 10/15 patients provided clinical data: 4 cases occurred in RA patients (none treated with TNFI) and no HBV-related complication occurred. Percent of 39.3 patients had received pre-emptive NAs. Similarly, a recent prospective study reported in a cohort of 37 HBsAg$^+$ patients with inflammatory arthritis a reactivation rate of 33.3% without pre-emptive antiviral prophylaxis with no cases in patients treated with NAs. Of the 6 CHB patients (HBV-DNA > 10^5 copies/mL, elevated ALT), both of the 2 patients not receiving oral NAs developed viral reactivation; and the other 4 treated with pre-emptive NAs showed no viral replication. In the 31 inactive HBsAg carriers (HBV-DNA < 10^4 copies/ml, normal ALT), out of the 22 patients not receiving NAs, a transient HBV-DNA increase was detected in 4 cases, with a gradual viremic normalization after therapy withdrawal. No cases were detected in the 9 inactive carriers receiving antiviral prophylaxis[80]. It is currently unknown whether different TNFI affect HBV reactivation to different extents. The risk may be lower with ETN, whose affinity for TNF-α is lower; whilst IFX seems to be more frequently associated with HBV reactivation[70]. A possible explanation could be that IFX administration scheme at intervals of 8 wk may result in a cytokine wash-out leading to a possible "immune-reconstitution" effect[82].

Patients with resolved HBV infection (HBsAg-/anti-HBc$^+$)

Data from oncology and hematology indicate that HBV reactivation occurs in < 5% of patients with resolved HBV infection treated with chemotherapy[83,84]. Similar data among rheumatic patients have cumulatively been published in recent years. In two prospective studies from Southern Europe no cases of HBV reactivation among 88 TNFI-treated patients with remote HBV infection were described[70,72] although none of these patients had detectable HBV-DNA at baseline. Similarly, in a retrospective taiwanese study[84] no reactivation was observed among 58 HBsAg$^-$/anti-HBc$^+$/anti-HBs$^+$ patients under TNFI, although one case of HBV reactivation was recorded between 12 HBsAg$^-$/anti-HBc$^+$/anti-HBs$^-$ patients, involving a patient with detectable baseline HBV-DNA. These data suggest that the risk of HBV reactivation is rather low in anti-HBc$^+$/anti-HBs$^+$ patients, although the likelihood of this event could be higher for HBV-DNA$^+$ and/or for anti-HBs$^-$ patients. A chinese prospective study did

not report any reactivation case in 50 TNFI-treated patients with resolved HBV infection and inflammatory arthritis[77]. Lee *et al*[79] have recently reported in a systematic review of 468 anti-HBc$^+$/HBsAg$^-$ rheumatic patients undergoing TNFI a reactivation rate of 1.7%. Out of 8 cases, 7 were RA patients, and in all cases clinical outcome was satisfactory. In a japanese prospective study of 50 RA HBcAb$^+$ patients treated with csDMARDs and/or TNFI, reactivation occurred in only 1 out of 45 HBsAg$^-$ patients (2.2%), treated with csDMARDs only. Interestingly, in TNFI-treated patients, anti-HBs titres decreased significantly in the middle- and low-titer groups (P = 0.032 and P = 0.007), remaining high in high-titer group (P = 0.875), but did not became negative in any patient[75]. It remains to be clarified whether a long period of TNFI therapy in RA patients induces the disappearance of anti-HBs leading to viral reactivation as well as in hematological field. A recent japanese retrospective study of 244 HBsAg$^-$/anti-HBc$^+$ RA patients, HBV-DNA was detected in three patients (5.3%), only one receiving a TNFI, particularly ETN (out of a total of 48 TNFI-treated patients indicating a 2.1% reactivation rate). Reactivation consisted in a subclinical, transient HBV-DNA elevation, subsequently turning undetectable within months[81]. Even if limited by a possible publication bias, these data indicate that HBV reactivation in patients with serological markers of past HBV infection is a quite rare event and that for these patients no specific prophylaxis is required.

Vaccinated patients

As suggested by a prospective study, a possible decrease in anti-HBs titer in TNFI-treated patients with remote HBV infection can occur[70], but this might not be systematically followed by viral reactivation such as in hematological field[85]. Similarly, in a prospective study of 19 HBV vaccinated patients a slight decrease in anti-HBs levels during TNFI treatment was reported[73], although a comparable decrease was observed in patients treated with methotrexate alone, indicating no specific effect of TNFI on HBV protective immunity.

RTX

A growing scientific literature indicates high rates of HBV reactivation in patients undergoing RTX for hematological diseases not receiving proper antiviral prophylaxis, ranging from 27% to 80% in HBsAg$^+$ patients[39] and from 3% to 25% in HBsAg$^-$/anti-HBc$^+$ patients, with higher risk for anti-HBs$^-$ individuals[86,87]. Reports of HBV reactivation in rheumatic patients treated with RTX are summarized in Table 4. There are limited data regarding the safety of RTX in rheumatic CHB patients[68]; although, the efficacy of pre-emptive NAs (mainly lamivudine) in preventing reactivation in HBsAg$^+$ patients treated with RTX appears to be similar to that observed in patients under chemotherapy, with a reactivation rate of 0%-13%[68]. Three recent reports from Southern Europe have described HBV reactivation in RA patients undergoing RTX, occurring not only in HBsAg carriers but also in two patients with resolved HBV infection. The first patient experienced HBV reactivation 1 mo after RTX administration, even though she had been receiving pre-emptive lamivudine for CHB. Lamivudine was thus switched to tenofovir with aminotransferases and HBV-DNA normalization[88]. In the second report, a RA patients with resolved HBV infection experienced HBV reactivation following 2 years of therapy, 3 mo after the last RTX infusion. RTX was then withdrawn and entecavir initiated, with a gradual amelioration of aminotransferase levels and HBV-DNA normalization[89]. The third report involved a HBsAg$^-$/anti-HBc$^+$/HBV-DNA- RA patient that developed HBV reactivation with subsequent acute hepatitis after 2 years of RTX + methotrexate combination therapy. Discontinuation of immunosuppressive treatment and antiviral therapy with entecavir resulted in the control of HBV infection within a few months[90]. A 2013 prospective study did not report any case of HBV reactivation under RTX neither in a series of 2 HBsAg$^+$/HBV-DNA$^-$ rheumatic patients treated with antiviral prophylaxis nor in a series of 12 HBsAg$^-$/anti-HBc$^+$ rheumatic patients, indicating a quite safe profile[91]. Interestingly, in the 4 patients with history of HBV vaccination, a slight decrease in antibody titers that did not reach statistical significance was noted. However, antibody titers did not fall below protective levels[92]. Droz *et al*[38] reported other 4 cases of HBV reactivation under RTX in patients affected with inflammatory diseases; indicating an earlier timing of viral reactivation for patient receiving anti-CD20 treatment.

NEWER BDMARDS

TCZ

There is limited experience with TCZ, a humanized anti-IL-6 receptor antibody, among HBV-infected RA patients (Table 5). Nagashima *et al*[92] reported that TCZ was administered safely and effectively in a RA patient affected with CHB, not recognised at baseline since serological screening for HBV had not been performed. In another case report, a patient with active RA stopped IFX due to HBV reactivation (pre-treatment viral status unknown). She was then treated with lamivudine until HBV-DNA turned undetectable, and later started TCZ; this resulted in a prompt disease control together with persistently normal serum aminotransferases and undetectable HBV-DNA[93]. A similar report regarding a CHB patient with adult-onset Still's disease indicates an excellent disease control with no viral reactivation by introducing TCZ after complete viral control with entecavir[94]. A recent retrospective study from Nakamura et al described 2 cases of HBV reactivation in RA patients undergoing TCZ without pre-emptive antivirals (out of a total of 18 TCZ-treated HBsAg$^-$/anti-HBc$^+$ patients, with a

Table 4 Case reports and studies of hepatitis B virus reactivation in rheumatoid arthritis patients undergoing rituximab

Ref.	Study design	Patients charachteristics	Therapy	Baseline virological status	Timing of HBV reactivation	Antiviral Therapy	Results	Comments
Pyrpasopoulou *et al*[88]	Case Report	56-year-old female RA pt starting RTX after 3 anti-TNF (ETN, IFX, ADA) and ABA. Previous diagnosis of CHB	RTX Antiviral prophylaxis with lamivudine	$HBsAg^+$, $anti\text{-}HBe^+$	1 mo after first RTX administration	Tenofovir + lamivudine RTX withdrawal	Good clinical, serological and virological response	-
Ghrénassia *et al*[89]	Case Report	78-year-old with RA test+ and erosive RA starting RTX after IFX because of a concomitant diagnosis of lymphoma	RTX monotherapy	$HBsAg^-$/$anti\text{-}HBc^+$	9 mo after RTX starts	Entecavir RTX withdrawal	Good clinical, serological and virological response	-
Gigi *et al*[90]	Case Report	64-year-old female RA pt starting RTX as a first-line bDMARD	RTX + methotrexate	$HBsAg^-$/$anti\text{-}HBc^+$	2 yr after RTX start	Entecavir RTX withdrawal	Good clinical, serological and virological response	-
Mitroulis *et al*[91]	Prospective study	41 rheumatic pts (34 RA; 7 others) who had received ⩾ 1 cicle of RTX and had ⩾ 6 mo of follow-up	17 pts treated with concomitant methotrexate, 35 with concomitant steroids. 2 pts treated with NAs without HBV reactivation	23 pts not HBV exposed; 4 vaccinated pts; 12 $HBsAg^-$/$anti\text{-}HBc^+$ (9 $anti\text{-}HBs^+$); 2 pts $HBsAg^+$ $anti\text{-}HBc^+$. HBV-DNA undetectable in all pts	No cases of viral reactivation observed	-	-	Slight decrease in anti-HBs titres in vaccinated pts ($P = 0.29$), never under protective levels
Droz *et al*[38]	Retrospective (subgroup analysis)	4 pts affected with immune-mediated inflammatory disease treated with RTX	Not specified	Not specified	4 cases of HBV reactivation a median of 35 wk after therapy start (global data)	Not specified	No cases of fulminant hepatitis	Early reactivation with RTX and in $HBsAg^+$/$HBV\text{-}DNA^+$ pts (global data)

Pts: Patients; ETN: Etanercept; IFX: Infliximab; ADA: Adalimumab; ABA: Abatacept; CHB: Chronic hepatitis B; bDMARD: Biological disease-modifying antirheumatic drug; NAs: Nucleot(s)ide analogues; RTX: Rituximab; HBsAg: Hepatitis B surface antigen.

reactivation rate of 11.1%): in both patients HBV-DNA rised but remained below quantitation limits (< 2.1 log copies/mL), and then it spontaneously turned undetectable, along with normal aminotransferases throughout the entire period of follow-up: such data might suggest the possibility of a transient HBV-DNA fluctuation during the first 3-6 mo of TCZ, which doesn't necessarily lead to *de novo* hepatitis and possibly resolves within a few months[81]. This evidence suggests that TCZ, if co-administred with pre-emptive NAs, could be a treatment option for RA HBV carrier patients when disease activity is uncontrolled with csDMARDs. However, periodic monitoring of liver function tests and HBV-DNA is mandatory.

Abatacept

Studies regarding HBV reactivation in rheumatic patients undergoing ABA are summarized in Table 6. A recent monocentric retrospective study has been performed including 8 ABA-treated RA patients showing active CHB ($n = 2$) or inactive HBsAg carriers ($n = 6$)[95]. All patients not receiving antiviral prophylaxis ($n = 4$), which were all inactive HBsAg carriers at baseline, experienced viral reactivation, with a > 10 fold increase of HBV-DNA. None of the NAs-treated patients (3 with entecavir and one with tenofovir) had viral reactivation. Moreover, patients who had been receiving both ABA and NAs showed a statistically significant improvement in DAS28 compared to those without prophylaxis ($P = 0.025$). This is the first study suggesting that the use of ABA in RA/CHB patients appears to be safe and efficacious as long as pre-emptive antiviral prophylaxis is properly given. A case of severe hepatitis due to HBV reactivation has been reported in a RA patient with resolved HBV infection ($HBV\text{-}DNA^-$) previously

Table 5 Case reports of hepatitis B virus reactivation in rheumatoid arthritis patients undergoing tocilizumab

Ref.	Study design	Patient charachteristics	Therapy	Basal virological Status	Timing of HBV reactivation	Antiviral Therapy	Results	Comments
Nagashima *et al*[92]	Case Report	60-year-old female RA pt, RA test and ACPA positive with erosive disease, starting TCZ after IFX and methotrexate	TCZ + steroids	HBsAg-10 yr before TCZ start, basal serological screening not performed	6, 5 years after TCZ start	Entecavir Ongoing TCZ	Subclinical, good serological and virological responses	Diagnosis made by detection of persistently high serological markers in an asymptomatic pt without liver function tests' alterations
Tsuboi *et al*[93]	Case Report	59-year-old female RA pt initially treated with IFX thus withdrawn for HBV reactivation	IFX and then TCZ (after HBV reactivation)	Serological screening not performed before IFX. At 5th IFX infusion $HBsAg^+/HBV\text{-}DNA^+/HBeAg^+$	32 wk after IFX start	Lamivudine Ongoing TCZ	Good clinical, serological and virological response until 2 years after TCZ start	-
Kishida *et al*[94]	Case Report	Adult-onset Still's disease pt affected with CHB	TCZ + ongoing entecavir	$HBsAg^+$	No reactivation observed	-	Good clinical, serological and virological response until end of follow-up	-
Nakamura *et al*[81]	Retrospective	Among 9 RA pts treated with TCZ (7 with TCZ alone and 2 with TCZ and ABA in sequence), 2 cases of HBV reactivation were detected: (a) 75-year old male pt starting TCZ as a first line therapy	TCZ monotherapy	$HBcAb^+$ Undetectable HBV-DNA	4 mo after TCZ start	-	Subclinical, subserological, good virological response	HBV-DNA fluctuated always < 2, 1 log copies/mL throughout 4 mo until it became persistently undetectable, even after switch to ETN (due to lack of efficacy)
		(b) 55-year-old female pt starting TCZ after IFX and ETN	TCZ + methotrexate	$HBcAb^+$ Undetectable HBV-DNA	2 mo after TCZ start	-	Subclinical, subserological, good virological response	HBV-DNA fluctuated always < 2, 1 log copies/mL throughout 5 mo until it became persistently undetectable, even after switch to ADA (due to lack of efficacy)
Droz *et al*[38]	Retrospective (subgroup analysis)	1 pt affected with immune-mediated inflammatory disease treated with TCZ	Not specified	Not specified	median of 35 wk after therapy start (global data)	Not specified	No cases of fulminant hepatitis	Early reactivation in $HBsAg^+/HBV\text{-}DNA^+$ pts (global data)

Pts: Patients; ACPA: Anti-citrullinated peptide antibodies; IFX: Infliximab; CHB: Chronic hepatitis B; ABA: Abatacept; ETN: Etanercept; HBsAg: Hepatitis B surface antigen; TCZ: Tocilizumab; HCV: HCV: Hepatitis B virus.

treated with a TNFI without antiviral prophylaxis and then with ABA, leflunomide and steroids. Nine month after the first ABA infusion, she experienced viral reactivation with liver function tests increase; this led to suspension of biological therapy. As expected, liver function tests continued to increase along with the gradual T-cell immune reconstitution, with a time lag of 2 mo between ABA withdrawal and viral flare. She was then treated with tenofovir with gradual amelioration of aminotransferases and undetectable HBV-DNA[96]. Another case of HBV reactivation in a RA patient with resolved HBV infection (basal HBV-DNA unknown) undergoing ABA was lately reported: 10 mo after treatment start, HBsAg turned positive along with a rise in HBV-DNA; treatment was then stopped and tenofovir was started, with a gradual amelioration of liver function tests and HBV-DNA within a few months[97]. In a recent italian case series of 9 RA patients treated with ABA (8 with resolved HBV infection and 1 chronic inactive carriers), one patient with comorbid HCV chronic infection started lamivudine for liver function tests elevation (< 2-fold ULN) occurring 2 mo after ABA initiation, with a gradual amelioration of lab levels

Table 6 Case reports of hepatitis B virus reactivation in rheumatoid arthritis patients undergoing abatacept

Ref.	Study design	Patients characteristics	Therapy	Basal virological status	Timing of HBV reactivation	Antiviral therapy	Results	Comments
Kim *et al*[95]	Retrospective	8 RA pts affected with CHB	ABA + pre-emptive NAs (4 pts: 3 with entecavir and 1 with tenofovir) ABA without antiviral prophylaxis (4 pts)	$HBsAg^+$ Detectable HBV-DNA in 3/8 pts	Not specified	Not specified	Among pts receiving NAs, no cases of HBv reactivation. Among pts without antiviral prophylaxis, all pts experienced HBV reactivation	Among pts receiving NAs, a statistically significant improve in DAS28-ERS was detected; which was not noted in the control group
Germanidis *et al*[96]	Case Report	72-year-old female RA pt starting ABA after ADA	ABA + leflunomide	$Anti\text{-}HBc^+$/ $HBsAg^-$/$anti\text{-}HBs^+$/$anti\text{-}HBe^+$	6 mo after ABA start	ABA and leflunomide withdrawal Consequent anviral treatment with tenofovir (12 about 1 yr later)	Good clinical, serological and virological response to tenofovir	2 mo time lag between ABA withdrawal and liver tests flare, suggesting that HBV reactivation evolved in parallel with T cell immune reconstitution
Fanouriakis *et al*[97]	Case report	68-year-old female RA pt with erosive disease	ABA + methotrexate	$HBsAg^-$/$anti\text{-}HBc^+$ Basal HBV^- DNA unknown	10 mo after ABA start	Tenofovir ABA withdrawal	Good clinical, serological and virological response	-
De Nard *et al*[98]	Case series	9 RA pts treated with ABA	ABA 8/9 pts treated with concomitant methotrexate Lamivudine in 2 pts (1 $HBsAg^+$ and 1 $HBsAg^-$) from baseline, and in 1 pt with comorbid HCV infection after ABA start	8 $HBsAg^-$/ $anti\text{-}HBc^+$ 1 $HBsAg^+$/ $HBV\text{-}DNA^-$	1 pt with comorbid HCV infection experienced aminotransferases elevation (< 2 x ULN) 2 mo after ABA start 1 pt with resolved HBV infection not receiving NAs developed HBV-DNA positivation 12 mo after ABA start	Lamivudine Ongoing ABA Ongoing ABA No prophylaxis	Subclinical, gradual amelioration of liver function tests, persistently undetectable HBV-DNA ConsecutiveHBV-DNA fluctuations; no flares in liver function tests even after entecavir initiation at 24 mo while switching to ADA (unpublished data)	No cases of HBV reactivation among pts receiving pre-emptive NAs
Droz *et al*[38]	Retrospective (subgroup analysis)	1 pt affected with immune-mediated inflammatory disease treated with ABA	Not specified	Not specified	median of 35 wk after therapy start (global data)	Not specified	No cases of fulminant hepatitis (global data)	Early reactivation in $HBsAg^+$/$HBV\text{-}DNA^+$ pts (global data)

Pts: Patients; CHB: Chronic hepatitis B; NAs: Nucleot(s)ide analogues; ADA: Adalimumab; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis B virus.

along with persistently undetectable viral load. Other 2 patients (1 chronic inactive carrier and 1 with resolved infection) underwent lamivudine before ABA with no HBV-related adverse event, whilst among the other 6 HBsAg- patients not receiving antiviral prophylaxis only 1 developed HBV-DNA positivity (85 UI/mL) without aminotransferase elevation at 12 mo[98], with subsequent fluctuations of viral load, turning initially negative at 18 mo and then rising again at 24 mo (290 UI/mL) without liver function tests alterations and without specific antiviral treatment. After infectivologist consult the patient thus started entecavir, concomitantly with switch to adalimumab for arthritis flare, with undetectable HBV-DNA 3 mo after TNFI start (unpublished data).

CONCERNS ABOUT HBV VACCINATION

Hepatitis B vaccine (HBVv) is a recombinant DNA vaccine that provides protection against HBV infection and its complications, including cirrhosis and HCC[99]. As more than 1 billion doses of vaccine have been used

since 1982, it is considered to be safe[100]. Vaccination scheme usually involves 3 booster injections and is currently indicated for high risk patients: newborns, health care workers and adults presenting conditions of immunosuppression or behavioral risk factors (multiple sexual partners, drug abusers, travelers to endemic areas)[100].

Some concerns have been raised on the possible development of autoimmune adverse events after HBVv: cases of multiple sclerosis, Guillain-Barre syndrome, idiopathic thrombocytopenic purpura, optic neuritis, glomerulonephritis, transverse myelitis, vasculitis, systemic lupus erythematosus diagnosis or flare-up[101,102] and even the so-called autoimmune/inflammatory syndrome induced by adjuvants[103] have been reported[104]. Conflicting data about a possible association with HBVv and arthritis (RA or other) diagnosis or flare-up have been reported in 32 cases and 3 controlled studies. In one study HBVv, compared to rubella vaccination, showed an increased risk of chronic arthritis incidence (attributable risk 5.1-9.0)[105]; whilst two studies comparing the risk of RA flare following HBVv with RA controls[106] or with RA prevalence in the same community[104] did not find a significant association between HBVv and the risk of arthritis. Interestingly, in one of these studies a decreased efficacy of HBVv in RA patients was noticed[106]. These results do not provide solid scientific data to support the existence of a causal link between arthritis and HBVv: the most likely explanation still remains the coincidental temporal association. However, in a panorama of decreasing worldwide incidence of hepatitis B, mainly due to immunization programs, a recent "anti-vax" misconception is concentrating public attention and the media on vaccine adverse events rather than on prevention and control, which may lead to lower vaccine coverage and subsequent community wide outbreaks.

Routine vaccination before biotherapy initiation in patients with negative screening tests seems to be obviously appropriate; nevertheless, in patients affected with autoimmune diseases some precautions should be taken[78]: the risk of infection must be weighed against the theoretical risk of vaccine side effects, according to each patient's profile (age, family history, risk factors, *etc.*)[107]. On the other hand, HBVv administration may delay biotherapy initiation, and the immune response may be blunted in patients affected with chronic arthritis taking immunosuppressants[107]: if we consider that a single injection is insufficient to induce protective immunization, if the following injections are administered under TNFI (most of all IFX and to a lesser degree ETN), they often fail to generate an immune response[108]. Since the risk of contracting HBV during adulthood is low, except for high-risk situation, HBVv might be postponed. In contrast, in high risk patients, particularly the younger ones, HBVv should be administered before treatment initiation[109]. In patients with resolved HBV infection and insufficient antibody protection (anti-HBc$^+$/anti-HBs$^-$), a booster injection might be given to strengthen immunization[109] and to induce the production of protective anti-HBs, whose titer should be then measured.

RECOMMENDATIONS FOR CLINICAL MANAGEMENT OF RA PATIENTS WITH CHRONIC/RESOLVED HBV INFECTION UNDERGOING BDMARDS

Since proper prospective studies comparing different screening and treatment options for HBV-infected RA patients starting bDMARDs therapy are lacking, only expert-opinion-based suggestions can be made[2-4,6,33,38,68,82].

Screening

As stated by all the recent recommendations, all patients starting bDMARDs should be screened for HBV infection with HBsAg, anti-HBc and anti-HBs antibodies, as well as HBV-DNA load and liver function tests in patients found to be positive[2-4,6,68,82]. Considering the cost of chronic bDMARD therapy and the potential for serious HBV-related complications, such screening results to be cost-effective. Recent studies have shown that only 69% of US rheumatologists routinely performs universal HBV screening before biologic therapy[110]. When the results indicate active/remote HBV infection, a full battery of liver tests must be obtained, and it is appropriate to consult an hepatologist to evaluate whether antiviral treatment or prophylaxis is indicated before starting bDMARDs therapy[68].

Vaccination

As stated before, a careful risk-benefit assessment should be made concerning HBVv administration before bDMARD treatment. According to current recommendations[111], HBVv is only recommended in patients "at risk" (*e.g.,* travel to or residence in endemic countries, medical profession, infected family member; only if protective antibodies against HBV are absent. Grade of evidence Ⅱ-Ⅲ; strength of recommendation B-D) and should ideally be administered during stable disease. Nevertheless, vaccination could also be considered in selected patients with active disease for whom the benefits of HBVv outweight the risks[99]. HBVv can be administered during the use of csDMARDs and TNFI but should ideally be administered before starting RTX (or, when treatment with RTX is already ongoing, at least 6 mo after the start but 4 wk before the next course[111]). Further studies are needed to establish indication and proper time of immunization in RA patients undergoing TCZ or ABA.

Antiviral pre-emptive therapy and follow-up

Given the risk associated with IFN-based schemes in patients with autoimmune diseases, only NAs are recommended for these patients. To date, no consensus or recommendations are available regarding the specific bDMARD that should be preferentially chosen according to HBV infection profile. Pre-emptive treatment must be started (at least 1 mo) before the biotherapy, and must be prolonged (as well as virological monitoring) until at least 6 mo after its discontinuation. The efficacy of pre-emptive lamivudine has been retrospectively assessed in 88 RA patients treated with TNFI showing markers of recent/remote HBV infection[77]: for $HBsAg^+$ patients, 5 of 8 untreated patients underwent HBV reactivation versus none of 10 treated patients. Regarding the 70 $HBsAg^-$/anti-HBc^+ patients, without antiviral prophylaxis, a single case of HBV reactivation was reported in a patient with OBI ($HBV\text{-}DNA^+$, anti-HBs^-). Nevertheless, newer NAs such as entecavir and tenofovir are considered the first choice for patients at high risk of HBV reactivation, due to lower risk of drug resistance. However, comparative data between lamivudine and newer NAs for pre-emptive therapy of HBV reactivation are lacking. In addition, the potential side effects of entecavir and tenofovir must be weighed against the excellent safety profile of lamivudine. The current recommendations are summarized below.

For $HBsAg^+$ patients, antiviral therapy should be initiated before any bDMARDs therapy[68]. The choice of the appropriate NA mainly depends on the duration of the scheduled therapy and on HBV serology status (CHB *vs* inactive carrier). In general, patients receiving long-term immunosuppression (> 12 mo) and/or suffering from CHB are placed on the newer NAs[34]. For patients with HBV-DNA < 2.000 IU/mL who are scheduled for short-term immunosuppression (< 12 mo) treatment with lamivudine should be considered. Biotherapy should not be started until the HBV-DNA levels become undetectable, and thus frequent (after 1 mo, then every 3-6 mo) monitoring of HBV-DNA and liver function tests is mandatory[35,81]. This recommendation might safely be applied to patients undergoing TCZ and ABA.

For $HBsAg^-$/anti-HBc^+ patients undergoing bDMARDs, additional risk factors should be taken into account: HBV-DNA positivity, presence of anti-HBs (which should protect against reactivation, although this effect is controvertial, mostly with RTX), and the degree of immunosuppression induced by different bDMARDs, particularly for RTX. Baseline screening for HBV-DNA is always recommended, and if found to be positive, it is appropriate to start newer NAs[34]. In patients with resolved HBV infection (undetectable HBV-DNA) undergoing TNFI, simple monitoring without pre-emptive treatment is recommended (particularly if also anti-HBs+): liver function tests, HBsAg and HBV-DNA should be assessed 1 mo after treatment start, and then every 3-6 mo. Pre-emptive treatment should also be strongly considered, regardless of HBV-DNA status, for patients undergoing RTX. Moreover, although there are few data concerning newer bDMARDs, we suggest that also for ABA-treated patients, regardless of HBV-DNA status, antiviral prophylaxis should be taken into account; whilst TCZ is relatively safe. In these cases, regular monitoring of aminotransferases, HBsAg and/or HBV-DNA every 3-6 mo of follow-up is mandatory.

When HBV reactivation is diagnosed, as soon as HBV-DNA becomes detectable and before hepatocellular damage starts, antiviral therapy must be promptly initiated and immunosuppressive therapy must be discontinued. If HBV-DNA becomes positive during lamivudine therapy, the patient should be switched to tenofovir, since cross-resistance has been reported between lamivudine and entecavir.

CONCLUSION

HBV infection is a relevant worldwide condition, affecting also rheumatologic patients. Immunosuppressive treatment with bDMARDs might interfere with HBV natural history, leading to an increasing risk of viral reactivation, with consequent liver damage, up to possible fulminant hepatitis and death. Rheumatologists should be well-aware about such risk in RA patients undergoing different classes of bDMARDs, in order to define drug by drug proper preventive and therapeutic strategies. More robust evidences about newer bDMARDs (TCZ and ABA) are still needed in order to better define their specific drug-related risk in HBV infected RA patients and to draw univocal recommendations. An integrated management of this subset of patients should be encouraged between rheumatologists and virologists.

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REVIEW

Diagnosis and treatment of hepatocellular carcinoma: An update

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies leading to high mortality rates in the general population; in cirrhotic patients, it is the primary cause of death. The diagnosis is usually delayed in spite of at-risk population screening recommendations, *i.e.,* patients infected with hepatitis B or C virus. Hepatocarcinogenesis hinges on a great number of genetic and molecular abnormalities that lead to tumor angiogenesis and foster their dissemination potential. The diagnosis is mainly based on imaging studies such as computed tomography and magnetic resonance, in which lesions present a characteristic classical pattern of early arterial enhancement followed by contrast medium "washout" in late venous phase. On occasion, when imaging studies are not conclusive, biopsy of the lesion must be performed to establish the diagnosis. The Barcelona Clinic Liver Cancer staging method is the most frequently used worldwide and recommended by the international guidelines of HCC management. Currently available treatments include tumor resection, liver transplant, sorafenib and loco-regional therapies (alcoholization, radiofrequency ablation, chemoembolization). The prognosis of hepatocarcinoma is determined according to the lesion's stage and in cirrhotic patients, on residual liver function. Curative treatments, such as liver transplant, are sought in patients diagnosed in early stages; patients in more advanced stages, were not greatly benefitted by chemotherapy in terms of survival until the advent of target molecules such as sorafenib.

Key words: Hepatocellular carcinoma; Surveillance;

Liver transplant; Sorafenib; Catheter ablation

Core tip: This paper reviews the most recent evidence on hepatocarcinoma including its molecular pathogenesis and prognosis, with special emphasis on its diagnosis, staging and treatment. The most recent Easter and Western international guidelines are also reviewed.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third cancer-related cause of death; it usually develops in patients with hepatic cirrhosis and is the primary cause of death in this patient group[1].

The prevalence of HCC varies worldwide, with a greater incidence in Asia (> 20 cases/100000) than in North America and Europe (< 5 cases/100000)[2]. Seventy to ninety percent (70%-90%) of patients with HCC also have cirrhosis although in Asia, there is a greater number of non-cirrhotic patients with HCC; their malignancy relates mostly to hepatitis B virus (HBV) and hepatitis C verus (HCV) infections[3].

There are several HCC staging systems but the most currently used is the Barcelona Clinic Liver Cancer (BCLC) staging system[4]. This system's advantage relies on its inclusion of early-stage patients in the therapeutic decision-making schema. BCLC is the system recommended by the American Association for the Study of Liver Disease (AASLD) and the European Association for the Study of the Liver (EASL)[5,6]. The diagnostic methods of choice are magnetic resonance imaging and computed tomography in patients with the classical late washout pattern[6]. If not detected, a diagnostic biopsy must be obtained.

The mortality due to HCC is very high, particularly in patients diagnosed in late-stages and in correlation with the underlying liver disease; however, with the implementation of screening programs in high-risk populations[7], early-stage diagnoses have increased and opened the possibilities to curative therapy. These include surgical resection and the treatment of choice, orthotopic liver transplant. Patients outside the realm of curative therapy are managed loco-regionally (radiofrequency ablation, percutaneous ethanol injection and trans-catheter chemoembolization) or with systemic therapy (sorafenib, doxorubicin and bevacizumab) that have been proven to decrease mortality[8].

MOLECULAR PATHOGENESIS

The molecular pathogenesis of HCC is a complex process involving numerous events and genetic abnormalities that provide oncogenic capacities to preneoplastic cells.

There are molecular abnormalities common to the various etiologies of hepatocarcinoma, the most relevant being mutations of the beta-catenin gene (*CTNNB1* gene), the TP53 tumor suppressor gene and deletion of the Axin 1 and *Axin 2* genes, both negative regulators of beta-catenin[9]. There is also *VEGF* gene overexpression (vascular endothelial growth factor) that correlates with the tumor's angiogenic capacity[10] and has led to attempts to develop target therapies against VEGF[11]. Other oncogenic factors include the overexpression of extracellular matrix metalloprotease inducers (EMMPRIN or CD147) that have been associated to increased vascularization, invasion, metastases development and tumor recurrence[12]. Moreover, up-regulation of the JAK/STAT pathway that activates phosphorylation of the STAT3 transcription factor, found in 50%-100% of all HCC, is also related to angiogenesis and cellular differentiation; this has also recently become a therapeutic target[13,14]. Chromosomal instability is one of the most frequent abnormalities in hepatocarcinoma, whereby amplification of chromosome 1q is the most common followed by amplification of 8q and 5p[15]; HCC has also been associated to deletions of 4q, 8p, 13q, 16q, and 17p[16]. Micro RNA (miRNA) involvement has also been recently described in the development of malignancies since they can act like oncogenes or tumor suppressor genes; specifically in hepatocarcinoma, the relation between miRNA down-regulation (miR-122, miR-141), the up-regulation of others (mi-R21, miR-221), angiogenic capacity, metastases development and apoptosis has been well documented[17,18].

Furthermore, there are specific mechanisms involved in the different HCC etiologies such as hepatitis B infection (HBV), in which viral integration into the human genome leads to the production of truncated proteins such as HBx and pre S2/S that in turn, modulate signaling pathways and induce gene activation fostering oncogenesis[19,20]. Unlike HBV, in hepatitis C (HCV) infection there is no genomic integration and HCV-associated oncogenes have not been identified; hence, all pro-oncogenic abnormalities appear to be cytoplasmic and are conditioned by chronic inflammation, replicative senescence resulting from telomere shortening, oxidative stress, liver steatosis and miRNA overexpression, such as that of

miR-155[21,22].

RISK FACTORS AND PREVENTION

Most cases of HCC develop in patients with chronic liver disease (70%-90%)[23]. Risk factors depend on the region where the studies are conducted; for instance, HCV is a major factor in Europe, Japan and North America (50%-70%), HBV accounts for 10%-15%, alcohol 20% and others, 10%. In Asia and Africa, HBV is associated to 70% of cases and HCV to 20%[1,24] although the synergistic effect of non-alcoholic liver disease is becoming more relevant[25,26]. Diabetes mellitus is an independent risk factor in HCC[27]. Obesity is associated with an increased risk of HCC in both males and females[28]. Tobacco use also increases the risk while coffee intake decreases it[29,30].

The most frequent risk factor for HCC (50% of cases), is chronic HBV infection - including occult infection - secondary to exposure to aflatoxin B1[23,31]. Depending on the study, the relative risk of developing a tumor is close to 100-fold in HBV carriers *vs* non-carriers; in patients with associated cirrhosis, the risk is even greater[32] fostered by the viral load and the duration of infection[33]. HBV-related HCC may be prevented by vaccination and in patients with chronic infection and viral replication, treatment with antiviral agents may prevent progression of the liver disease and possibly, the long-term development of HCC, although recent evidence reveals that despite adequate viral suppression the risk remain high[34,35].

The incidence of HCC in individuals with cirrhosis due to HCV, is 3%-5% per year[36]. There is currently no available vaccine as in HBV, but preventing the progression of the acute infection to chronic hepatitis and finally cirrhosis with antiviral agents, prevents cancer development; however, the risk of HCC remain higher[37]. In randomized controlled trials, treatment has not been shown to modify disease progression rates or HCC development in patients with chronic HCV and advanced fibrosis[38,39]. There are recent studies showing that elimination of HCV in patients with compensated cirrhosis, decreases the risk of developing the tumor after 10 years[40]. Alcohol has an important influence on tumor development since it acts synergistically in individuals with chronic HBV and/or HCV infection[36]. HIV and HBV or HCV co-infection is an important risk factor, fostering faster liver disease progression than in individuals without HIV; if cirrhosis develops as a result, the risk for HCC is further increased[41].

SCREENING

At-risk population and benefit of early detection

Screening patients for HCC is recommended in high-risk populations in order to decrease associated mortality if detected in a curable stage[8]. Unfortunately, most detected cases are diagnosed in advanced stages since less than 20% of patients with cirrhosis are screened for HCC[42]; this is due, in great measure, to the first contact physicians' lack of knowledge of the recommended clinical guidelines although they care for 60% of these patients[43].

The decision to begin screening depends on the individual's risk and on whether they wish to be treated if diagnosed with HCC. Screening recommendations include: (1) patients with cirrhosis of any etiology, with conserved liver function (Child-Pugh A and B), lacking severe comorbidities; (2) decompensated cirrhosis (Child-Pugh C) on a transplant waiting list; (3) non-cirrhotic chronic HBV infection with active hepatitis or a family history of hepatocarcinoma; and (4) non-cirrhotic HCV infection and advanced liver fibrosis (F3)[5].

Screening methods

Liver ultrasound: Liver ultrasound twice a year is the screening procedure of choice since it is not an invasive method, it is easily available and its cost is moderate. Its sensitivity is 60%-80% and its specificity is above 90%[44]. A recent randomized prospective study revealed that its diagnostic yield was comparable to that of an annual triphasic computed tomography, and at a lower cost[45].

Serum alpha fetoprotein (AFP): Serologic tumor markers are of limited use: although more sensitive than other biomarkers with a cut-off point of 10.9 ng/mL[46], its diagnostic yield is inferior to ultrasound since its concentration depends on the tumor size and thus, preferentially detects tumors in advanced stages.

Ultrasound + alpha fetoprotein: If both strategies are combined, serum alpha fetoprotein levels only add 6%-8% to the number of cases undetected by hepatic ultrasound (HUS)[47]. The combination of these strategies increases the number of false positives as well as costs. There is currently insufficient evidence to support or refute the use of both methods in HCC screening/surveillance in the population with hepatitis B infection[44,48].

DIAGNOSIS

Pathology studies have revealed that most nodules < 1 cm detected in cirrhotic livers, are not HCC[49]. To date, HUS follow-up every 3-4 mo of lesions under 1 cm is recommended. If they grow, evaluation should be conducted according to the size of the lesion; if it remains stable, HUS is recommended every 4 mo[5,6]. In lesions greater than 1 cm, non-invasive diagnostic strategies should be followed with imaging methods; if a HCC diagnosis is not established, a liver biopsy is warranted. If this is inconclusive, the patient should be followed every 4 mo, but if the lesion grows or imaging patterns change, a second biopsy should be obtained[5].

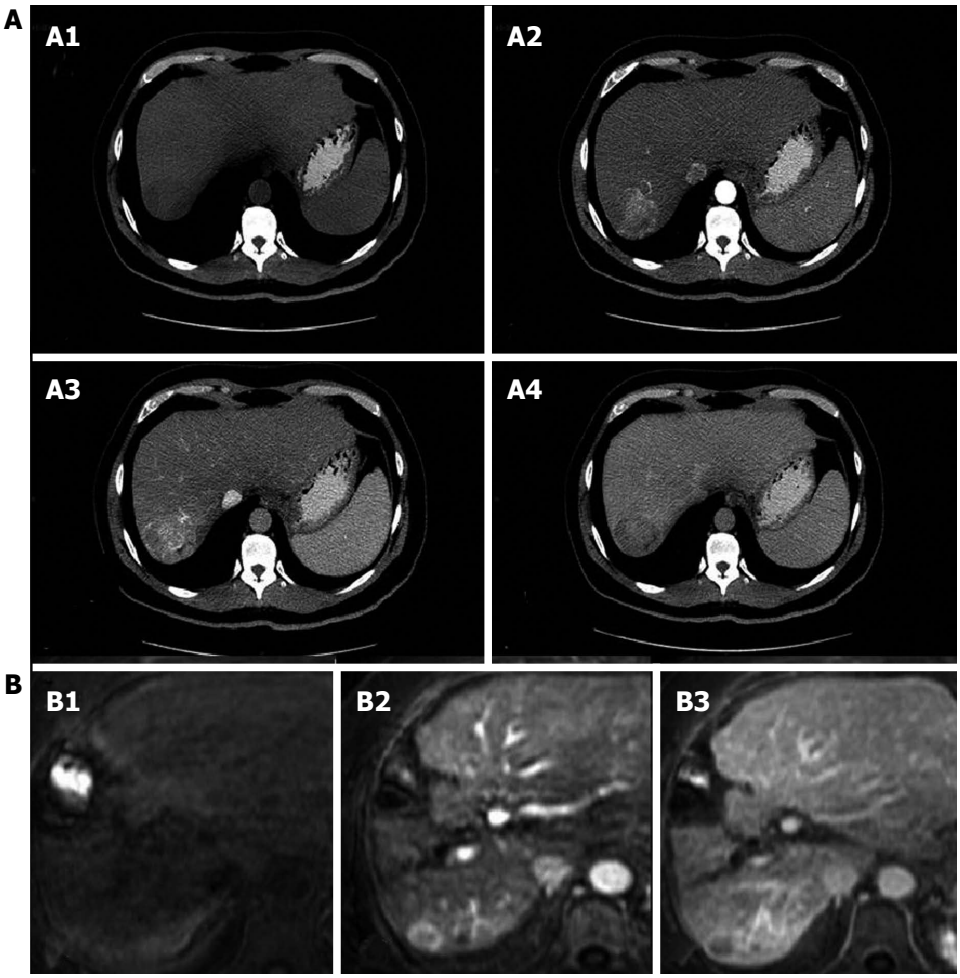

Figure 1 Contrast - enhanced computed tomography and Dynamic contrast-enhanced magnetic resonance imaging. A: Classical imaging pattern of hepatocellular carcinoma in contrast-enhanced computed tomography; A1: Simple phase, hypodense lesion in segment Ⅶ; A2: Arterial phase; A3: Enhanced portal; A4: 3 min late-phase washout; B: Diagnostic dynamic-contrast magnetic resonance imaging with classical pattern; B1: Simple phase; B2: Portal phase; B3: late-phase washout.

The clinical and economic impact of using guidelines in the diagnosis of HCC, such as those proposed by the AASLD and EASLD, has been recently prospectively evaluated. The sequential approach to hepatic lesions leads to a decreased need for liver biopsies when evaluating nodules between 1 and 2 cm, and also reduces costs when compared with lesions > 2 cm[50].

Non-invasive methods

There are some differences in terms of non-invasive diagnosis between Western and Eastern countries; these differences are reflected in different international guidelines pertaining to each geographical area: EASL[5], AASLD[6], Asian Pacific Association for the Study of the Liver (APASL)[51] and Japanese Society of Hepatology (JSH)[52].

Western guidelines (AASLD and EASL)

Imaging: Contrast - enhanced computed tomography and Dynamic contrast-enhanced magnetic resonance imaging: The diagnosis of HCC with non-invasive methods should be based on computed tomography (CT) and magnetic resonance imaging (MRI) results showing the characteristic pattern of early arterial enhancement followed by a contrast medium "washout" (Figure 1) phase in late venous phases; it is applicable to lesions > 1 cm[5,6].

Nodules between 1 and 2 cm have a malignancy rate of 14%-23%[53]. If this type of nodule has a characteristic contrast agent-mediated enhancement, the study's positive predictive value is close to 100% and its sensitivity is 71%, as long as it was performed in a center with sophisticated equipment[6]. If not characteristic, continued evaluation will require the use of two accepted imaging modalities: four-phase CT with contrast medium or dynamic contrast MRI. If these two methods do not reveal the characteristic HCC pattern, the lesion must be biopsied (Figure 2)[54].

Western liver societies do not consider contrast-enhanced ultrasound (CEUS) an appropriate study in the diagnostic approach to HCC due to the theoretical qualm in differentiating HCC from cholangiocarcinoma[55].

Eastern guidelines (APASL and JSH)

The guidelines proposed by the APASL and the JSH recommend following an algorithm that begins by

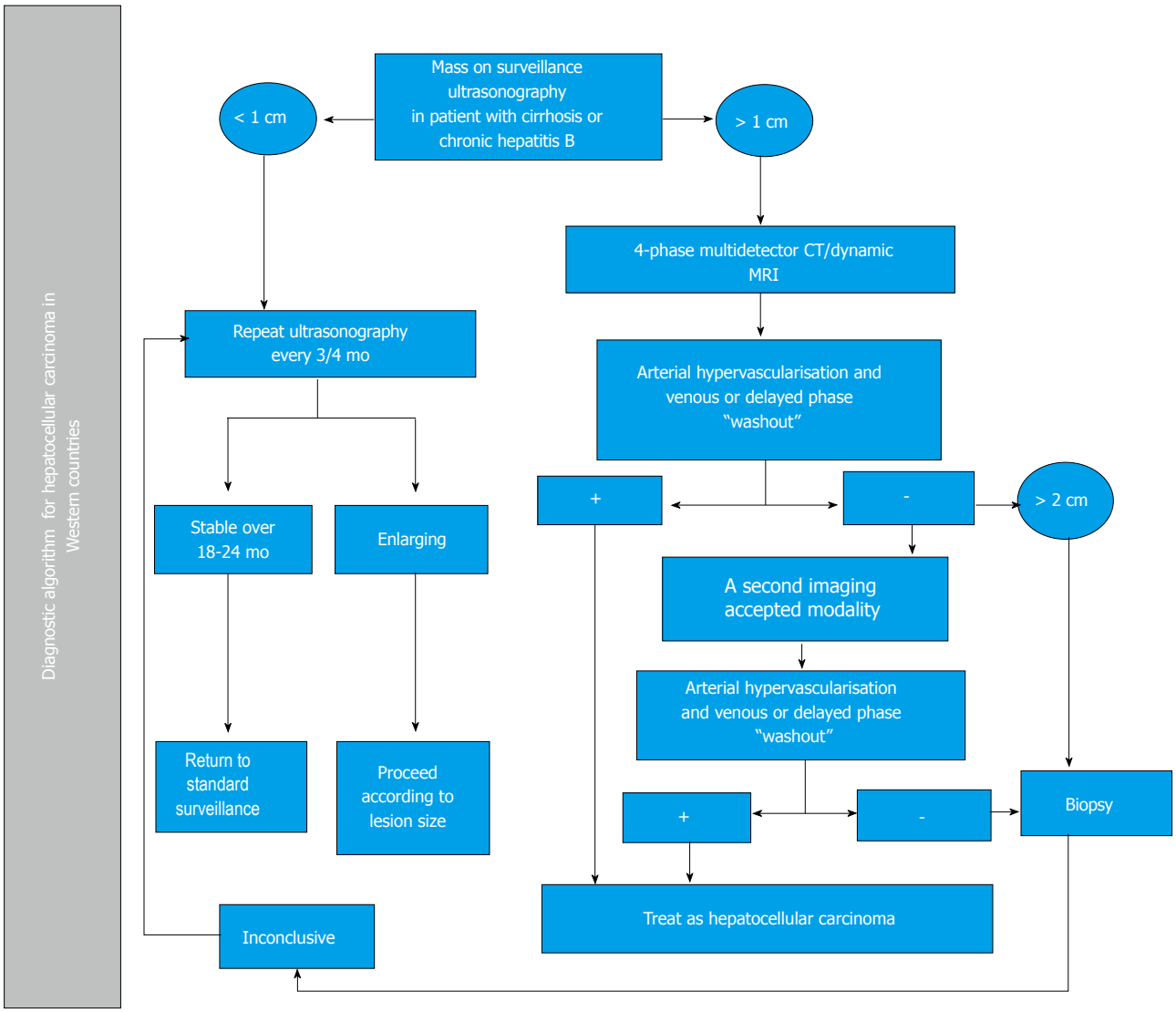

Figure 2 Diagnostic algorithm for hepatocellular carcinoma in Western countries. Modified from Bruix *et al*[6], with permission of the author and John Wiley and Sons. CT: Computed tomography; MRI: Magnetic resonance imaging.

evaluating the contrast medium pattern in the arterial phase of the imaging study and classifying it as hypervascular or hypovascular. Diagnostic tools include CT, dynamic contrast MRI and CEUS; hence, before the lesion can be classified as hypovascular, more than one study must be performed and should always include CEUS. Hypervascular lesions detected in the arterial phase as well as in the venous washout phase (classic pattern) or hypovascular lesions in the post-vascular phase of the CEUS with Sonazoid® as a contrast agent (in JHS guidelines), are diagnostic of HCC (Figure 3)[51,52]. None of the guidelines suggest that the use of positron-emission computed tomography (PET-CT) is pertinent in the diagnostic approach.

CEUS

This imaging method is accepted as part of the diagnostic approach of patients with HCC[56-58] in Eastern countries[51,52] but not in the West. Some of its advantages when compared with other imaging methods, include the fact that the microbubbles make it amenable to imaging patients in renal failure and also captures the arterial enhancement phase in real time. Moreover, the washout period has apparently been reported more consistently than with CT or MRI[57,58].

Histopathology

Liver biopsy should only be considered when evaluating nodules greater than 2 cm, if radiological findings are not compatible with HCC, or if findings in any nodule are inconclusive after a thorough work-up. But biopsies can yield false negative results even with immunohistochemical techniques[59]. Alpha fetoprotein is not a useful tissue marker due to its low sensitivity (25%-30%)[60]. Some strategies such as biopsying nodules showing arterial hypervascularity in at least one imaging study or the presence of typical synchronic lesions, have proven to increase sensitivity (62%) and specificity (79%) in the diagnosis of

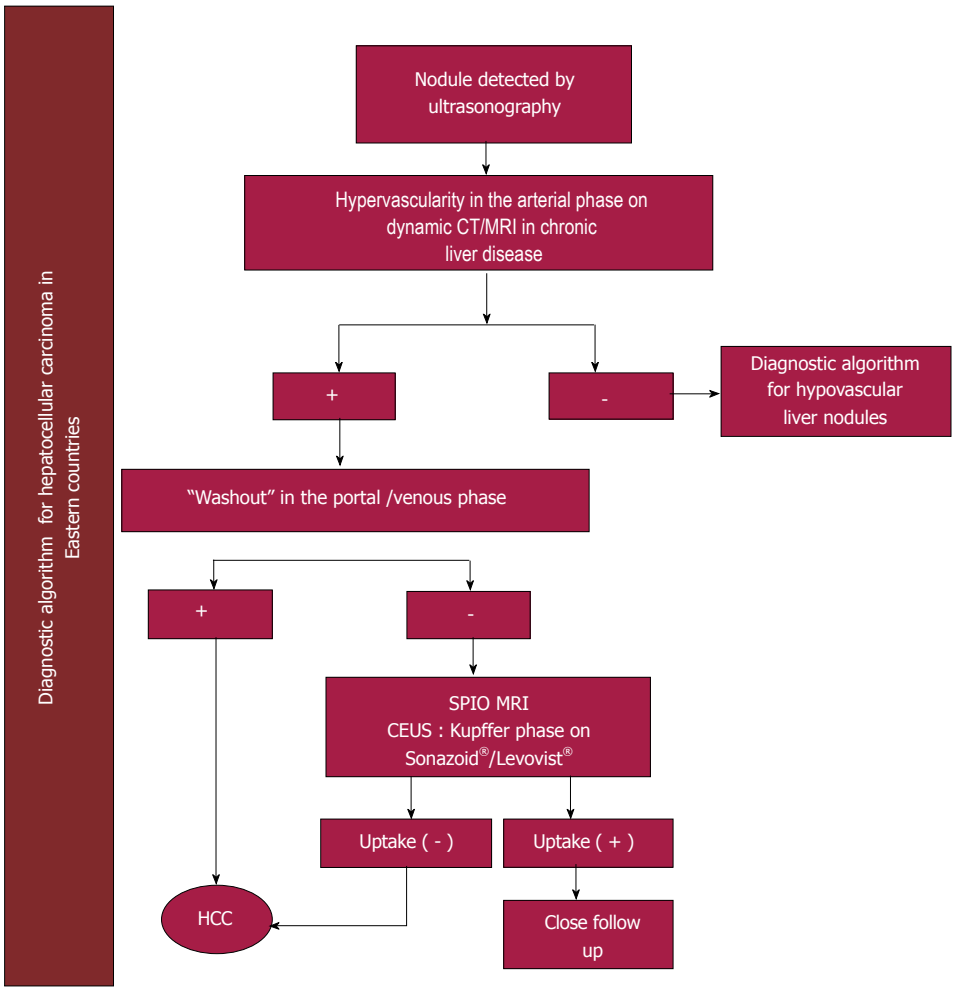

Figure 3 Diagnosis algorithm for hepatocellular carcinoma in Eastern countries. Modified from Omata *et al*[51], with permission of the author and Springer. HCC: Hepatocellular carcinoma; CT: Computed tomography; MRI: Magnetic resonance imaging; SPIO: Super paramagnetic iron oxide; CEUS: Contrast-Enhanced Ultrasonography.

malignancy in nodules between 1 and 2 cm and classified as indeterminate[53].

A histopathological diagnosis is established if the sample is positive for glypican 3, heat shock protein 70 (Hsp70) and glutamine synthetase. Positivity of at least two of these three markers has a diagnostic sensitivity of 72% and a specificity of 100%[60].

However, a negative biopsy does not preclude a HCC diagnosis since the rate of false negative results may reach 30%. This is due to sampling error or to the lack of specific histological findings[60].

Comparison of international guidelines

The main international societies studying the liver (AASLD, EASL, APASL and JSH) have similarities and differences in terms of HCC screening and diagnosis. The most relevant differences in the HCC diagnostic guidelines[5,6,51,52] in the West and the East hinge on the non-invasive diagnostic algorithm. All four guidelines accept the contrast medium enhanced classic pattern as definitively diagnostic of HCC. Western guidelines (AASLD and EASL) only consider acceptable the following imaging studies: four-phase computed tomography and dynamic-contrast magnetic resonance. Eastern groups propose algorithms that begin by evaluating the size of the lesion. The APASL and JSH recommend initiating the evaluation by analyzing the lesion's arterial vascularity (hyper or hypovascular). There are important differences between the Western and Eastern guidelines in terms of the non-invasive diagnosis of HCC.

STAGING

Determining the prognosis of patients with HCC is a crucial step in the management of these patients. An early diagnosis and effective treatment is associated with survival beyond 5 years[5,6].

Several classifications have been proposed in order to stratify patients according to their expected outcomes[4]. Obviously, although there are established guidelines and recommendations, therapy decisions should be individualized taking into account the available scientific evidence and the patient's personal profile.

Most cases of HCC develop in patients with cirrhosis

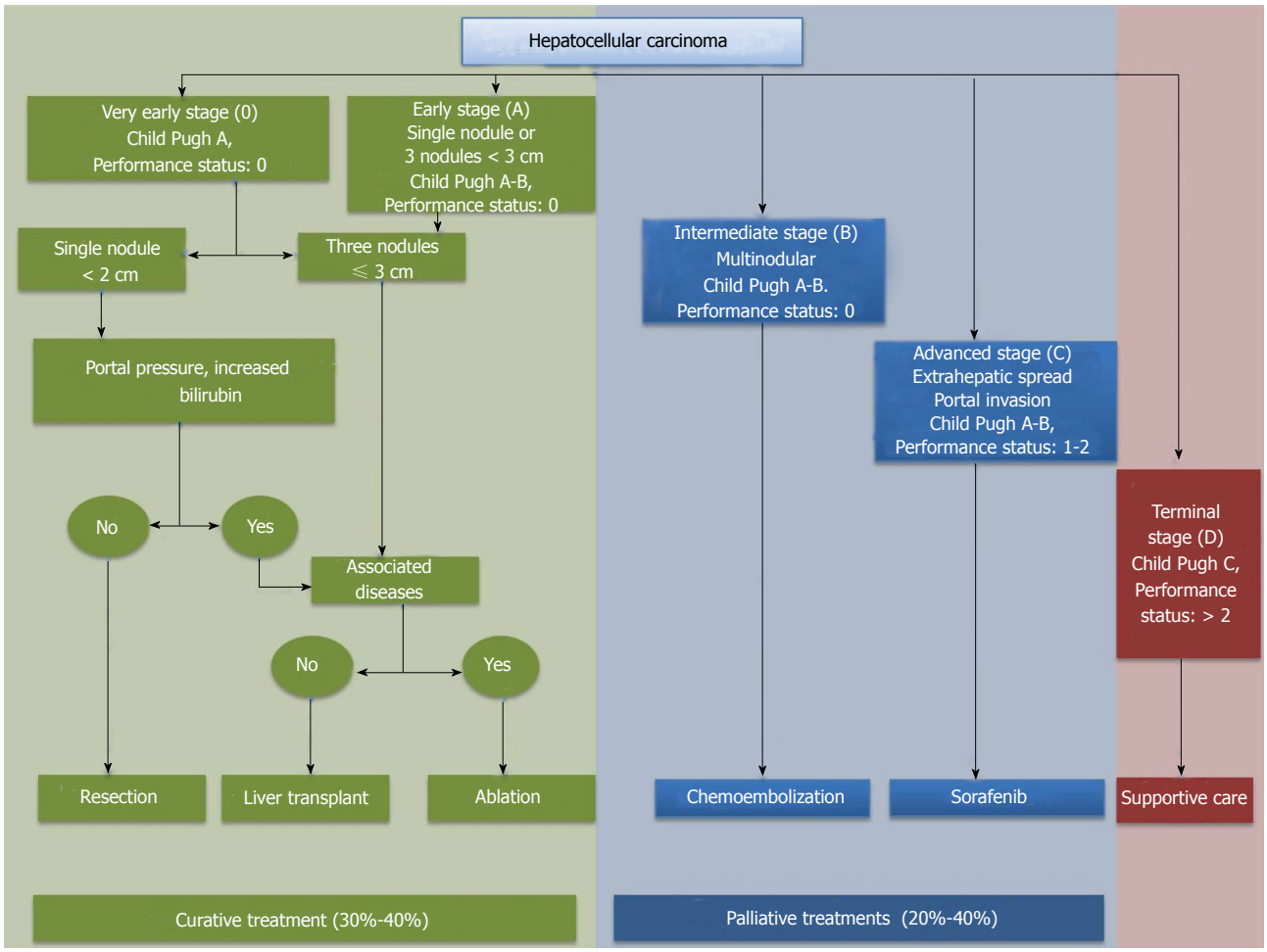

Figure 4 Barcelona Clinic Liver Cancer staging system and treatment strategy.

so for now, determining the patient's prognosis and therapy should consider the baseline degree of liver damage as well that due to HCC.

Several strategies have been proposed for prognostic staging and decision-making in patients with HCC: Child-Pugh[61], MELD[62], TNM classification[63], tumor volume estimation[64], evaluation of the patient's performance status (ECOG)[65], all characterized and limited by their one-dimensional assessment.

The most used classification is that developed by the Barcelona Clinic Liver Cancer group[66], a multidimensional strategy. This strategy has been validated in different scenarios and has established recommendations for each stage of the disease[67,68].

For now, the BCLC system is the recommended staging system by international guidelines (Figure 4)[5,6], since it stages the disease and proposes treatment according to the stage:

Very early stage (0)

Very early stage is patients with cirrhosis and compensated liver function (Child-Pugh A), with no signs of portal hypertension and with a single lesion ⩽ 2 cm (carcinoma "*in situ*"). The performance status according to ECOG must be 0. If treated by resection, these patients' 5-year survival is > 90%[69] and the tumor rarely recurs.

Early stage (BCLC A)

Early stage is patients with a single HCC lesion > 2 cm or three nodular lesions, each ⩽ 3 cm in diameter. Liver function should be evaluated according to the Child-Pugh classification and should be limited to groups A and B. The lack of significant portal hypertension and normal serum bilirubin levels are survival predictors in patients with a single lesion that undergo resection[70]. The determined size of the tumor is a criterion when considering liver transplantation as established in the Milan criteria[71]. If these criteria are not fulfilled other therapies are less effective[72]. The risk of vascular invasion is directly proportional to the size of the tumor. Five-year survival in these patients is over 50% after curative transplant[73,74].

Intermediate stage (BCLC B)

Intermediate stage includes patients with one, large HCC lesion as well as asymptomatic patients with multifocal disease and no vascular invasion or extrahepatic lesions. Their reported survival has been approximately 16 mo. Liver function must be

preserved (Child-Pugh A and B). These patients may undergo trans-catheter arterial chemoembolization (TACE) which is associated with an increased survival[75]. A recent meta-analysis of randomized clinical trials, suggests that ascites (a contraindication to TACE), is the most important adverse prognostic factor in this sub-group of patients[76].

Advanced stage (BCLC C)

This stage includes patients who do not fulfill BCLC B criteria. They are symptomatic (pain, general malaise or ECOG 1-2), they have vascular invasion or extrahepatic HCC involvement. Their survival has recently increased (10.7 mo) with sorafenib, a tyrosine kinase inhibitor[67,77].

Terminal stage (BCLC D)

This stage includes patients with severe hepatic dysfunction (Child-Pugh C) that are not liver transplant candidates and those patients with an ECOG score greater than 2. They have a dire prognosis and a survival under 6 mo while benefitting from conservative therapy (no intervention)[6].

Molecular classification

Evaluating a tumor's molecular classification provides a biological sub-classification that can optimize molecular therapies. These biomarkers allow improved staging. Increased alpha fetoprotein levels are associated to a poor or dire prognosis. Although an optimal cutoff point has not been established, it appears that high alpha fetoprotein levels predict an increased risk of HCC progression while the patient is on the liver transplant waiting list[78].

TREATMENT

HCC can be cured by surgical resection or liver transplant if it is diagnosed at an early stage; however, only 15% of cases are selected for management with these treatment modalities[79].

Liver resection

Deciding to perform a liver resection depends on three conditions: tumor size, tumor location and liver function. Resection is considered the treatment of choice in patients with solitary tumors limited to the liver, with no radiological evidence of vascular invasion and with normal liver function (normal total bilirubin, hepatic venous pressure gradient $\leqslant$ 10 mmHg, platelets > 100000 and no esophageal varices on endoscopy)[80]. The 5-year survival rate after tumor resection varies between 41% and 77%. Resection is also an option in multifocal HCC, fulfilling or not the Milan criteria or if the patient has mild portal hypertension and is not a liver transplant candidate[81,82]. Loco-regional therapy should be preferably considered in this group of patients, avoiding subsequent liver decompensation. The perioperative mortality after HCC resection in cirrhotic patients is approximately 2%-3%, greater than in patients with no cirrhosis. As a general rule, patients with some manifestation of decompensation (bleeding, ascites or portal hypertension), hepatic reserves are insufficient to consider surgical resection. Ideally, resection should only be considered in patients with tumors $\leqslant$ 5 cm in diameter[80,83], although there is consistently more evidence that size may not be a strict criterion in candidate selection; regardless, one must not ignore the fact that the greater the tumor mass, the greater the risk of vascular invasion or dissemination and the recurrence rate increases up to 70% at 5 years[79,84,85]. *De novo* tumor development may arise after primary resection, although most recurrences appear after 1 or 2 years as a result of dissemination of the primary tumor. The approach to post-resection has not been well studied yet, but repeating the resection is known to be of no value. Rescue liver transplantation or loco-regional therapies with or without multikinase inhibitors may be a viable alternative[86].

Liver transplant

In patients with unresectable tumors, the most feasible surgical option is orthotopic liver transplant (OLT) in conjunction with adjuvant therapies such as TACE or percutaneous ablation[80,86]. However, OLT is not an optimal choice in all patients and in spite of a necessary and prudent evaluation, patients should be well selected when dealing with a scarce resource such as organ donation[80]. In 1996, Mazzaferro *et al*[87] published a prospective cohort study including 48 patients transplanted because of HCC and in accordance with the Milan criteria (a single lesion $\leqslant$ 5 cm or 3 lesions $\leqslant$ 3 cm each); their survival rate at 4 years was 75%. Therefore, deceased donor liver transplant is a real option in these patients. Over time, experience with this treatment modality has increased and current 5-year survival is above 70% with a 15% recurrence rate, a similar survival to OLT without HCC[5,6,88].

There are several studies investigating the expansion of the Milan criteria, so as to not restrict the tumor size. The University of California proposed the San Francisco criteria that include patients with a single nodule $\leqslant$ 6.5 cm or 3 nodules $\leqslant$ 4.5 cm and with a total volume no greater than 8 cm; there are also other retrospective and prospective studies with very similar results to the Milan criteria[89]. In spite of these results, international guidelines insist on adhering to the Milan criteria while awaiting more solid data[5,6,51,52].

Interest in down-staging has recently increased targeting patients with HCC exceeding the OLT criteria and that are treated with loco-regional therapy (TACE and/or ARF) in order to decrease the tumor's size and then fulfill the OLT criteria[90,91]. Current data has led to conflicts, with some experts

recommending OLT only in patients that are down-staged effectively while others favor liver transplant as rescue therapy in spite of not having achieved the desired response[92,93]. Yao *et al*[91] published a study on a down-staging protocol using TACE and/or radiofrequency ablation, and reported a 1-year survival of 96.2% and 92.1% at 4 years, in patients who underwent OLT; they were also recurrence-free after an average follow-up of 25 mo.

The down-staging approach is controversial: some experts believe that large or multifocal tumors have the same recurrence risk in spite of successful down-staging[92]. One of the main poor response and recurrence biomarkers after transplantation is AFP. With a cut-off limit above 1000 ng/mL it could indicate microvascular invasion although further studies are required for confirmation[94].

Upon HCC diagnosis, this group of patients usually has stable liver disease, a disadvantage when awaiting an OLT. In this context, the United Network for Organ Sharing determined that patients fulfilling the Milan criteria should have a MELD score of 22 when added to the transplant waiting list, and the score should increase every 3 mo (the equivalent to a 10% increase in mortality); this is established after computed tomography or magnetic resonance confirmation of Milan criteria fulfillment[95]. This is turn, depends on the study region and on the number of patients on the waiting list, since some remain with stable liver disease and on the list for up to two years. Hence, the Living Donor Living Transplant program is a viable alternative; the risk of donor death and developing complications is 0.3% and 2% respectively. This option is limited to centers of excellence. Whether this group of patients has the same long-term survival as recipients of deceased donor livers remains to be established with certainty.

Loco-regional treatments

Loco-regional treatments in patients with HCC are chosen based on their oncological stage, performance status and underlying liver disease(s).

Early stage (BCLC A): Currently, the most commonly used ablation methods are percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA). PEI consists of the direct injection of ethanol into the HCC. This was the first treatment modality used before the development of RFA[96,97]. The curative capacity of PEI in tumors > 2 cm is limited and requires multiple injections over several sessions[98]. PEI can lead to complete tumor necrosis in 70% of nodules < 3 cm and in approximately 100% of nodules < 2 cm.

RFA is currently considered the safest ablation method and yields better results in BCLC A patients[97]. Complete response rates can reach 80% in patients with tumors < 3 cm, 50% in those with tumors between 3 and 5 cm and 25% in tumors > 5 cm. RFA is associated with a 5-year survival of 76%[98].

The available data is sufficient to conclude that RFA significantly improves survival and decreases local recurrence when compared to PEI. PEI use should be limited to circumstances when RFA is unavailable or technically not possible[99,100].

Intermediate stage (BCLC B)

Intra-arterial chemoembolization is the main treatment modality in unresectable HCC. This procedure requires the endovascular placement of a catheter until it reaches the hepatic artery and a microcatheter is guided to the segmental and sub-segmental branches. The chemotherapeutic agents most commonly used are cis-platinum and doxorubicin mixed as an emulsion with lipiodol, an oily radio-opaque contrast agent concentrated in the tumor and that promotes the exposure of neoplastic cells to the drugs. This emulsion is distributed in the affected segments or lobes and selectively infused in the tumor[99]. Survival rates are 82%, 47% and 26% at 1, 3 and 5 years, respectively. Therapy leads to tumor necrosis in 30%-50% of patients but rarely leads to a complete response especially after only one session[98]. Embolizing agents are administered after the chemotherapy emulsion following the same procedure. The most commonly used are: Gelfoam®, polyvinyl alcohol microparticles and trisacryl gelatin microspheres. Vascular obstruction thus decreases the chemotherapeutic agents' washout[98].

The soft embolization technique is very similar to TACE but without the administration of the chemotherapy emulsion with lipiodol. After diagnostic angiography, embolizing particles are injected directly into the tumor's afferent artery in order to produce tumor ischemia and necrosis. This technique is useful in patients with a significant tumor load and in whom future progression may lead to no viable treatment options. It has also been associated with less adverse effects[101].

Most advantages of soft embolization are shared with TACE and the debate continues on which technique offers the greatest benefits. Among the few controlled trials comparing TACE/soft embolization *vs* conservative treatment, survival was the greatest at 1 and 2 years with chemoembolization, 82% and 63% *vs* 75% and 50% with soft embolization and 63% and 27% with conservative management. Currently, the most commonly used standard technique is TACE. There is recent evidence that TACE in combination with sorafenib may decrease by 35% the risk of death in patients with intermediate and advanced HCC[102].

Terminal stage (BCLC D): This stage includes patients with Child-Pugh C and some with high score B liver disease associated to other comorbidities and terminal stage oncological symptoms. They must be very carefully evaluated and in most cases, loco-regional therapies are not an option since they can lead to the development of severe and even fatal

adverse effects[99].

Another application of intra-arterial embolization is in patients in an early HCC stage and in whom ablation therapy is precluded due to the tumor's location (close to the gallbladder, main bile ducts or main portal vein branches) or other contraindications.

Combined treatment

The combination of chemoembolization and radiofrequency ablation has proven to better control tumor growth in lesions between 3 and 5 cm.

The advantages of combined therapy include the fact that hypoxic aggression from embolization and the effects of the chemotherapy agents are synergistic in decreasing the tumor's blood flow and impedance. Moreover, a disruption of the intra-tumoral septa after chemoembolization, may foster the distribution of heat within the tumor and decrease perfusion-mediated tissue cooling, resulting in a greater ablated area. The suggested protocol is to first perform the selective chemoembolization followed by radiofrequency ablation within the subsequent 14 d[103].

An increase in survival has been demonstrated with combined treatment *vs* RFA with rates of 92%, 66% and 61% *vs* 85%, 59% and 45% at 1, 3 and 4 years, respectively. Recurrence-free survival rates have been reported as 79%, 60% and 54% *vs* 66%, 44% and 38% throughout the same follow-up periods[104].

Targeted system therapy

The molecular pathways involved in the pathogenesis of HCC are manifold but there are few therapeutic modalities specifically directed to these molecular targets that have yielded relevant results; the most studied and validated is the use of sorafenib. This molecule acts by inhibiting multiple kinases, including the Raf-1 and B-Raf serine-threonine kinases, VEGFR 1, 2 and 3 and PDGFR-β[105]. In the initial phase Ⅰ studies, sorafenib led to partial responses in various solid tumors and among them, one hepatocarcinoma case[106].

The SHARP study focused on the Western population. They assigned 602 patients with Child A cirrhosis and good performance status (ECOG 0 - 1 in over 90%), that had never received systemic therapy; they were randomized into a group treated with sorafenib 400 mg *bid* and a placebo group. Their main outcome was overall survival (OS) and symptomatic progression-free survival. Overall survival was significantly greater in the sorafenib arm, with a survival rate of 10.7 mo *vs* 7.9 mo (HR = 0.69, 95%CI: 0.55-0.87; $P < 0.001$) and there was no difference in terms of symptomatic progression (4.9 mo *vs* 4.1 mo; $P = 0.77$) although radiological progression did decrease when evaluated by RECIST (5.5 mo *vs* 2.8 mo, HR = 0.58, 95%CI: 0.45-0.74, $P < 0.001$). No patient had a complete response, only 2% of the patients had partial response in the sorafenib group and 1%in the placebo group. Up to 80% developed an adverse event, almost all grade 1 or 2. Grade 3 events not found in the placebo group included diarrhea and hand-foot syndrome, each in 8% of cases; there were no grade 4 events[67]. In an Asian phase Ⅲ study of 226 patients fulfilling similar selection criteria, results were very similar. The increase in OS was a little less marked, 6.5 mo *vs* 4.2 mo (HR = 0.68, 95%CI: 0.50-0.93; $P = 0.014$) and in terms of disease progression, 4.2 *vs* 2.8 mo (HR = 0.57, 95%CI: 0.42-0.79; $P = 0.0005$). Although survival in this study was not as good as in SHARP, this difference was attributed to the fact that they included patients with worse performance status and more advanced disease; HR were very similar[107]. A SHARP sub-analysis revealed that patients with HCV benefitted more from sorafenib (14 mo *vs* 7.4 mo, difference of 6.6 mo) when compared with patients with HBV (10.3 mo *vs* 8 mo, difference of 2.3 mo); one must emphasize that almost 75% of patients in the Asian study were infected with HBV while only 20% were so in the Western study, another possible explanation for the observed difference between studies.

In the United States, the Food and Drug Administration approved sorafenib without specifying the severity of liver disease, but in patients with Child B cirrhosis its benefits are much less evident. A retrospective analysis of 59 patients (26 Child A, 23 Child B, 10 Child C) revealed an OS of 8.3, 4.3 and 1.5 mo, respectively; grade 3-4 adverse events were present in 15% of Child A patients *vs* 30% in Child B[108].

In the GIDEON (Global Investigation of therapeutic DEcisions in hepatocellular carcinoma and Of its treatment with sorafenib), there were also more reported G3-4 adverse events in Child B patients (67% *vs* 42%) and a greater possibility of abandoning treatment due to these adverse effects (40% *vs* 25%)[109].

Other strategies such as combining sorafenib with chemotherapy have been attempted. In a phase Ⅱ study of 96 Child A patients, two groups were defined: sorafenib 400 mg bid and doxorubicin 60 mg/m^2 *vs* doxorubicin and placebo, OS was 13.7 mo *vs* 6.5 mo ($P = 0.006$) and progression-free survival was 6.0 mo *vs* 2.7 mo[110]. A phase Ⅱ study is currently being conducted (CALGB 80802). Other studies of targeted therapy plus chemotherapy have yielded controversial results.

Other treatments such as sunitinib have been attempted but a phase Ⅲ study revealed worse survival (7.9 mo *vs* 10.2 mo when compared with sorafenib) and more frequent and severe toxicity[111]. Other molecules such as cetuximab, erlotinib and everolimus have also not proven to be superior to sorafenib or have not been studied comparatively.

Sorafenib is currently considered first-line systemic therapy due its effectiveness and toxicity profile. Some clinical markers (rash, hypertension) as well as molecular markers (VEGF genotypes, VEGF

polymorphisms, Mcl-1 expression, pERK) may reflect its efficacy, but none have been validated[109].

PROGNOSIS

In spite of advances in treatment, mortality in HCC remains high. In untreated patients, 1-year survival is 17.5% and 7.3% at 2 years[76]. Due to patient heterogeneity, their clinical status, the available therapeutic options and particularly the presence or lack of liver disease, prognosis is difficult to establish unlike in other neoplasias in which prognostic factors are solely determined by the tumor.

There are currently numerous staging systems[4] and although there is no consensus, the AASLD and EASL guidelines recommend the use of the BCLC system; according to this classification, 5-year survival of stage A patients is 50%-70% after curative treatment, 16-20 mo in stage B, 6-10 mo in stage C and 3-4 mo in stage D[66,73,74,76]. However, several factors of great impact on mortality are not considered in this classification.

HCV and HBV infection also compromise survival in non-cirrhotic patients undergoing curative surgery by conferring an increased and earlier risk of recurrence. Persistent HBV viremia also fosters an increased recurrence risk[4,35,112-115].

In patients without liver disease, HCC tends to be diagnosed at a more advanced age than in patients with cirrhosis and it is usually detected in latter stages (BCLC D in 51.6% *vs* 42% in patients with cirrhosis) due to the lack of screening; however, mortality in patients in intermediate stages is lower than that in patients with cirrhosis. In this group of patients, the BCLC classification correlates best with survival than other staging systems and their survival rates are better due to the possibility of providing curative treatment of larger lesions in turn, leading to decreased recurrences (27% *vs* 73%) and greater survival (81% *vs* 23%)[116,117].

Upon recent inclusion of molecular markers such as wtER, IGF and VEGF-1 in prognostic scoring systems such as CLIP, their precision has been favorable although they are not currently routinely used[118-120].

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REVIEW

Angiogenesis and liver fibrosis

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Abstract

Recent data indicate that hepatic angiogenesis, regardless of the etiology, takes place in chronic liver diseases (CLDs) that are characterized by inflammation and progressive fibrosis. Because anti-angiogenic therapy has been found to be efficient in the prevention of fibrosis in experimental models of CLDs, it is suggested that blocking angiogenesis could be a promising therapeutic option in patients with advanced fibrosis. Consequently, efforts are being directed to revealing the mechanisms involved in angiogenesis during the progression of liver fibrosis. Literature evidences indicate that hepatic angiogenesis and fibrosis are closely related in both clinical and experimental conditions. Hypoxia is a major inducer of angiogenesis together with inflammation and hepatic stellate cells. These profibrogenic cells stand at the intersection between inflammation, angiogenesis and fibrosis and play also a pivotal role in angiogenesis. This review mainly focuses to give a clear view on the relevant features that communicate angiogenesis with progression of fibrosis in CLDs towards the-end point of cirrhosis that may be translated into future therapies. The pathogenesis of hepatic angiogenesis associated with portal hypertension, viral hepatitis, non-alcoholic fatty liver disease and alcoholic liver disease are also discussed to emphasize the various mechanisms involved in angiogenesis during liver fibrogenesis.

Key words: Liver fibrosis; Angiogenesis; Cirrhosis; Fibrogenesis; Hepatic stellate cells; Vascular endothelial growth factor; Hypoxia; Chronic liver disease

Core tip: Hepatic angiogenesis is closely associated with the progression of fibrosis in chronic liver diseases (CLDs). Recent evidences demonstrated that blocking angiogenesis means also prevention of fibrosis progression. Hypoxia plays a crucial role in eliciting angiogenesis together with hepatic stellate cells being the most prominent sources of vascular endothelial growth factor and Angiopoietin-1. Adipokines, endoplasmic reticulum stress and related unfolded protein response; neuropilins; might be future therapeutical target in the progression of fibrosis in CLDs. Moreover studies on non-alcoholic steatohepatits demonstrated that of angiotensin and renin inhibitors could be effectively used as a new treatment strategy against angiogenesis in the prevention of fibrosis in CLDs.

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INTRODUCTION

Angiogenesis, the formation of new vessels from preexisting vasculature, is an active, growth factor dependent and hypoxia induced event that takes place in several organs during growth and repair of injured tissues[1,2]. It should always be distinguished from other characteristic mechanisms of vessel growth that include vasculogenesis, arteriogenesis, and collateral vessel growth[3,4].

Although it is crucial for tissue growth and regeneration, accumulated evidence indicated that angiogenesis develops in many organs during multiple pathologic situations. Angiogenesis is a fundamental part of tumor progression and contributes to the pathogenesis of different inflammatory, fibroproliferative and ischemic diseases[2]. Recent studies demonstrated that chronic liver diseases (CLDs) can not be excluded from this rule, emerging angiogenesis as a promising therapeutic target[5-11].

As a matter of fact angiogenesis does not solely takes place in CLDs but has been clearly documented in many conditions including liver regeneration (after acute liver injury or partial hepatectomy), ischemia, in primary (hepatocellular carcinoma) and metastatic tumors[3,12]. It is still unclear whether angiogenesis represents a simple response to maintain homeostasis or one that exerts a pathological role leading to liver injury. However, recent data in CLDs have been releaved that angiogenesis might contributes to the progression of fibrosis during the wound healing process in chronic liver damage[3,5,7,9,10,12]. Consequently, efforts are being directed to revealing the mechanisms that are involved in angiogenesis in CLDs with different etiology.

In this review first, a consideration of the basic mechanisms and events in angiogenesis will be described. The following section will be focused to give a clear view on the relevant features that communicate angiogenesis with progression of fibrosis in CLDs towards the-end point of cirrhosis. I recommend to the intrested reader to refer to more detailed comprehensive articles on the role of angiogenesis in liver regeneration or liver tumors.

ACTORS THAT STIMULATES ANGIOGENESIS

In general two main pathways are determined in the progression of angiogenesis in all tissues: inflammation and hypoxia.

In physiological angiogenesis, an immune response triggered by tissue damage provide the extravasation of immune cells from peripheral blood into the injured tissue leading to the restoration of tissue homeostasis[13]. However, the persistence of tissue damage and accompanying inflammation perpetuates the activation of endothelial cells (ECs) resulting an increase of vascular permeability and promoting chemokine-mediated recruitment of inflammatory cells[13-15]. These cells can produce angiogenic cytokines and growth factors that induce the proliferation and migration of ECs that are necessary for the formation of new vessels[14,16-18]. Besides, during chronic inflammation the accumulation inflammatory cells together with fibrosis may contribute to hypoxia, by increasing the resistance of damaged tissue to blood flow and oxygen (O_2) supply[15,19,20]. On the other hand accumulated evidences indicate hypoxia alone could be important in the stimulation of angiogenesis and can also stimulate inflammation leading to a viscous circle between inflammation and angiogenesis[3,21,22] (Figure 1). Hypoxia activates angiogenesis as a result of signaling mediated by hypoxia-inducible factors (HIFs)[12,21-23]. By definition, these critical molecular mediators are transcription factors which promote cells to react to the reduced levels of pO_2 in the site of injury by up-regulation of several genes carrying the hypoxia response elements (HRE) sequences in their promoter or enhancer[12,22-24]. HIFs are heterodimers formed by an oxygen sensitive and inducible α subunit and an oxygen-independent β subunit[12,22-24]. Three α subunits, named hypoxia inducible factor-1-α (HIF-1α), HIF-2α and HIF-3α have been described and all bind to a common β subunit named, the aryl hydrocarbo nuclear receptor translocator (ARNT), alternatively, HIF-1β[23,24]. The best-characterized member of this family is HIF-1, is regarded as a main regulator of homeostasis [12,22-24]. Under the normal levels of O_2, HIF-1α is incessantly hydroxylated by several enzymes [prolyl-hydroxylases (PHD1,PHD2 or PHD3) and asparaginyl hydroxylase (FIH1)] whose activity is O_2 dependent[23,24]. This modified HIF-1α scaffolded on a multimeric protein complex including von Hippel-Lindau protein(VHL) leading to rapid ubiquitination and proteasomal degradation[23,24]. Hypoxia inhibits the activity of O_2 dependent enzymes and HIF-1α forms a heterodimer with HIF-1β subunit then phosphorylated and stabilized to form a transcriptional complex able to bind HRE sequences in the promoter region of target genes in the nuclei[23,24]. HIFs activate transcription of a broad range of genes[12,19,22-24]. Although a comprehensive list of HIF targets exist, only oxygen dependent regulation of HIF-1α in normal and hypoxic conditions is in the scope of this article. The target genes in mediating hypoxia-induced angiogenesis are demonstrated in Figure 1.

PHASES OF ANGIOGENESIS AND MOLECULES INVOLVED

The development of new functional vessels is closely related to precise orchestration of the molecular effectors that stimulate different processes. It comprehends consecutive phases and a large spectrum of proangiogenic mediators. These phases and mediators that are involved in angiogenesis are described in Figure 2.

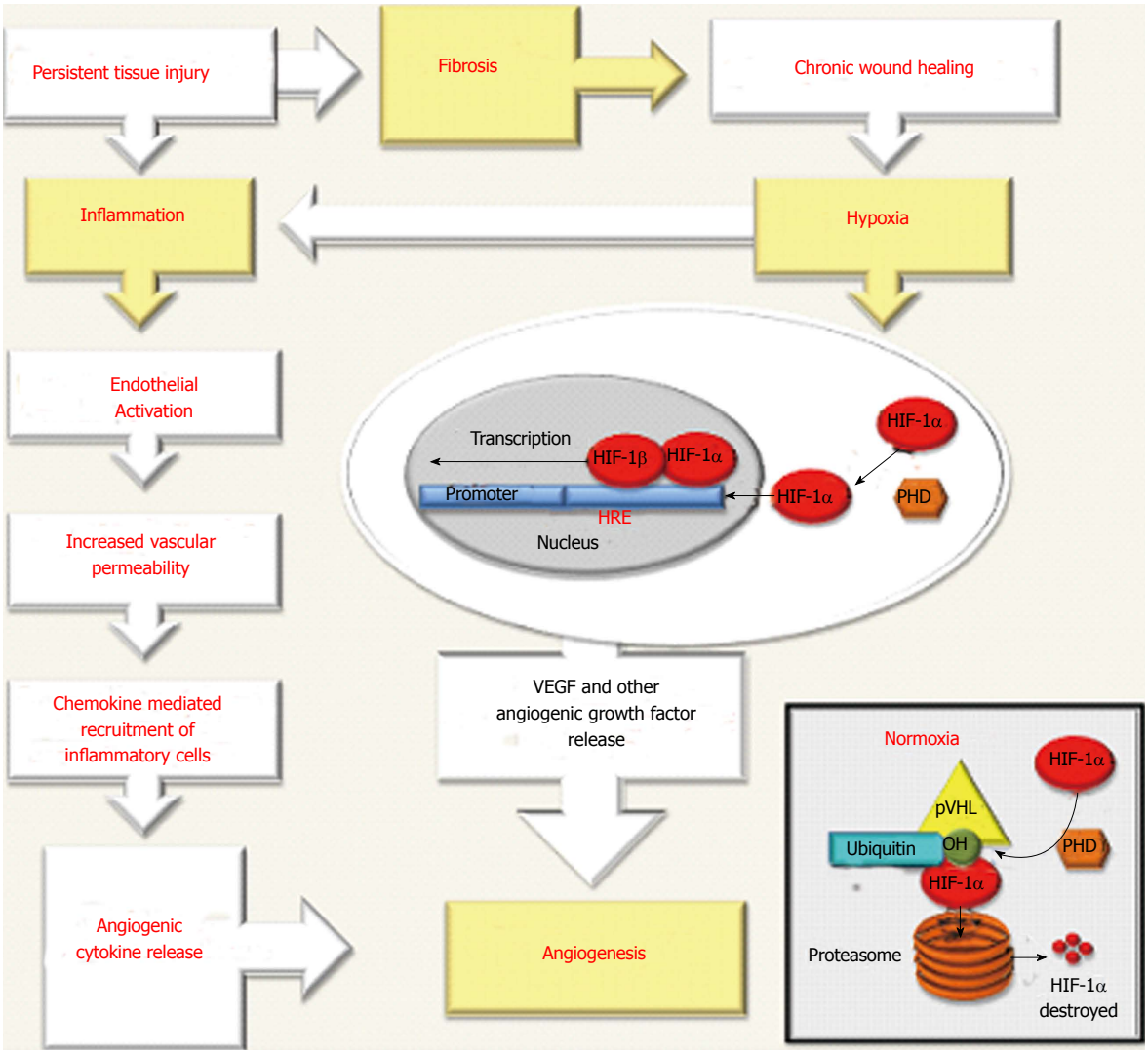

Figure 1 Link between angiogenesis, inflammation and fibrosis. Hypoxia plays a crucial role in the the activation of HIF-1. HIF-1α: Hypoxia inducible factor 1α; HIF-1β: Hypoxia inducible factor 1β; HRE: Hypoxia responsive elements; VHL: Von Hippel Lindau protein; PDH: Prolyl hydroxylated domain; VEGF: Vascular endothelial growth factor.

Sprouting and budding of ECs

This is the fist step of angiogenesis that involves the changes in structural organization in terms of intercellular and matrix interactions of ECs. In quiescent state ECs adhere to each other and to extracellular matrix (ECM) through inter-endothelial junctions (IEJs) and integrin receptors that provide together a mechanical strength and tightness to establish a barrier, as well as allow intercellular communications[25,26]. IEJs comprise tight junctions, gap junctions and adherens junctions[25-27]. While occludin, claudins, and junctional adhesion molecules are the keystones of tight junctions, VE-cadherin is necessary for formation of adherens junctions and connexins constitute gap junctions[25-27]. The contacts are also relevant through CD31 (PECAM1)[25,26]. The link between ECs with ECM is provided by the connection of integrin receptors with matrix proteins [fibronectin (FN) or vitronectin (VN)][26,27] (Figure 3).

After an initiating stimulus (mainly hypoxia) NO-dependent vasodilation and the influence of Ang-2 and vascular endothelial growth factor (VEGF) on increased vascular permeability with loosening of all those inter-endothelial contacts result in leakiness from vessels[26-28]. The extravasation of plasma proteins together with ECM components constitute a scaffold for migration of ECs[26-28]. The main antagonist to these starting events is represented by Angiopoietin-1 (Ang1), which tightens inter-endothelial contacts[26-29].

ECM degradation and EC migration

In order to allow migration, proliferation of ECs to form new sprouts, the ECM network has to be submitted to a process of proteolytic remodeling. This remodeling are related to the coordinated activity of matrix metalloproteinases (MMPs), plasminogen activators (mainly urokinase plasminogen activator or uPA) and their inhibitors [tissue inhibitors (TIMPs) and plasminogen activator inhibitor (PAI-1)][27,28,30]. Other proteinases, including heparinases and cathepsins

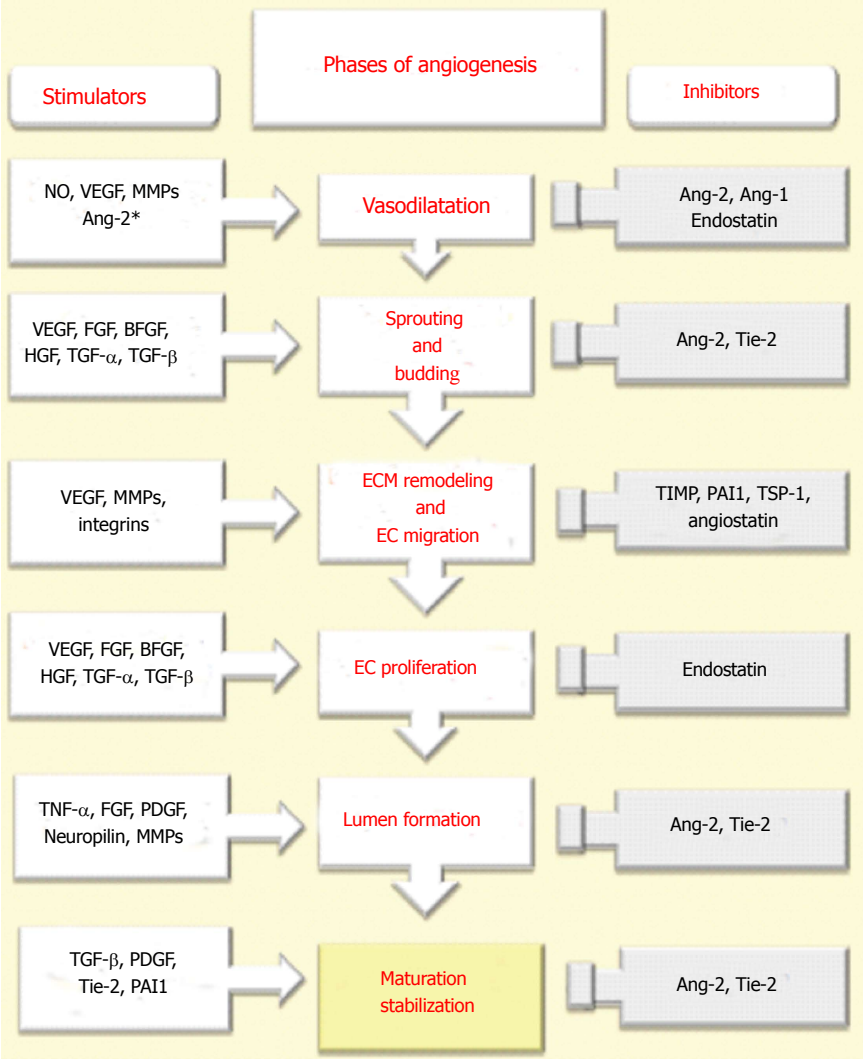

Figure 2 Phases of angiogenesis and the agents involved. NO: Nitric oxyde; VEGF:Vascular endothelial growth factor; MMPs: Matrix metalloproteinases; Ang-2: Angiopoietin 2; FGF: Fibroblast growth factor; BFGF: Basic fibroblast growth factor; HGF: Hepatocyte growth factor; TGF-α: Transforming growth factor-α; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α; PDGF: Platelet derived growth factor; PAI: Plasminogen activator inhibitor; Ang-1: Angiopoietin-1; TIMP: Tissue inhibitor of metalloproteinase; TSP-1: Thrombospondin-1.

are also involved[27,30]. The proteoliytic degradation of ECM gives rise to the exposure of cryptic epitopes and to disruption of integrin-mediated contacts between ECs and ECM leading to migration of ECs. vβ3 and vβ5 integrins regulate the connection of ECs to the ECM and provide their communication with their microenvironment[2,30]. On the other hand they (vβ3 and vβ5) may also act as anti-angiogenic factors by inhibiting VEGF and VEGF receptor Type 2 [VEGFR-2 (Flk-1)][3,31,32]. It should be noted that proteolysis during angiogenesis should be well balanced[32-34]. Insufficient or inadequate proteolysis prevents migration of ECs[32-34]. However, an exaggerated degradation of ECM impairs the migration of ECs through the disorganisation of supporting structures and results in inhibiton of angiogenesis[32-34]. Proteinases can also mediate the release of ECM-bound proangiogenic factors [VEGF, basic fibroblast growth factor (bFGF) and transforming growth factor 1 (TGF 1)] or proteolytically activate other factors, as such facilitate the migration of ECs[30].

Endothelial cell proliferation, tube formation and branching

Several angiogenic growth factors that are secreted both by ECs or surrounding cells induce the proliferation of ECs. The most relevant angiogenic factor is VEGF, that act mainly on cells expressing two tyrosine kinase receptors, VEGF-R1 (FLT-1) and VEGF-R2[34,35]. Other growth factors including transforming growth factor- (TGF-), fibroblast growth factor (FGF), TGF- and hepatocyte growth factor (HGF) also up-regulate EC proliferation[36]. In addititon, cytokines also provide positive stimuli for proliferation of ECs[17,37]. Certain chemokines, lipid mediators and hormones may stimulate proliferation of ECs[18]. In contrast, angiostatin, endostatin, interferon, platelet-derived growth factor 4, leukemia inhibiting factor and Antithrombin Ⅲ are potent inhibitors of EC proliferation[37]. Following proliferation of ECs, signaling pathways and mediators

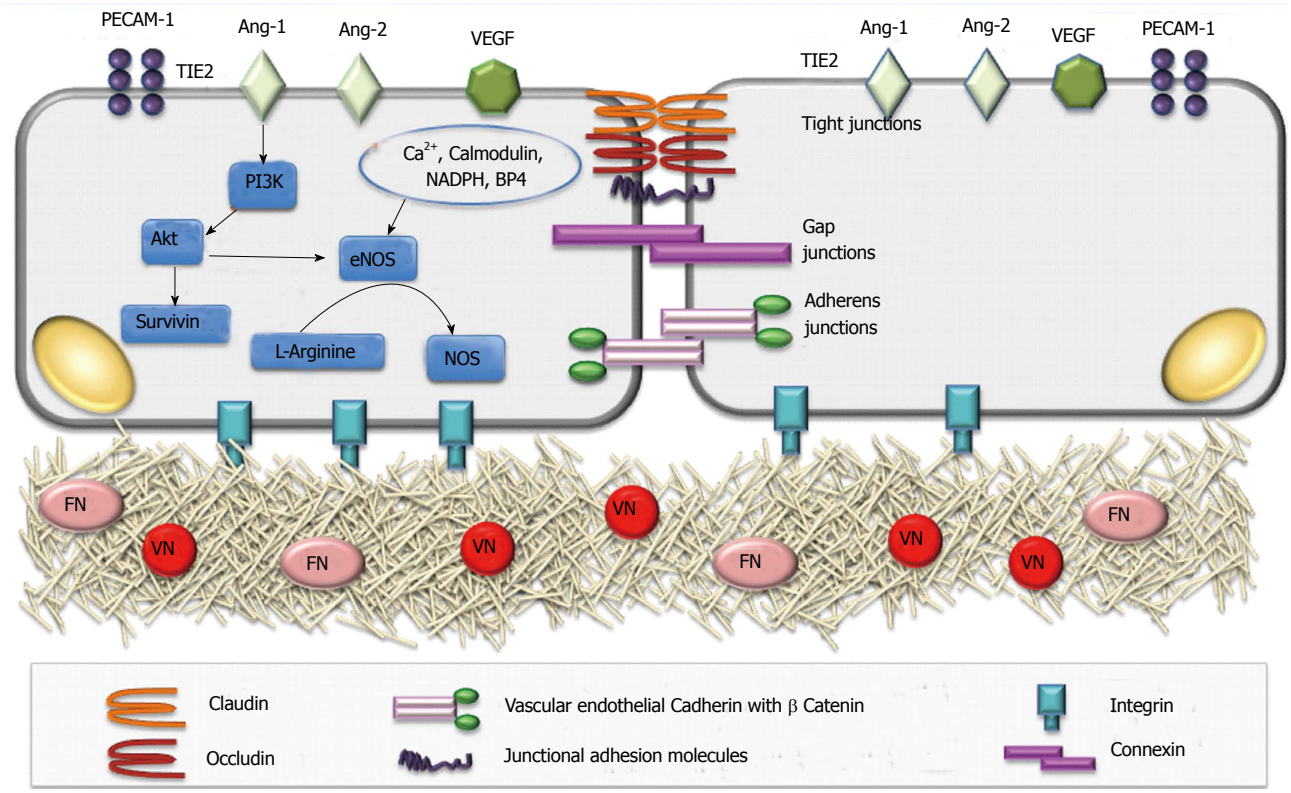

Figure 3 Structure and function of endothelium. In endothelial cells an increase in Tie-2 signaling *via* the Ang-1 receptor initiates phosphorylation of Akt, which in turn phosphorylates eNOS and survivin. Enzymatic activity of eNOS is also regulated by calcium, calmodulin, NADPH, and BH4. The conversion of L-arginine to NO by eNOS leads to the cyclic-GMP-mediated relaxation of smooth muscle cells. Ang: Angiopoietin; BH4: 5,6,7,8 tetrahydrobiopterine; eNOS: Endothelial nitric oxide synthase; FN: Fibronectin; NO: Nitric oxide; PECAM-1: Platelet/endothelial cell adhesion molecule 1; VN: Vitronectin; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; VEGF: Vascular endothelial growth factor.

that determine tube formation and branching are activated[36,37]. ECs accumulate in the form of tubular structures. VEGF, Ang-1, vβ3 and vβ5 integrins are mainly responsible in the regulation of both the diameter and length of these structures[36]. The most potent antagonist of this phase is thrombospondin[36,37].

A three dimensional of an efficient vessel network requires precise orchestration of signaling pathways that influence branching of new vessels, deposition of ECM and formation of basement membrane[36-39]. Branching is mainly monitored by ephrins and neuropilins[38,39]. TIMPs and MMPs regulates of ECM deposition and formation of basement membrane.

Vessel maintenance, maturation and stabilization

For nascent vessels to mature and to stabilize the recruitment of pericytes is required. This is regulated mainly by the secretion of platelet derived growth factor (PDGF)-BB[40,41]. PDGF-BB and its receptor PDGF-b subunit play a crucial role in the stabilization of nascent vessels[40,41]. PDGF-BB is released by ECs and contributes to recruit of PDGFR- b expressing mesenchymal cells to nascent vessels and leads to their proliferation[40,41]. Besides its role in the regulation of vessel maturation through stimulation of ECM, TGF-1 also induces differentiation of mesenchymal cells into pericytes[42]. Pericytes release Ang-1 that interacts with the corresponding receptor Tie-2 expressed on ECs facilitates the formation of junctions between ECs and pericytes eventually leading to the stabilization of nascent vessels[43]. Whereas lack of migration of mural cells into nascent vessels results in fragile and permeable vessels that results in hypoxia, an excess of Ang-1 ending up with the formation of tightened vessel and prevents angiogenesis[41,43].

It should be noted that Ang-2 acts differently in angiogenesis. In the presence of angiogenic signals (VEGF, Ang-1, PIGF, PDGF-BB) Ang-2 can activate Tie-2[3,36]. However, in the absence of angiogenic signals or in an excess of anti-angiogenic factors in the microenvironment Ang-2 can block Tie-2 allowing to EC death and vessel regression[3,36] .

HEPATIC ANGIOGENESIS

In liver, angiogenesis proceeds with steps and molecular mechanisms that mostly coincide with those demonstrated in other part of the body. However, a number of differences in liver render angiogenesis more complex[3,5,44,45]. These differences are: (1) liver parenchyma possesses two different microvascular structures: Large vessels such as portal vessels that are lined by a continuous ECs lying on a basement membrane and liver sinusoids that are lined by

fenestrated and discontinuous Ecs; (2) the presence of liver derived angiopoietin-like peptide 3 (ANGPTL3). Although no data are available at present on its role in liver angiogenesis, this peptide do not bind to the angiopoietin receptor Tie-2 but can bind αvβ3 integrin, inducing EC adhesion and migration and function to manipulate angiogenesis[45,46]. ANGPTL3 also regulates lipid, glucose, and energy metabolism independent from angiogenic effects[46]; and (3) the "stars" of liver fibrogenesis, hepatic stellate cells (HSCs), especially in their activated and myofibroblast like phenotype may contribute to angiogenesis and vascular remodeling in liver[3,5,15]. Whereas HSCs regarded as liver-specific pericytes, they differ from their microcapillary counterparts because they play an active role in modulating angiogenesis[3,5]. The role of activated HSCs in angiogenesis during liver fibrosis will be described in more details in following sections.

Mechanisms of hepatic angiogenesis

During chronic liver injury, angiogenesis can be interpreted by two basic phenomena. First, many liver diseases are characterized by inflammation and fibrosis leading to progressive tissue hypoxia which in turn stimulates angiogenesis[3,5]. Second, wound healing typical for CLDs is defined by an increase in the expression of some cytokines, growth factors with proangiogenic action[3,5,12,14]. Both pathways contribute to structural and functional chages in liver angioarchitecture[3,5,12,14].

Hypoxia and hepatic angiogenesis: After the first evidence about the parallel development of angiogenesis and fibrosis during liver injury their association with hypoxia has been described by many studies[5,19,24,35,44,47]. Indeed both in humans and in experimental models, chronic liver damage is defined by an increase in EC numbers and microvessels, the latter being particularly prominent in portal tracts and fibrotic septa[3,5,35,47,48]. On the other hand, the co-localization of VEGF-A expressions with hypoxic areas and a parallel increase of VEGF-A expression and hypoxic areas during progression of fibrosis have been supported that hypoxia; angiogenesis and fibrosis are closely related[3,5,19]. Moreover, the response to hypoxia and VEGF expression not only encountered in ECs but also in hepatocytes as well as HSCs in the progression of fibrosis. Currently with accumulated data, it is possible to conclude that hypoxia is one of the most important stimulus to switch on the transcription of pro-angiogenic genes through the action of HIFs[1-4]. This is not surprising because angiogenesis is frequently encountered in any kind of wound healing response and, as previously suggested, in liver chronic activation of this response represents the principal impulsive force for deposition of ECM components, leading ultimately to cirrhosis[3,5,7,12,15,22,49,50].

In chronic liver injury progression of fibrosis by it self can favor the development of hypoxia. During this progression the deposition of fibrillar collagen (type Ⅰ) instead of sinusoidal collagen (type Ⅳ) leads to the formation of regenerative nodules of parenchyma, encircled and divided by fibrotic septa, and closely related with prominent changes in angioarchitecture[12,48,51]. The anatomical changes that follow the progression of fibrosis with an increased participation of the hepatic artery to the generation of sinusoidal blood allows to arterialisation of sinusoidal blood flow with higher oxygen concentration[48-51]. Accordingly, continuous capillarisation of sinusoids occurs and causes the loss of specific endothelial fenestrations[48-51]. This process together with the accumulation of fibrotic tissue provokes vascular resistance and diminishes the transport of oxygen to the parenchyma leading to up-regulation of pro-angiogenic mechanisms *via* hypoxia[22,48]. Recently it has been noted that the pattern of fibrosis (bridging fibrosis, peri-cellular fibrosis, centrilobular fibrosis) can affect the extent of angiogenesis and favor progression of liver injury, representing at the same time a key limiting factor for fibrosis reversibility[49,50].

In liver, inflammation is a biological response for activation of healing process following cellular injury[51]. However prolonged inflammation in chronic liver injury may affect the extent of angiogenesis and favors fibrosis progression. During injury HSCs may be activated and release inflammatory mediators[3,5,21,49-51]. These mediators can elicit angiogenesis *via* the induction of HIF-1α and HIF-1-dependent transcriptional activity[3,5,15,21,49-51].

All of these findings reveal the strong relation between angiogenic and inflammatory pathways during chronic liver injury. In hypoxia, HIF-1 not only induces angiogenesis but also stimulates the NF-k pathway, thus induces inflammation[24,51]. Moreover, both events are capable of supporting each other[51] (Figure 1). Therefore neovessels themselves express chemokines as well as adhesion molecules and stimulate the recruitment of inflammatory cells that allows to the prolongation of the inflammatory response[48,51]. Consequently angiogenesis in the earlier phases of liver damage contributes to the progression from acute to chronic inflammation[48,51].

Inflammatory cells in hepatic angiogenesis: Activated Kupffer cells that reside in hepatic sinusoids may contribute to angiogenesis by their ability to release of reactive oxygen species (ROS) and platelet-activating factor (PAF)[51]. An increase of ROS and nitrogen species may induce new vessel formation by the stimulation of TNF-, NO, HIF-1 and VEGF expressions[51-54]. TNF- an inflammatory cytokine that is primarily produced by macrophages can also stimulates the mitogen-activated protein kinase (MAPK)/ERK pathway that can also stimulates angiogenesis[51]. PAF induces nuclear factor (NF)-k activation that stimulates angiogenic factors including VEGF[51,53,54].

Mast cells are involved in the regulation of angio-

genesis by releasing mediators (histamine, heparin, tryptase, TNF, TGF-1, cytokines, interleukins) and they also affect the number of ECs , including ECs covering liver sinusoids[51,55,56].

The inflammatory response is not solely induced *via* the activation of cells that reside in the liver[48,51]. As mentioned above during tissue injury increased vascular permeability promotes chemokine-mediated recruitment of inflammatory cells. Besides their role in angiogenesis chemokines may also regulates the influx of leucocytes[51]. Leucocytes can produce angiogenic factors [VEGF, PlGF, PDGF, FGF, Ang-2, TGF-1, epidermal growth factor (EGF)] and various interleukins[51,55]. Several mediators generated during chronic liver injury such as hepatocyte growth factor (HGF) can also contribute to angiogenesis[51,54,55,57].

HSCs in hepatic angiogenesis: During chronic liver inflammation activated HSCs express different chemokines which are also capable to stimulate angiogenesis[49,51,57-59]. Therefore, the cellular and molecular relations between fibrosis and angiogenesis involve the role of activated HSCs[48,60]. They constitute a crossroad between inflammation, fibrosis and angiogenesis[22]. HSCs are sensitive to hypoxia, can be modulated by both cytokines and chemokines during liver injury[22,35,61]. The expression of chemokines by HSCs is controlled by products of oxydative stress, proteases, growth factors and pro-inflammatory cytokines[51]. HSCs can also regulate sinusoidal calibre and blood flow thereby modulates hepatic microvascular dynamics[51]. Once activated HSCs act as proangiogenic cells and may respond to hypoxia in a HIF-1α related pathway through the increase of VEGF, Ang-1, and their receptors VEGFR-2 and Tie-2[22,35,48,61]. On the other hand, they can be activated by both VEGF and Ang-1[22,48]. VEGF stimulates their proliferation, migration, and chemotaxis[48]. In particular, oriented migration of activated HSCs in response to either hypoxia or proangiogenic mediators has been described as a biphasic mechanism[21,22,35,48,62]. This mechanism first switched on by ROS and proceeds through activation of Ras/ERK and c-Jun-NH2-terminal kinase isoforms (JNKs)[21]. This is followed by a delayed stage of migration that is related to up-regulation of VEGF expression, leading to the chemotactic action of extracellularly released VEGF[21]. These findings are in accordance with the results of a previous study that showed activated HSCs that express both VEGF and Ang-1 were not found in the established bridging septa which is in contrast to the presence of such cells at the edge of incomplete fibrotic septa[22,48]. This finding demonstrates the presence of two different phases of angiogenesis in CLDs[48]. An early phase that is regulated by activated HSCs and a later phase in the large and mature septa with expression of proangiogenic agents only in ECs reflecting the stabilization of newly formed vessels[21,22,48]. Accumulating evidences suggest that HSCs may operate their pro-angiogenic role in a hypoxia-independent manner by responding to a number of stimuli[21,22,48,51]. These include pro-fibrogenic polypeptide mediators like mainly PDGF and leptin (see in adipokines section)

PDGF can promote an angiogenic phenotype of HSC. This phenotype regulates the formation of vascular tube and enhanced coverage of sinusoids *in vitro* and *in vivo*, respectively[61,62]. HSCs together with PDGF contribute to the modulation of microvascular structure and function in liver parenchyma[63,64]. PDGF might play also an additional pro-angiogenic role in vascular remodeling in cirrhosis[62]. A previous study in experimental biliary cirrhosis model emphasized that cholangiocytes and HSCs in response to PDGF have been produced and released Hedgehog (Hh) ligands that contained in microparticles[64]. In normal circumstances the effect of low level of Hh ligands released from immature ductular cells antagonized by Hh interacting protein (HIP) expression by quiescent HSCs and sinusoidal ECs[64]. During chronic liver damage HIP expression is supressed and stimulation of ductular-type progenitor cells may lead to PDGF-BB release[22,64]. This event may ends with the production of Hh ligands by HSCs and ductular cells, which may influence the gene expression of sinusoidal ECs resulting in capillarisation of sinusoids and release of NO, then contributing to vascular remodeling in cirrhosis[22,64]. Activated HSCs also induce some inflammatory mediators and the recruitment of inflammatory cells, together with hypoxia stimulate the proangiogenic factor expression from cells in the microenvironment[48]. Moreover HSCs are capable to express a large number of chemokines[65]. These include the CC chemokines (CCL2, CCL3, CCL5)and the CXC chemokines (CXCL8, CXCL9, CXCL10, CXCL12)[65,66]. Many of them have been related to liver fibrosis in CLDs[65-67]. The CXC chemokines also manipulate angiogenesis during iniation and progression of fibrosis[65-67]. Whereas CXC chemokines containing the ELR motif (ELR+) stimulate angiogenesis, chemokines devoid of this motif (ELR-) inhibit it[65-67]. For instance the direct interference of angiostatic CXCR3 ligand CXCL4 with VEGF signaling have been demonstrated in liver angiogenesis during the progression of fibrosis[68]. The evidences indicating the counter-regulation of VEGF-driven angiogenesis by CXCR3 ligand CXCL9 also decribed both *in vivo* and *in vitro* experimental studies[69,70]. As pointed out by Yasar *et al*[65] all of these new findings set the stage for additional investigation of ELR- chemokines as promising therapeutical targets in CLDs.

Other contributors of hepatic angigogenesis

Adipokines: In CLDs, besides their roles in the regulation of fibrogenesis, metabolic and inflammatory processes, adipokines have been shown to be important regulators of angiogenesis[9,10,48,71-77]. They manipulate and induce the agents responsible for the modulation of angiogenesis[48,72,78,79]. Although, Leptin, visfatin [pre-B cell colony-enhancing factor

1/nicotinamide phosphoribosyltransferase (PBEF-1/Nampt)], chemerin [retinoic acid receptor responder protein 2 (RARRES2), tazarotene-induced gene two protein (TIG2) or RAR-responsive protein (TIG2)], and resistin have been found to stimulate angiogenesis, adiponectin attenuates the new vessel formation[48,71-77,80]. Until now, the effect of vaspin (visceral adipose tissue-derived serine protease inhibitor, SERPINA12) on ECs has been described in a few study[48,81].

Leptin can directly up-regulate VEGF, Ang-1 and monocyte-chemoattractant protein 1 (MCP-1 or CCL2) in human HSCs[71,80]. An experimental study has been demonstrated that leptin has been operated the pro-angiogenic actions by recruitment/stabilization of HIF-1α and nuclear translocation of HIF-1[71]. Leptin increases gene expression of the VEGF and Ang-1[71]. After induction of fibrosis in rat the specific leptin receptor ObR was found to be co-localized with VEGF and αSMA[71,82]. More recently both leptin and PDGF-BB up-regulated VEGF in HSCs[48,71,82]. Pro-angiogenic role of leptin involves both activation of the mammalian target of rapamycin (mTOR) pathway and generation of ROS *via* NADPH-oxidase, the latter being relevant for HIF-1α stabilization but not for mTOR activation[71,82,83].

An interesting adipokine, is apelin that has been reported to be markedly increased in cirrhotic livers[84,85]. It is overexpressed by HSCs and the use of an antagonist of the apelin receptor inhibits both angiogenesis and hepatic fibrosis[85]. In HSCs exposure to recombinant apelin allows to an increased synthesis of type Ⅰ collagen and PDGF-β receptor. It expression is also up-regulated by hypoxia[86].

Endoplasmic reticulum stress and consequent unfolded protein response: The changes in microenvironment, such as hypoxia provoke to cell and consequently endoplasmic reticulum (ER) stress that result in the unfolded protein response (UPR) to maintain cellular homeostasis[87-89]. This response takes place in a number of processes in liver including angiogenesis[88,89]. The role of angiogenesis and ER stress in the pathogenesis of CLDs is reported[89,90]. Although the relation between the UPR and angiogenesis is not fully described evidences indicate that ER stress induces angiogenesis in CLDs[88-90]. It is suggested that further studies concerning the role of the UPR in new vessel formation could provide new anti-angiogenic targets that inhibit specific ER stress mediators[90].

Neuropilins: Neuropilins (NRPs) were originally identified as receptors for class 3 semaphorins, polypeptides with key roles in the nervous system[91-94]. Following studies have been revelead that NRPs are also contribute in many signaling pathways such as PDGF, TGF- and VEGF signaling[91,93,94]. NRP overexpression was also observed in specimens from CLDs and some studies revealed a role for NRP-1 as a regulator of angiogenesis (see above) and may be involved in the progression of liver cirrhosis[39,93]. In some experimental models of liver fibrosis a NRP-1 neutralizing antibody diminished VEGF responses of ECs in cultures[94]. Because antibodies to NRP-1 are under investigation in phase Ⅰ trials, it is suggested that these antibodies might be available for future antifibrotic therapies that targets PDGF, TGF-β and VEGF in CLDs[91,94,95].

ANGIOGENESIS IN CLDS

Chronic viral hepatitis

Althought in chronic viral hepatitis (CVH), the molecular mechanisms of angiogenesis have not been fully discovered, accumulated evidences suggest that angiogenesis plays an important role in the progression of disease[44]. The stimulation of migration and proliferation of ECs by sera from patients with CVH [especially in hepatitis C virus (HCV infection)]; the frequent occurence of angiogenesis in CVH when compared to normal tissues and a positive correlation between the intensity of angiogenesis and activity of inflammation are important findings that support an active role of angiogenesis in CVH progression[6-8,96-98]. Other evidences about the contibution of angiogenesis in the progression CVH are the up-regulation of HGF (that induce proliferation of ECs *via* VEGF pathway), the increase of PDGF expression and its receptors in sinusoidal and HSCs, the overexpression of inducible NO synthase leading to NO overproduction[44,48,78,99,100]. Moreover the amount of angiogenesis and hepatic VEGF expression is associated with the grade of fibrosis indicating the potential role of angiogenesis in the progression of fibrosis in CVH[47,72,97,99]. In patients with CVH as well as in cell cultures an increase in Hh activity was found to be related with poor prognosis suggesting that blocking Hh pathway may be another approach in the inhibition of fibrosis in CVH[101].

During hepatitis, HCV may activates several pathways and systems that are implicated in angiogenesis. The core E1, NS3 and NS5A proteins induce mithochondrial dysfunction resulting to generation of new ROS that leads to induction of HIF-1 and up-regulation of VEGF and placental growth factor (PlGF)[102,103]. The first evidence about the modulation of HIF-1α in HCV infection has been described in 2007 by Nasimuzzaman *et al*[104] that demonstrated non-structural proteins have the capacity to induce HIF-1α, even in normoxia. The induction of oxydative stress activates several kinase pathways (such as PI3K and MEK) leading to an increase of HIF-1α synthesis that may results in angiogenesis[104,105]. More recently Abe *et al*[106] observed that under hypoxia, transfection with the HCV Core protein activated NF-kβ signaling (by an unknown mechanism) resulting in transcriptional up-regulation of HIF-1α mRNA and eventually augmented HIF-1α. However, these results were found in cell lines already containing HCV. Therefore the relevance of the Core protein overexpression in a natural HCV infection

remains to be elucidated. It is also demonstrated that proangiogenic markers are increased in sera from patients with HCV hepatitis and their concentrations significantly diminished after therapy (pegylated interferon and ribavirin)[48,107,108]. These findings indicate that proangiogenic markers may be valuable in the follow-up of the disease and response to treatment in HCV infection[48]. Besides, these data also highlight the close association of angiogenesis, inflammation and fibrosis during the progression of HCV hepatitis. Recently it is reported that VEGF-A expression promoted by HCV results in the loose of cellular polarization and increased viral entry[109,110]. In HCV infected cell cultures, hepatocytes exposed to VEGF-A inhibitors regain their polarization and viral infection was reduced suggesting that VEGF-A inhibitors may be another treatment option in HCV hepatitis[107-111]. Interestingly, Rowe *et al*[112] observed a reduced VEGFR-2 activation of sinusoidal ECs and a concomitant increase in bone morphogenetic protein 4 (BMP4) expression in patients with HCV highlighting that EC-hepatocyte crosstalk may reduces the activity of VEGF inhibitors in HCV infection.

Although it will be describted later, it is worhty to note that steatosis that is frequent during the course of HCV hepatitis, by provoking the cellular injury evokes a wound healing which is closely related with angiogenesis. Indeed CD34 expression are more frequent in liver tissues with steatosis from HCV infected patients[6,48]. The number of microvessels are increased parallel to the severity of steatosis[6,48]. In high grade steatosis, angiogenesis is higher in steatotic areas of parenchyma and primarily observed in periportal zone (zone 1)[6,48]. As HCV hepatitis is also recognised as metabolic disease as well, various adipokines are considered to be involved in their progression[48,96,111,113]. Besides their role in the regulation of fibrogenesis, metabolic events and inflammatory conditions adipokines are also important in the manipulation of angiogenesis[48]. Serum leptin levels are higher in HCV infection when compared to controls[96,111]. There is also a positive correlation between level of leptin and the grade of fibrosis[60,99]. These results point out that, leptin contributes to angiogenesis that takes place during the evolution of HCV hepatitis. Besides, a few studies in HCV hepatitis with new adipokines (visfatin and chemerin and vaspin) that exert pro-angiogenic activities also performed[79,99,114,115]. Interestingly, although a negative correlation between visfatin and the severity of angiogenesis described in females patients, such an association did not observed in males[115]. On the other hand, vaspin was significantly down-regulated, but increased in higher stages of fibrosis[114]. In these patients vaspin was positively related to angiogenesis with a strong association in males[114]. Accordingly, it is suggested that the function of some adipokines in neovessel formation may be different depending on gender[115].

HBV virus it self can influence angiogenesis. Of the different proteins coded in HBV genome, the regulatory protein X (Hbx) was found to stimulate angiogenesis directly through the both activation and stabilization of HIF-1[116]. HbX also promotes the induction of Ang-2 and contribute to angiogenesis[116]. More recently in viral induced HCC Yen *et al*[117] observed that Hbx protein also activates m-TOR and VEGF-A pathways *via* up-regulation of IKKβ[103]. Although the precise mechanism of IKKβ stimulation by Hbx needs to be further investigated it seems to represents a potential new pathway in angiogenesis that occurs in HBV infection[103,118].

Angiogenesis and portal hypertension

In addition to gross hepatic structural disorders related to diffuse fibrosis and formation of regenerative nodules, the morphological and functional rearrangements of the hepatic vasculature are shown to contribute in portal hypertension (PHT). A hyperdynamic splanchnic circulation is an important constituent of the portal hypertensive syndrome[51,119-121]. An increase of blood flow in splachnic organs that drain into the portal vein leading to the augmentation of portal venous flow provokes the portal hypertensive syndrome[119,120]. Traditionally, it is accepted that this increase has been related to an increased production of endogenous vasodilators together with a decreased vascular reactivity to vasoconstrictors and the development of collaterals has been considered a mechanical consequence of an increased blood pressure[120,122]. However, recent studies object to these opinions by demonstrating that angiogenesis, contributes to the evolution and perpetuation of splanchnic hyperemia and the development of portal-systemic collaterals[120-124]. Many studies revealed that angiogenesis induced by VEGF, PDGF and PIGF actively modulates the occurence of hyperdynamic splanchnic circulation[51,125,126]. It has been also observed that the formation of portal–systemic collaterals are also induced by VEGF and PlGF dependent angiogenesis[51,125,127].

It should be noted that in mice with PHT rapamycin by inhibiting mTOR pathway has been prevented mesenteric neoangiogenesis and reduced the splanchnic blood flow[51,128]. Recently, the effect of PPAR activation in the improvement of hepatic EC dysfunction and the dimunition of fibrosis in PHT developed in cirrhotic rats, suggested that PPARα may represent as a new therapeutic strategy[51,129].

Cannabinoids (CBs) inhibit angiogenesis, but little is known about their influences in CLDs. In an experimental study a CB2 agonist alleviated PHT, severity of angiogenesis, portosystemic shunts, and liver fibrosis[130]. It is suggested that the vascular effects might be mediated by COX and NOS down-regulation and concluded that targeting CB2 receptors might be useful in the control of PHT[129].

Briefly angiogenesis participates in the pathogenesis of PHT by modulating activation of HSCs and fibrogenesis, splanchnic vasodilation and formation of

portal-systemic collaterals.

Angiogenesis in fattly liver diseases

Fatty liver diseases (FLDs) possess a broad spectrum of histopathological findings that ranges from steatosis through non-alcoholic fatty liver disease(NAFLD) to non-alcoholic steatohepatitis (NASH) that can cause cirrhosis[22,48,131]. Although the mechanism of NASH is not completely understood, currently it is pointed out that its pathophysiology should be recognized as multifactorial, including a link between changes in microvasculature and the progression of fibrosis[107,131,132].

Tissue damage related both to lipotoxicity and to fat accumulation results in reduced sinusoidal perfusion as well as changes in sinusoidal architecture. Cytokines activated by lipotoxicity mediates the migration of the inflammatory cells leading to inflammation[107,133]. In inflammatory foci these cells can contribute to angiogenesis. As the disease progress large, fatty hepatocytes, together with inflammation and related perivascular fibrosis constrict the sinusoidal lumens and impair sinusoidal perfusion leading to hypoxia[48,133]. All of these events provoking liver damage can innate angiogenesis.

Recent studies have been demonstrated that angiogenesis in NASH is significantly increased when compared to steatosis and control tissues[72,134]. Moreover, the grade of angiogenesis has been found to be related to the grade of fibrosis both in humans and in experimental studies[73,134]. It is also observed that during the evolution of disease the progression patterns of fibrosis and angiogenesis are parallel[73,134]. All of these findings support that angiogenesis contributes to the progression of fibrosis in NASH[132]. Besides, in a recent study by employing hepatocyte-specific-VHL and HIF-1 or HIF-2 mouse mutants demonstrated that mice with liver conditional disruption of VHL have spontaneous fatty liver and inflammation progressing to fibrosis in a HIF-2 dependent way[135,136]. These observations suggest that in addition to its role in angiogenesis hypoxia together with HIF-2 play a critical role in inflammation that is also necessary for the evolution of FLD, as proposed by the two hit hypothesis[135-137]. More recently in an elegant study, Ciupińska-Kajor *et al*[10] detected that in morbid obesity the activation of angiogenesis took place at an early stage, when compared to nonobese patients in whom the activation took place at the level of NASH. Besides, in this group the expression of angiogenic factors has been correlated with the progression of fibrosis independently from the development of NASH. These interesting findings highlight that in morbid obesity the progression of liver fibrosis is leaded by angiogenesis independently from steatosis[48].

Recently it is observed that similar to HCV hepatitis, patients with simple steatosis had low level of serum leptin when compared to patients with NASH[10,48]. It has been indicated that leptin could mediate angiogenesis during the progression of steatosis[48]. Increased serum level of new angiogenic adipokines, vaspin and chemerin have been also described in NAFLD[10,138,139].

Recently a role of angiotensin-Ⅱ (AT-Ⅱ) in the progression of CLDs, including NASH has been suggested[140-143]. In an experimental model of NASH , inhibition of AT-Ⅱ by a AT-Ⅱ-type-Ⅰ receptor blocker (ARB) was significantly decreased the formation of new vessels[140,144]. Moreover it is observed that liver fibrogenesis might be impaired by both angiotensin converting enzyme inhibitor (ACE) and ARB along with inhibiton of the HSCs[142]. These results indicate that anti-fibrotic effects of these agents were provided by their dual influence both in angiogenesis and HSCs[144,145]. It is also demonstrated that in liver the expressions of AT-Ⅱ, TGF-β and VEGF were inhibited by DRI (direct renin inhibitor-Aliskiren) indicating an important role of renin during liver fibrogenesis in NASH[144]. Because these agents are frequently used in practice, it is suggested that they could be effectively used as a new strategy against angiogenesis that takes place in the fibrogenic progression of steatohepatitis in the future.

Angiogenesis in alcoholic liver disease

Evidences also indicate that long-term ethanol consumption is closely related to angiogenesis by means of finely coordinated action of various mediators in liver[146,147]. Ethanol up-regulates VEGF and VEGFR-2 and stimulates angiogenesis in the rat liver after 36 weeks of consumption[146,147]. Moreover, higher concentrations of Ang2 and VEGF-A in alcoholic liver disease (ALD) patients as compared to controls were found. It is proposed that angiogenesis-related biomarkers are useful in the noninvasive monitoring of the ALD course. More recently it is observed that alcohol also induces angiogenesis *via* PECAM-1[147]. It is suggested that these molecular insights could contribute to the evolution of new anti-angiogenic treatment strategies.

In conclusion, angiogenesis contributes to the progression of fibrosis in CLDs. Vascular remodeling leading to capillarization of the sinusoids with generation of intrahepatic shunts characterize hepatic angiogenesis. These changes in angioarchitecture give rise to an increase in vascular resistance and a decrease of hepatocyte perfusion leading to hypoxia. This is the one of the most important stimulus to switch on the transcription of pro-angiogenic genes through the action of HIFs. Cellular and molecular mechanisms implicated in the interactions between angiogenesis and fibrosis and liver cells including hepatocytes, ECs, and activated HSCs have been described. HSCs may constitute a crossroad at the interaction between inflammation, angiogenesis, and fibrosis. Metabolic abnormalities, steatosis and adipokines, may also influence angiogenesis, consequently inflammation and fibrosis.

The relationship between angiogenesis and fibrosis progression in CLDs indicates that determination of

proangiogenic factors may be a useful noninvasive approach in follow-up of both disease progression and response to therapy. Although anti angiogenic therapy may be a promising in the prevention of fibrosis in CLDs, it should be well balanced because an excessive blockage may prevent wound healing response. Finally there are still large gaps in our understanding and ability to treat angiogenesis during the progression of CLDs and clinical trials on a large number of patients are needed.

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REVIEW

Cirrhosis in children and adolescents: An overview

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Abstract

Several conditions, especially chronic liver diseases, can lead to cirrhosis in children and adolescents. Most cases in clinical practice are caused by similar etiologies. In infants, cirrhosis is most often caused by biliary atresia and genetic-metabolic diseases, while in older children, it tends to result from autoimmune hepatitis, Wilson's disease, alpha-1-antitrypsin deficiency and primary sclerosing cholangitis. The symptoms of cirrhosis in children and adolescents are similar to those of adults. However, in pediatric patients, the first sign of cirrhosis is often poor weight gain. The complications of pediatric cirrhosis are similar to those observed in adult patients, and include gastrointestinal bleeding caused by gastroesophageal varices, ascites and spontaneous bacterial peritonitis. In pediatric patients, special attention should be paid to the nutritional alterations caused by cirrhosis, since children and adolescents have higher nutritional requirements for growth and development. Children and adolescents with chronic cholestasis are at risk for several nutritional deficiencies. Malnutrition can have severe consequences for both pre- and post-liver transplant patients. The treatment of cirrhosis-induced portal hypertension in children and adolescents is mostly based on methods developed for adults. The present article will review the diagnostic and differential diagnostic aspects of end-stage liver disease in children, as well as the major treatment options for this condition.

Key words: Cirrhosis; Liver diseases; Nutrition; Pediatric patients; Portal hypertension

Core tip: The investigation and management of pediatric cirrhosis presents several challenges. The etiology of the condition may vary according to patient age. In many cases, cirrhosis is a predictable consequence of the progression of several chronic liver diseases, such as biliary atresia, although it may also be detected when splenomegaly is discovered on routine examination, or during the investigation of conditions such as hypersplenism, anemia, thrombocytopenia, leukopenia, petechiae and/or ecchymosis. The present article will discuss the diagnostic and treatment aspects of cirrhosis in children and adolescents.

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INTRODUCTION

Harvey Cushing - "A physician is obligated to consider more than a diseased organ, more than even the whole man - he must view the man in his world".

General considerations

Cirrhosis is a diffuse process characterized by fibrosis and nodular regeneration, which lead to the disorganization of liver architecture. Cirrhosis was long thought to be irreversible and associated with limited life expectancy. However, it is now considered a dynamic condition, which can be reversed if adequately treated.

According to data from the Brazilian Unified Health System, between 2001 and 2010, liver diseases were the eighth leading cause of death in Brazil[1]. Cirrhosis was the main cause of hospital admissions and death from liver disease in Brazil, especially in the South region of the country. Little is known about its incidence in children.

Studies of the natural history of cirrhosis have found that the disease tends to present with a silent clinical course, followed by the onset of liver dysfunction and portal hypertension. The most important predictor of decompensation is the increase in hepatic venous pressure gradient (HVPG), which is seldom measured routinely in children and adolescents. In clinical practice, mortality risk is generally estimated on the basis of albuminemia, MELD (Model for End-Stage Liver Disease)/PELD (Pediatric End-Stage Liver Disease)/Child-PughTurcotte scores and body mass index. Advances in diagnostic and treatment technology, especially liver transplant surgery, have contributed significantly to the management of these cases. Currently, the majority of children diagnosed with cirrhosis in the first years of life can grow, develop and reach adulthood.

Biliary atresia and inherited syndromes of intra-hepatic cholestasis are the most frequent causes of chronic liver disease in children[2]. The most common causes of cirrhosis in the first years of life are biliary atresia and genetic-metabolic diseases, whereas in older children, cirrhosis is usually caused by chronic viral hepatitis and autoimmune diseases (Table 1). Cirrhosis can be classified in several ways, based on morphological, histological, etiological and clinical criteria. As cirrhosis is the final stage of several types of progressive liver disease, etiological classification is often crucial for treatment planning. Despite the long list of possible etiologies shown in Table 1, the cause of cirrhotic disease is not always possible to determine.

Table 1 Diseases potentially resulting in cirrhosis in children and adolescents

Biliary obstruction
Biliary atresia
Choledochal cysts
Gallstones
Bile duct stenosis
Familial intrahepatic cholestasis
Alagille syndrome
FIC1 deficiency (ATP8B1)
BSEP deficiency (ABCB11)
MDR3 deficiency (ABCB4)
Defects of bile acid synthesis
Hepatotropic viral infections
Hepatitis B and D
Hepatitis C
Hepatitis E
Inherited genetic-metabolic diseases
α-1-antitrypsin deficiency
Glycogenosis type III and IV
Galactosemia
Fructosemia
Tyrosinemia type 1
Wilson's disease
Mitochondrial hepatopathies
Late cutaneous porphyria
Cystic fibrosis
Hemochromatosis
Wolman disease
Drugs and toxins
Total parenteral nutrition
Isoniazid
Methotrexate
Vitamin A intoxication
Autoimmune diseases
Autoimmune hepatitis
Primary sclerosing cholangitis
Vascular alterations
Budd-Chiari syndrome
Veno-occlusive disease
Congenital cardiopathy
Congestive heart failure
Constrictive pericarditis
Other: Fatty liver disease, Neonatal hepatitis, Zellweger disease

As a result, the condition is considered cryptogenic in 5%-15% of cases. Cryptogenic cirrhosis in pediatric patients may result from the progression of fatty liver disease or from the effects of complex metabolic syndromes, such as mitochondriopathies[3].

Biliary atresia

Biliary atresia (BA) occurs exclusively in childhood, and is the most common cause of chronic cholestasis and liver transplantation in children. It occurs in the first weeks of life and is characterized by complete obstruction of the biliary tract. Portal hypertension and biliary cirrhosis tend to occur early in the course of illness, and can be detected by 2 to 3 mo of age. Two forms of the disease have been identified: the congenital or "fetal" form, which accounts for 10% to 20% of cases of BA, and the perinatal or "acquired" form, which is responsible for 80% to 90% of cases. The fetal form usually manifests as jaundice at birth, and,

in 15%-30% of patients, may also involve extrahepatic anomalies (vascular malformations, variant abdominal organ positioning, heart disease, *etc.*). In acquired BA, jaundice occurs later, after the first or second week of life, resulting in a jaundice-free period between the onset of physiological jaundice and biliary obstruction. Congenital extrahepatic anomalies are rare in this form of the disease. Studies involving the surgical removal of biliary obstruction have revealed that, in most cases, the preformed bile ducts are affected by inflammatory processes, the causes of which are still poorly understood. BA may represent a phenotype resulting from factors which lead to biliary obstruction and include developmental anomalies, infections, vascular alterations and exposure to toxins[2,4,5]. Treatment for this condition is surgical (Kasai portoenterostomy and its modifications, performed before the first 8 wk of life). The degree of restoration of biliary flow is inversely proportional to the age when surgery is performed. Without treatment, patients do not generally survive past 18-24 mo.

Choledochal cysts

This condition consists of congenital dilatation of the biliary ducts. Choledochal cysts are rare, with a prevalence of 1 per 13000-15000 live births in Western countries, although they are more common in Japan. Choledochal cysts are more common in females (5:1) and can be diagnosed antenatally by ultrasound. The cysts can be classified into five types: Ⅰ, cystic dilatation of the common bile duct; Ⅱ, diverticulum of the common bile duct; Ⅲ, choledochocele; Ⅳ, multiple cysts; and Ⅴ, intrahepatic fusiform dilatation[6]. Most patients present with the typical symptom triad of abdominal pain, jaundice and palpable masses in the right upper quadrant. Surgical treatment consisting of cyst excision and bilioenteric anastomosis have produced excellent results, although a small percentage of patients may develop cholangiocarcinoma in the remaining biliary tract.

Alagille syndrome

Alagille syndrome is the most common cause of familial progressive intrahepatic cholestasis[7], and occurs in approximately 1:100000 live births. Most patients have mutations in the *JAG1* gene, located on the short arm of chromosome 20. The syndrome is diagnosed on the basis of clinical symptoms, which can be difficult to detect in the first months of life, especially if the clinical picture is not yet clear. Histological examination may reveal a reduction of interlobular bile ducts in addition to cholestasis. Its main clinical features are cholestasis, a characteristic facies, cardiac abnormalities, vertebral arch defects and posterior embryotoxon. Supportive management is required in approximately 50% of cases. Alagille syndrome progresses to secondary biliary cirrhosis in 20%-25% of cases. The differential diagnosis must include other causes of ductopenia such as alpha-1-antitrypsin deficiency; the Zellweger, Ivemark and Williams syndromes; cystic fibrosis; chromosomal alterations (trisomy 18 or 21); and HIV.

Inherited progressive cholestatic syndromes

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of hereditary autosomal diseases characterized by mutations in genes governing intrahepatic biliary transport, comprising PFIC 1 (ATP8B1), PFIC 2 (ABCB11) and PFIC 3 (ABCB4). These conditions manifest in the first year of life, usually as cholestasis and its associated consequences. These are rare but universally occurring conditions, whose exact prevalence is unknown. Although phenotypes may vary, the following clinical characteristics are often present: cholestatic jaundice, choluria and hypocholia, severe itching in the first months of life, delayed weight gain, malnutrition and progression to cirrhosis and associated complications (Table 2).

Viral hepatitis

Hepatitis B/D are the most common viral causes of cirrhosis in children and adolescents. Although Hepatitis C may also be acquired in childhood, it only tends to lead to cirrhosis later in life[8]. According to a study of the population-based prevalence of infections by the hepatitis B and C viruses (HBV and HCV), conducted between 2005 and 2009 in all Brazilian capitals and the Federal District, the frequency of viral hepatitis B and C in people between the ages of 10 and 69 was 7.4% and 1.38% respectively, which is consistent with low endemicity of these conditions[9].

Few studies have attempted long-term outcome of children with hepatitis B[10,11]. At least 50% of children infected by the vertical route (mother-to-child) test positive for viral replication in adulthood. The use of medication (telbivudine, lamivudine or tenofovir) by HBsAG-positive mothers with high levels of viremia (serum HBV DNA 10^{5-7} IU/mL) during the last trimester of pregnancy reduces the risk of intra-uterine and perinatal transmission of HBV if given in addition to hepatitis B immunoglobulin (HBIg) and HBV vaccine[12].

The hepatitis delta virus consists of an RNA genome enveloped by the HBV surface antigen (HBsAg). The rates of progression to chronicity in hepatitis delta are similar to those observed in hepatitis B, since HDV depends on the persistence of HBV. Chronicity rates are high when infection is acquired in the neonatal period (80%-90%), intermediate (25%-50%) when it occurs between 1 and 5 years of age, and low when acquired in later childhood (2%-6%).

The natural history of hepatitis C in children is very different from that seen in adults, and although the progression of chronic hepatitis to cirrhosis is unlikely, it may even progress to hepatocellular carcinoma[13]. Since viral screening began to be used routinely in blood donors, vertical transmission became the main cause of hepatitis C in children. Vertical transmission occurs in approximately 5% of HIV-negative mothers and in up to 25% of mothers co-infected with HIV[14].

Table 2 Characteristics of progressive familial intrahepatic cholestasis

Disease	Relevant clinical aspects	Laboratory findings	Chromosome
PFIC1	Early jaundice and increasing pruritus. Extrahepatic clinical manifestations: chronic diarrhea, pancreatitis, deafness. Early cirrhosis and liver transplantation in the first years of life	GGT: Normal ALP: high Cholesterol: ↑	18q21-q22
PFIC2	Early jaundice. Progression to cirrhosis and ductopenia in the first years of life. Frequent cholelithiasis. Possible complications include liver and bile duct cancer. No extrahepatic symptoms. Liver transplantation in the first years of life	GGT: Normal ALP: v. high Cholesterol: ↑	2q24
PFIC3	Variable phenotype and progression to cirrhosis in adolescence. Cholelithiasis. Liver transplantation in the first years of life. No extrahepatic symptoms	GGT: High ALP: v. high Cholesterol: normal	7q21

PFIC: Progressive familial intrahepatic cholestasis; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase.

In a study conducted by Bortolotti *et al*[15], only 6 (1.8%) of the 332 children evaluated developed cirrhosis after a period of 10 years. The incidence of hepatitis symptoms and severe liver disease was low[15]. In a separate study performed in 80 Spanish and Italian children, only one presented with cirrhosis[16]. More recently, a study of 121 children with chronic hepatitis C and a mean age of 10 years found that cirrhosis was only present in 2% of the sample[17].

Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin (AAT) is a glycoprotein produced in large quantities in the liver to inhibit the neutrophil proteases associated with inflammation. The classical form of the disease is caused by homozygosity for the Z mutation ("PiZZ" genotype for *SERPINA1*). Over 100 mutations in the *AAT* gene have been described, although the Z mutant is that most closely associated with liver disease[18]. The clinical course is variable, and may involve neonatal cholestasis, liver dysfunction, liver failure and cirrhosis. Liver transplantation is often required. Approximately 20% of "PiZZ" patients develop cholestasis in the first weeks of life, and have a 5% risk of developing more severe forms of the disorder in childhood/adolescence. These patients are also more likely to develop hepatocellular carcinoma. There is no specific treatment or prevention against this condition, save for liver transplantation.

Wilson's disease

This autosomal recessive disease affects 1 in every 30000 live births. It is caused by mutations in the *ATP7B* gene (chromosome 13), which encodes the protein responsible for the metabolism, transport and biliary excretion of copper. The clinical presentation of Wilson's disease (WD) involves abnormal liver function tests, acute hepatitis, liver failure, portal hypertension, gallstones and cirrhosis. Copper accumulates in the liver, brain and cornea, and hepatic manifestations occur after the first 3 years of life. In adolescents, the diagnosis of WD is often based on signs and symptoms of chronic liver disease. Patients often have a family history of WD, and approximately 25% of cases are diagnosed as a result of the investigation of asymptomatic relatives. If left untreated, WD usually carries a bad prognosis.

Cystic fibrosis

This autosomal recessive disease occurs in approximately 1 in 2000-4000 live births in Caucasian populations. Cystic fibrosis (CF) is caused by mutations in the *CFTR* transmembrane regulator gene, which is expressed in the apical membrane of biliary epithelial cells. This condition leads to severe and progressive impairment in the respiratory and digestive systems. Most patients do not present with liver damage in the early phase of the disease, although hepatic impairment is present in 10%-30% of cases. In older children and adolescents, CF liver disease manifests as hepatosplenomegaly and/or portal hypertension. Gallstones and micro-gallbladder are the most common biliary abnormalities in these patients. Portal hypertension is the major hepatic complication in CF.

Fatty liver disease

Given the high prevalence of obesity and metabolic syndrome in the general population, non-alcoholic fatty liver disease has become the most common cause of liver disease in both adults and children[19]. It is a common, albeit under diagnosed, condition. Most cases are associated with obesity and type 2 diabetes mellitus. The risk of liver disease tends to increase with weight[20]. Most patients are asymptomatic, and the disease is often discovered during routine examinations (by ultrasound or biochemical screening). Progression to cirrhosis is not common in childhood, but may occur in young adults.

Autoimmune diseases

Autoimmune liver diseases include sclerosing cholangitis, primary biliary cirrhosis, and autoimmune hepatitis (AIH), with the latter being the most autoimmune liver condition in children and adolescents. Primary biliary cirrhosis is rare in this age range, although sclerosing cholangitis has been increasing in prevalence, often accompanied by inflammatory bowel disease.

AIH is a chronic inflammatory liver disease, with

variable onset and duration. The prevalence of this condition in children is not yet known. There are few publications regarding this disease in patients from South America. In Brazil, AIH is still considered unusual, accounting for only 5%-10% of cases of liver disease in the major medical centers of the country[21]. There are particular human leukocyte antigen (HLA) specificities associated with AIH in Latin Americans. HLA-DQ2 and DR52 seem to be risk factors, whereas DR5 and DQ3 appear to have a protective effect in this population[22]. The trigger of the autoimmune process is not yet known. AIH can be classified into two subtypes, according to the non-organ specific antibodies present: type Ⅰ (AIH-1) is associated with anti-nuclear (ANA) and anti-smooth muscle (ASMA) antibodies, whereas type Ⅱ (AIH-2) is associated with positivity for anti-liver/kidney microsomal (LKM-1) antibodies. AIH-2 manifests earlier, in the first years of life, while AIH-1 generally occurs in older children and adolescents[23].

Primary sclerosing cholangitis (PSC) is a chronic, progressive liver disease of unknown cause. There is strong evidence to suggest that PSC is the result of alterations in the immune response to certain antigens. Cases with a well-defined etiology (*e.g.*, cytomegalovirus infection, trauma, ischemic lesion) are diagnosed as "secondary sclerosing cholangitis". PSC is characterized by obliterative fibrosis of the intra- and/or extrahepatic biliary tree. Concentric fibrosis is very common, and may lead to narrowing of the bile ducts and, eventually, to biliary cirrhosis and its complications. Sclerosing cholangitis may present soon after birth, in which case it must be distinguished from other causes of neonatal cholestasis, or later, with manifestations ranging from asymptomatic to decompensated cirrhosis[24].

Mitochondrial diseases

Mitochondrial diseases, or mitochondriopathies, are inherited disorders of energy metabolism associated with a vast range of presentations, symptoms, severities and outcomes. Combined, they form one of the commonest groups of inherited metabolic diseases[25]. Mitochondrial diseases occur due to dysfunction of the respiratory chain (RC) with resultant cellular ATP deficiency, increased production of reactive oxygen species and toxic metabolites and, ultimately, cell death. Disorders of mitochondrial energy metabolism result from mutations of nuclear or mitochondrial DNA. The exact prevalence of hepatic mitochondrial disease is not known, although it has been estimated that 10%-20% of patients with RC deficiencies have hepatic dysfunction. The three main defects associated with liver disease are almost always fatal: deficiency of RC enzymes, DNA depletion syndrome and Alpers syndrome[26]. Liver involvement occurs frequently in childhood-onset mitochondrial disease, particularly in cases presenting in the neonatal period and early infancy[27]. Liver transplantation is contraindicated in children with severe, life-threatening multiorgan extrahepatic mitochondrial disease due to poor post transplant neurological outcome.

EVALUATION OF CHILDREN AND ADOLESCENTS WITH CIRRHOSIS

The assessment of pediatric cirrhosis involves a thorough investigation of the child's clinical history and physical examination findings (Table 3).

CLINICAL HISTORY

Investigation of the medical history of a child with chronic liver disease must cover not only the presence of previous hepatic disease, but also the history of blood or plasma transfusions, use of drugs and neonatal intercurrent clinical conditions such as cholestasis, infections, surgery and prolonged parenteral nutrition (PN). Neonatal cholestasis has been described in patients with alpha-1 antitrypsin deficiency. Prolonged PN can cause several types of liver damage which may progress to cirrhosis, especially in patients with intestinal failure[28]. The source of patient referral must always be investigated, as knowledge of the regional prevalence of certain diseases can facilitate investigation of the etiological factors involved in cirrhosis. The maternal history of systemic diseases such as hepatitis B or C must always be investigated. Additionally, in adolescents, the presence of tattoos or piercings must be investigated due to their association with hepatitis C virus infection. In patients with inflammatory bowel disease, the presence of sclerosing cholangitis and/or AIH must also be considered. When investigating the clinical history of older children, it is also important to inquire as to the family history of neuropsychiatric diseases and/or hemolytic anemia. A family history of such conditions may raise suspicion of WD, which tends to affect children older than 5 years[29]. Consanguinity and familial liver diseases must also be investigated. A history of jaundice in relatives may suggest the presence of PFIC. In children with pruritus associated with cholestasis and normal GGT levels, PFIC type 1 or 2 must be considered once other common causes of cholestasis have been ruled out.

CLINICAL FINDINGS

The clinical presentation of cirrhosis depends on the primary cause of liver disease and on whether the cirrhosis is compensated or decompensated. In up to 40% of cases, patients may be asymptomatic before liver failure occurs[30]. In decompensated cirrhosis, there may be a cascade of progressive complications such as gastrointestinal bleeding, ascites and hepatic encephalopathy[31]. The diagnosis is often predictable,

Table 3 Evaluation of children and adolescents with cirrhosis

Clinical history
Age, sex, ethnicity
Pregnancy and birth data: Adverse events during pregnancy, maternal serologies, birthweight, neonatal cholestasis, surgery, TPN
Signs and symptoms of systemic disease: anorexia, fatigue, muscle weakness, failure to thrive
Nausea, vomiting, abdominal pain, diarrhea, dyspepsia
Jaundice, pruritus, discoloration of urine and feces
Abdominal distension
Peripheral edema
Bleeding - nose, gums, skin, gastrointestinal tract
Bone pain, fractures
Adolescence: Menstrual history
Previous medical history: Jaundice, hepatitis, drug use, blood transfusions, inflammatory bowel disease
Social behaviors (adolescence): Use of alcohol or other drugs, tattoos, piercings
Family history: Consanguinity, liver disease, autoimmune disease
Physical examination:
General: Anthropometric data (malnutrition or obesity), fever
Skin and extremities: jaundice, flushing or pallor, spider nevi, telangiectasias, palmar erythema, clubbing of the nails, xanthoma, Terry's nails
Abdomen: Distension, prominent blood vessels, liver and spleen alterations (reduced liver size, splenomegaly)
Neurological alterations: Academic performance, sleep, asterixis, positive Babinski sign, mental status changes
Miscellaneous: Pubertal delay, gynecomastia, testicular atrophy, feminization

Adapted from: McCormick[57], 2011; Högler *et al*[35], 2012; Hsu[31], 2014. TPN: Total parenteral nutrition.

since it is part of the natural progression of chronic liver conditions such as BA. However, cirrhosis may already be present when diseases such as AIH are diagnosed. Approximately 44%-80% of children with AIH present with cirrhosis[32].

The symptoms of cirrhosis in children and adolescents are similar to those experienced by adults. Poor weight gain is a common early symptom of cirrhosis in pediatric patients, who may also present with nonspecific symptoms such as anorexia, fatigue, muscle weakness, nausea and vomiting. Abdominal pain may also be present as a result of ulcers, gastritis or gallstones[33]. The liver may be normal or reduced in size, and be covered by hardened or nodular tissue. The presence of ascites may also cause abdominal distension. The collateral vessels observed on the abdomen develop as a result of portal hypertension. Wide pulses and warm extremities are often indicative of high cardiac output[30]. The identification of classical signs of chronic liver disease, such as spider nevi, visible abdominal circulation and palmar erythema, may also contribute to the diagnosis. Other cutaneous manifestations of cirrhosis include susceptibility to bruising, telangiectasias of the face and back and recurring epistaxis[34]. Digital clubbing may also be detected by physical examination. Patients with chronic cholestasis may also present with pruritus, which can be so severe as to affect quality of life, as in the case of PFIC type 2 or Alagille syndrome. Several endocrine abnormalities may also be caused by the absence of hormonal conjugation or alterations in hormone metabolism, such as hepatic osteodystrophy, which may lead to fractures; rickets due to vitamin D deficiency; and spinal abnormalities[35]. A heart condition associated specifically with cirrhosis, termed "cirrhotic cardiomyopathy", has also been recently described[36]. Some patients may also present with structural heart abnormalities, especially those with BA, Alagille syndrome, glycogen storage disorders and mitochondrial disease[26].

INVESTIGATION

In children and adolescents with cirrhosis, clinical investigations should be performed so as to determine the cause of the disease and identify any complications. The investigation techniques employed may vary according to patient age, as etiological factors vary widely between age ranges. In infants, cirrhosis is most often caused by BA and genetic-metabolic diseases, while in older children, it tends to result from AIH, WD, alpha-1-antitrypsin deficiency and PSC[37].

Laboratory investigation of cirrhosis should be comprehensive and designed to detect both infectious and genetic-metabolic diseases. Imaging modalities - abdominal ultrasound, computed tomography, and magnetic resonance imaging (MRI) - can be used to detect more advanced liver disease, but are not sensitive for detection of hepatic fibrosis. Esophagogastroduodenoscopy (EGD) and abdominal ultrasound can identify both gastroesophageal varices and portal hypertension. Liver biopsy is still the "gold-standard" method for diagnosis of cirrhosis, and can also contribute to etiological investigations. HVPG measurements are not usually included in the assessment of pediatric patients[38]. Noninvasive methods such as transient elastography can also be used for the detection of fibrosis in patients with chronic liver disease[39,40]. Studies of pediatric patients have produced favorable evidence regarding the use of these techniques[40,41-43]. A study evaluating the use of transient elastography in children with chronic liver disease found that this method had good capacity to discriminate between significant fibrosis, severe fibrosis and cirrhosis[40]. Pediatric MR elastography has also begun to be used in recent years[44,45]. However, the reproducibility of these tests has yet to be evaluated in patients with cirrhosis of different causes[39].

ROUTINE BIOCHEMISTRY

Laboratory examinations are important for the assessment of liver function, the detection of hypersplenism and the identification of the causal factors underlying liver disease (Table 4). Aminotransferases are sensitive indicators of hepatocellular lesions. Alanine aminotransferase has been used as a specific

Table 4 Investigation of chronic liver disease and cirrhosis in childhood and adolescence

Hematology
Hemoglobin, leukocyte and platelet count, prothrombin time (INR)
Coombs test, blood type, Rh factor
Biochemistry
Bilirubins
Transaminases
Alkaline phosphatase
Gamma-glutamyl transferase
Albumin and globulin
25-OH vitamin D, parathyroid hormone, calcium, phosphorus, magnesium
Urea, creatinine
Lactic acid, fasting blood glucose, uric acid
Serum transferrin and ferritin saturation
Serum ceruloplasmin and copper, 24 h urinary copper (if age > 3 yr)
Alpha-1-antitrypsin phenotype
If ascites present
Paracentesis (in case of fever or sudden-onset ascites):
Cell count, albumin, total protein, neutrophil count
Amylase, cytology, PCR and mycobacterial culture (according to clinical suspicion)
Serum sodium, potassium, bicarbonate, chloride, urea and creatinine
Urinary sodium excretion
Immunology
Smooth muscle, mitochondrial, anti-nuclear, anti-LKM-1 antibodies
Hepatitis B antigen
Anti-HCV
α-fetoprotein
Immunoglobulins
HIV serology
Genetic-metabolic diseases
Metabolic screen (urine and serum amino acids, urine organic acids)
Genetic tests (if alpha-1-antitrypsin deficiency, Alagille syndrome, *etc*., suspected)
Sweat electrolytes test
Urine and serum analysis for bile acid and acid precursors (if PFIC suspected)
Bone marrow examination and skin fibroblast culture (if glycogen storage disease suspected)
Other:
Endoscopy (if prophylactic treatment is considered)
Abdominal ultrasound (computed tomography or MRI in selected cases)
Needle liver biopsy (if blood coagulation permits)
EEG (if neuropsychiatric changes present)

Adapted from: McCormick[57], 2011; Högler *et al*[35], 2012. LKM-1: Liver/kidney microsomal; PFIC: Progressive familial intrahepatic cholestasis; MRI: Magnetic resonance imaging; INR: International normalized ratio; HIV: Human immunodeficiency virus.

marker of hepatocyte injury[46]. In obstructive liver damage, levels of canalicular enzymes - alkaline phosphatase and gamma glutamil transferase (GGT) - are usually elevated, as are bilirubin concentrations. However, these enzymes are not associated with hepatic synthesis and have no prognostic value[31]. Presence of hypoalbuminemia and deficiency of coagulation factors correlate well with reduced hepatic synthesis, and are better predictors of survival[39]. An increased prothrombin time, despite vitamin K administration, suggests impaired liver synthesis and decompensated hepatocellular disease. Low levels of factors Ⅴ, Ⅶ, XIII or plasminogen are indicative of poor prognosis[31].

Abdominal ultrasound

Ultrasound is the ideal imaging method for the initial investigation of chronic liver disease in children and adolescents. The size of the spleen may provide indirect evidence of the presence or absence of portal hypertension, although it is not directly associated with measures of portal hypertension and is not an accurate indicator of the presence or absence of varices[31]. Ultrasound examination may also contribute to the diagnosis of gallstones, choledochal cysts and Caroli disease[47]. The use of Doppler techniques can complement ultrasound evaluations and help determine the perfusion and direction of blood flow in the portal system and hepatic artery. This method also allows identification of portal malformations. Cavernous transformation of the portal vein is a diagnostic feature of portal vein thrombosis.

Endoscopy

Endoscopy is the best method for evaluation of the presence, size and extension of gastric, esophageal and, more rarely, duodenal varices, and can help diagnose hypertensive gastropathy[48,49]. A prospective study of endoscopic findings in children with BA found that the presence of red signs and gastric varices was associated with increased risk of gastrointestinal bleeding[50]. Gastric mucosal damage, or hypertensive gastropathy, is characterized by the dilatation or ectasia of vessels in the mucosa and submucosa in the absence of inflammatory alterations, as identified by endoscopy or histological examination[51,52]. Although these criteria are not always used in children, a study of endoscopic findings in 51 children with portal hypertension found this condition in 59% of 28 children with cirrhosis[53]. Endoscopy is also important to exclude other causes of gastrointestinal bleeding, such as gastric or duodenal ulcers and Mallory-Weiss tears[31]. In a study of 76 children with cirrhosis candidates for liver transplantation, gastric or duodenal ulcers were diagnosed in 8/21 (38%) of children with gastrointestinal bleeding[54]. Studies of noninvasive methods to identify high risk of esophageal varices in children with chronic liver disease found that splenomegaly and hypoalbuminemia[55], as well as platelet counts, Z scores of spleen size and albumin levels, predicted the presence of varices in patients with cirrhosis[56].

Liver biopsy

Liver biopsy is still considered the gold-standard diagnostic method for cirrhosis. When required, it should be performed after through laboratory tests and imaging. The biopsy specimen should be evaluated by a pediatric hepatology specialist. The interpretation of results may be limited by several factors, especially

when suction biopsy is performed. These include small specimen size, sampling error or fragmented biopsy specimens[57].

Other tests

In adults, HVPG measurements are the best method of assessing the presence and severity of portal hypertension, and can be used to monitor the efficacy of medical treatment[58,59]. However, this is still not a routine procedure in children.

Miraglia *et al*[60] consider multidetector computed tomography scans and abdominal MRI crucial for the pretransplant assessment of patients with BA[60]. These imaging modalities permit identification of congenital anomalies or cirrhosis-related alterations (portosystemic shunts, portal thrombosis) which may require modification of surgical techniques[60]. Most of these methods have not been studied extensively in children due to their invasive nature. Angiographic examinations are usually only performed in children as part of the preoperative assessment of surgical portosystemic shunts or liver transplantation[61].

COMPLICATIONS OF PEDIATRIC CIRRHOSIS AND THEIR MANAGEMENT

Nutritional alterations

Malnutrition is an important prognostic factor, which may influence the clinical course of chronic liver disease[62] and is associated with greater morbidity and mortality in both the pre- and posttransplant periods[63]. In children and adolescents, the increased energy demands associated with anorexia and nausea may complicate the management of malnutrition[64-66].

A comprehensive clinical history and general physical examinations of the child/adolescent must be included in routine clinical practice[67], and special attention must be paid to changes in muscle mass and body fat depots, both of which reflect important aspects of patient nutritional status. In clinical practice, anthropometry is the most widely used method for nutritional diagnosis. Regular patient follow-up also enables early detection of nutritional impairments. Given the high prevalence of water retention and organomegaly in pediatric cirrhosis, body weight is an unsatisfactory marker of nutritional status. In addition to measuring the weight, height/length and head circumference of children younger than 3 years, it is important to follow their long-term growth using reference curves. Triceps skinfold thickness and upper arm circumference measurements are also important to assess fat and protein reserves and can allow early detection of alterations in the nutritional status of pediatric patients with liver disease[68,69]. Subjective global assessments of nutritional status, performed on the basis of interview and physical examination findings, can also help identify factors which may influence the progression or regression of nutritional abnormalities[70].

Nutritional treatment

Children or adolescents with chronic liver disease have increased nutritional needs. Patients at risk of malnutrition require 20%-80% more calories than healthy children to achieve normal growth[26]. These recommendations aim to ensure that children have sufficient energy to meet daily requirements, address the nutritional deficits caused by the increased energy demands of cirrhotic liver disease and prevent protein catabolism[71].

Protein intake should not be restricted in the absence of hyperammonemia[26]. Cirrhotic infants with cholestasis require a protein intake of approximately 2-3 g/kg per day to achieve normal growth and endogenous synthesis. Supplementation with up to 4 g/kg per day is generally safe and necessary to maintain normal growth and avoid excessive catabolism.

Lipids are an especially important dietary component in children with liver disease, and should account for approximately 30%-35% of total calories in the diet. Medium-chain triglycerides (MCTs) should account for 30%-50% of lipid intake, as these are absorbed directly by the intestinal epithelium and do not require bile salts for digestion and absorption[65-72]. Although MCT supplementation is crucial for the nutritional management of children with cholestasis, long-chain triglycerides should not be eliminated from the diet, as these substances provide essential fatty acids and contribute to the absorption of lipid-soluble molecules.

Deficiency of lipid-soluble vitamins is a common problem, especially in children with cholestasis; therefore, levels of these nutrients must be carefully monitored[73,74].

Oral nutrition should always be preferred, although enteral or parenteral supplementation may be necessary if not all nutritional requirements can be met by oral feeding[69]. Enteral supplementation is generally recommended when oral intake provides less than 60% of recommended energy needs or in cases of severe malnutrition[75].

Infections

Patients with cirrhosis are especially susceptible to infection, the most common of which is spontaneous bacterial peritonitis (SBP)[76]. Urinary and respiratory infections are also common[31]. In patients with cirrhosis, infections can lead to complications such as encephalopathy, ascites and hepatorenal syndrome.

Recommended preventive measures include nutritional supplementation, vaccination and prophylactic antibiotics for invasive procedures[37]. Pneumococcal and meningococcal vaccines are recommended for children with functional asplenia due to portal hypertension[37]. In children with cirrhosis candidates for liver transplantation, accelerated vaccination programs

should be considered[77].

Gastroesophageal varices and gastrointestinal bleeding

Rupture of gastroesophageal varices is the most common cause of gastrointestinal bleeding in children with cirrhosis. It is the most severe complication of the disease, and is considered a medical emergency[33]. Gastrointestinal bleeding caused by gastroesophageal varices in children and adolescents is generally treated using statements developed for adults. These statements were adapted for use in children by an expert committee (Baveno V Consensus), which also proposed a set of guidelines for the treatment of children with portal hypertension[78]. Treatments can be classified as pharmacological, endoscopic, mechanical and surgical[78,79].

Ascites

Ascites is a common complication in pediatric cirrhosis, especially in younger children with terminal liver disease[37]. This is generally associated with a poor prognosis[80]. Pediatric patients with a sudden increase in ascites or new episodes of water retention should undergo paracentesis[34]. Analysis of the ascitic fluid enables differentiation of ascites from portal hypertension of other causes of ascites. A serum-ascites albumin gradient - calculated by subtracting the albumin concentration of the ascitic fluid from the serum albumin level - greater than 1.1 g/dL can diagnose portal hypertension with 97% accuracy[81]. Tests such as amylase, cytology, polymerase chain reaction and mycobacterial cultures should also be performed in case of diagnostic uncertainty or when pancreatic ascites, malignant tumors or tuberculosis are suspected[81].

Management

In most cases, cirrhotic ascites is resolved through dietary sodium restriction and the use of diuretics[31]. However, children and adolescents ingesting low-sodium diets must be carefully monitored, since these restrictions often make the diet unpalatable and reduce food intake. Fluid restriction is strongly recommended in case of hyponatremia with serum sodium levels below 125 mEq/L[31]. When diuretics are required, spironolactone (1-6 mg/kg per day) should be preferred and, if necessary, combined with a loop diuretic such as furosemide (1-6 mg/kg per day). The combination with furosemide lowers the risk of hyperkalemia due to increased potassium excretion. Thiazide diuretics can be used for treatment maintenance[31]. During diuretic treatment and until the patient is stable, frequent laboratory testing should be performed to verify serum electrolytes, creatinine, blood urea nitrogen and urinary sodium levels. Excess fluid loss can lead to plasma depletion and deterioration of renal function.

Patients with refractory ascites or respiratory impairment can be treated by large-volume paracentesis. Up to 100 mL/kg of liquid can be removed at one time with the help of a post-paracentesis albumin infusion (25% albumin at 1 g/kg)[31]. Albumin and furosemide infusions can be used to treat patients with serum albumin below 2 mg/dL[33]. In severe or recurrent refractory ascites, a transjugular intrahepatic portosystemic shunt may be performed as a bridge to liver transplantation[31]. However, this procedure may be associated with increased risk of renal failure and encephalopathy[31].

Some medications should be avoided by patients with ascites. Aminoglycoside antibiotics, for instance, may increase the risk of renal failure, and non-steroidal anti-inflammatory drugs pose a high risk of sodium retention, hyponatremia and renal failure[81].

SBP

SBP refers to a bacterial infection of ascites without evidence of intestinal perforation or other intra-abdominal sources of infection[31]. Infection is usually monomicrobial and caused by *E. coli*, *Klebsiella* spp. and *Enterococcus faecalis*[31]. Polymicrobial infections are indicative of intestinal perforation or secondary peritonitis[31]. SBP is a relatively common and potentially fatal complication in children with ascites[33]. The presence of portal hypertension in patients with cirrhosis increases susceptibility to bacteremia and SBP[82]. These phenomena are likely caused by the translocation of intestinal bacteria and of immune system deficits associated with cirrhosis, such as alterations in complement fixation and opsonization, decreased Kupffer cell function and neutropenia[34]. Bacterial overgrowth and alterations in intestinal permeability probably play a role in bacterial translocation[83]. Studies have found that detection of bacterial DNA in the ascitic fluid of children with portal hypertension is superior to cell cultures in diagnosing SBP, but cannot differentiate between infection and ascitic fluid colonization[84].

SBP must always be considered in children with cirrhosis and ascites who present with fever, abdominal pain or leukocytosis[33]. Risk factors for SBP include ascites, hypoalbuminemia, gastrointestinal bleeding, pediatric intensive care unit admission and recent endoscopic examinations[34].

Management

Children with SBP are usually treated with a third-generation intravenous cephalosporin, such as cefotaxime, for 14 d. As a preventive measure, antibiotics should always be used during invasive procedures. If SBP recurs, the use of oral prophylactic antibiotics such as co-trimoxazole, ciprofloxacin or norfloxacin can be considered[34].

Hepatic encephalopathy

Hepatic encephalopathy (HE) is an alteration in brain

function caused by liver insufficiency and/or porto-systemic shunts[85]. Infection is the main cause of HE. Gastrointestinal bleeding, excessive doses of diuretics, electrolyte imbalance and constipation are also commonly associated with this condition[85]. HE can also be caused by excess protein intake, anesthetics and sedatives[86]. Patients with HE may present with several neurological and psychiatric manifestations, ranging from subclinical alterations to coma[85]. In children and adolescents, the first symptoms of HE are subtle, and include developmental delays, academic difficulties, lethargy or sleep inversion. Older children may also exhibit personality changes, intellectual impairments, obtundation (clouding of consciousness), stupor and coma.

Diagnosis of HE in children involves a high index of suspicion. Mild HE is even more difficult to diagnose, given the difficulty of administering psychometric tests to children and the absence of measures validated for use in this age range. Children are usually diagnosed on the basis of clinical symptoms. Psychometric tests evaluate memory and neuromotor function, and have been widely used to assess patients with mild encephalopathy. In children and adolescents, the most commonly used such tests are the Wechsler Intelligence Scales and the Dutch Child Intelligence test[87]. The critical flicker frequency test, a simple and reliable test for the assessment of low-grade HE, can also be used in children older than 8 years[87].

Neuroimaging studies are important to exclude other causes of encephalopathy, but cannot be used to diagnose the condition[88]. MR spectroscopy has proved to be as useful as neuropsychometric tests for the diagnosis of mild HE in adults[89]. According to Foerster *et al*[90], the alterations in cerebral metabolism observed in pediatric patients with suspected mild HE are similar to those observed in adults.

Management

Sedatives (especially benzodiazepines and opiates) should be avoided, as these drugs may worsen encephalopathy. Identification and treatment of the underlying cause of HE are crucial for the cure of the disease in approximately 90% of cases[85]. Prolonged use of low-protein diets should be avoided. When protein restriction is required, protein intake should be reduced to 2-3 g/kg per day[37]. Nonabsorbable disaccharides are the treatment of choice for patients with HE[31,87]. In children, the optimal dose of lactulose is 0.3-0.4 mL/kg, two to three times a day[31]. In adolescents and adults, the ideal dose can range from 10-30 mL three times a day, although treatment may begin with 25 mL/kg twice daily[85]. A study of lactulose therapy in 22 children with cirrhosis and HE revealed complete recovery in 73% of patients[91]. Several antibiotics, such as neomycin, vancomycin, metronidazole and rifaximin, have been used to reduce the number of ammonia-producing bacteria in the gastrointestinal tract[87]. A case report of a 9-year-old girl with cirrhosis and HE described positive clinical results with the use of rifaximim[92].

Acute-on-chronic liver failure

Acute-on-chronic liver failure (ACLF) is the acute deterioration of liver function in patients with cirrhosis resulting from extra- or intrahepatic causes. Data on the epidemiology of ACLF are rare[93]. In a study that evaluated a cohort of 192 patients admitted to the emergency department of a Brazilian tertiary hospital due to acute decompensation of cirrhosis, 46 (24%) fulfilled the EASL-CLIF Consortium criteria for ACLF. Bacterial infections were observed in 50%[94]. In children, the most common precipitating factor is infection. ACLF can progress to decompensation and multiple organ failure, resulting in high mortality rates. Mortality among inpatients with cirrhosis is strongly associated with infection. Patients with cirrhosis who acquire viral hepatitis, for instance, exhibit very rapid deterioration of liver function.

Hepatopulmonary syndrome

Hepatopulmonary syndrome (HPS) is a common complication in patients with portal hypertension and cirrhosis. It is characterized by intrapulmonary vasodilation, which results in insufficient oxygenation[95]. The diagnosis of HPS should only be made when clinical suspicion is high, since clinical manifestations are subtle in the early stages of the disease. The presence of three symptoms – liver dysfunction, arteriovenous shunts and reduced O2 saturation - is required for diagnosis[95]. The clinical picture can be reversed by liver transplantation, although the presence of HPS may interfere with tolerance to anesthesia and preclude transplant[96].

HPS is distinct from pulmonary hypertension, another underdiagnosed circulatory condition associated with pediatric cirrhosis. Pulmonary hypertension (also known in this setting as portopulmonary hypertension) has a vasoconstrictive etiology. Mild to moderate hypertension can be corrected by liver transplantation[97].

Hepatorenal syndrome

Patients with cirrhosis exhibit a progressive deterioration of renal function. Two types of hepatorenal syndrome (HRS) have been described: Type 1 HRS is associated with rapidly progressive kidney failure and a very low survival expectancy, the median survival time being only 2 wk if it is not treated; type 2 HRS is associated with slowly progressive kidney failure and has a better prognosis than type 1 HRS. Children also could exhibit alterations in renal function after liver transplantation, especially due to treatment with calcineurin inhibitors[98].

Hematological alterations

The basic laboratory tests of coagulation used to evaluate the risk of hemorrhage, such as prothrombin time and partial activated thromboplastin time, are only weakly associated with the incidence or duration

of bleeding after liver biopsy or other potentially hemorrhagic procedures[99]. Nevertheless, patients with cholestatic disease and prolonged prothrombin time should receive parenteral vitamin K supplementation. The recommended dosage for children is 2-10 mg Ⅳ once daily for 3 d, or 5 to 10 mg IM per week[100]. Fresh frozen plasma infusions (5-10 mL/kg) and cryoglobulin and/or platelet transfusions can also be used as treatment for bleeding episodes or prophylaxis against bleeding during procedures such as liver biopsy[100].

Chronic anemia is common in patients with cirrhosis, and can be caused by blood loss, iron deficiency and low folic acid levels due to sodium and water retention and hemolysis secondary to hypersplenism[31].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is also observed in patients with chronic liver disease. In pediatric patients, HCC can result from the progression of cholestatic, metabolic or viral diseases[101]. Its pathogenesis is complex and involves both genetic and environmental factors. The usual symptoms are abdominal discomfort and distention and eating difficulties. Abdominal masses may also be detected on physical or ultrasound examinations[102]. Alpha-fetoprotein levels may be elevated[102].

An Italian study found HCC to be present in 2% of 103 children with cirrhosis who underwent primary liver transplantation. Despite the early age of onset, prognosis after liver transplantation was excellent, and recurrence was not observed[103]. HCC has also been reported in children with BA and Alagille syndrome[104,105] and in adolescents with chronic hepatitis B[106,107] or C[108]. Children with cirrhosis of any etiology should undergo abdominal ultrasound examinations and alpha-fetoprotein measurements every 6 mo or at least on a yearly basis[101,109].

CONCLUSION

End-stage liver disease or cirrhosis in children and adolescents has a multifactorial etiopathogenesis, and is usually the result of longstanding disease. Causes also vary in consequence of different factors, including patient age and prevalence of specific diseases in different regions. In clinical practice, the majority of cases are due to a limited repertoire of etiological factors, mainly BA, genetic-metabolic disorders and viral and AIH. In pediatric patients, special attention should be paid to the nutritional alterations caused by cirrhosis, as children and adolescents have higher nutritional requirements for growth and development. Malnutrition can have severe consequences for both pre- and post-liver transplant patients. Treatment of the complications of cirrhosis in children and adolescents is mostly based on methods developed for use in adults. Future research should focus on gaining a better understanding of the pathophysiology of cirrhosis in children, as well as improve non-invasive diagnostic tests for hepatic fibrosis and the management of pediatric patients with cirrhosis.

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REVIEW

Staging systems for hepatocellular carcinoma: Current status and future perspectives

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Abstract

Hepatocellular carcinoma (HCC) is a major health concern worldwide and the third cause of cancer-related death. Despite advances in treatment as well as careful surveillance programs, the mortality rates in most countries are very high. In contrast to other cancers, the prognosis and treatment of HCC depend on the tumor burden in addition to patient's underlying liver disease and liver functional reserve. Moreover, there is considerable geographic and institutional variation in both risk factors attributable to the underlying liver diseases and the management of HCC. Therefore, although many staging and/or scoring systems have been proposed, there is currently no globally accepted system for HCC due to the extreme heterogeneity of the disease. The aim of this review is to focus on currently available staging systems as well as those newly reported in the literatures since 2012. Moreover, we describe problems with currently available staging systems and attempts to modify and/or add variables to existing staging systems.

Key words: Hepatocellular carcinoma; Staging system; Scoring system; Prognosis

Core tip: Hepatocellular carcinoma is a major health concern worldwide with extreme heterogeneity of the disease. This makes it difficult to identify globally accepted staging systems or treatment algorithms for hepatocellular carcinoma. Clinicians should use currently available staging systems or treatment algorithms carefully while understanding their features and limitations.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health concern worldwide and the third cause of cancer-related death[1,2]. Approximately 90% cases of HCC

Table 1 Okuda

Stage	Ascites		Tumor size		Albumin		Bilirubin
	(+)	(-)	> 50% (+)	< 50% (-)	< 3 g/dL (+)	> 3 g/dL (-)	> 3 mg/dL (+)
Ⅰ (mildly advanced)	(-)		(-)		(-)		(-)
Ⅱ (moderately advanced)	One or two (+)						
Ⅲ (very advanced)	Three or four (+)						

are attributable to underlying liver diseases, such as chronic hepatitis B, chronic hepatitis C, alcohol abuse, nonalcoholic steatohepatitis (NASH) or aflatoxin exposure[3]. Approximately 80% of HCC cases arise in eastern Asia and sub-Saharan Africa, where the main risk factor is chronic hepatitis B in addition to exposure to aflatoxin B1. In contrast, in North America, Europe and Japan, chronic hepatitis C is the main risk factor, in combination with alcohol abuse[1]. NASH has also recently emerged as a relevant risk factor[2].

Despite advances in treatment, such as the use of surgical resection, transplantation, percutaneous ablation and transarterial chemoembolization (TACE) and the administration of multikinase inhibitor sorafenib, as well as careful surveillance programs, the mortality rates in most countries are very similar to the incidence of HCC, thus reflecting the poor prognosis of this disease and subsequent lack of effective treatments[2].

In contrast to that observed for other cancers, the prognosis and treatment of HCC depend on the tumor burden in addition to patient's underlying liver disease and liver functional reserve, both of which affects survival and treatment selection. Moreover, there is considerable geographic and institutional variation in both risk factors attributable to the underlying liver diseases and the management of HCC. This background highlights the extreme heterogeneity of HCC. Therefore, developing a robust staging system and/or identifying prognostic marker for HCC is urgently required.

Staging systems and/or prognostic scores for cancer can be used to evaluate the extent of the tumor burden in the primary organ and the degree of spread to the lymph nodes or other organs. It is thus necessary to accurately predict the patient's prognosis, determine the optimal therapeutic approach and group patients homogenously for objective comparisons in clinical trials. Moreover, such markers should be simple, reliable and reproducible for clinical use based on clinically available data[4-7].

With regard to HCC, due to the aforementioned heterogeneity, staging systems and/or prognostic scores must account for the tumor burden, underlying liver disease and liver functional reserve, thus indicating the unique required characteristics of such markers. As a result, although a number of staging systems for HCC have been proposed and developed, there is currently no globally applicable staging system.

In this review, we focus on currently available staging systems as well as those newly proposed in the literatures since 2012. Moreover, we describe attempts to modify and/or add variables to existing staging systems.

CURRENTLY AVAILABLE STAGING SYSTEMS

Okuda staging system (Table 1)

The Okuda staging system was proposed by a Japanese group in 1984[8]. This system is derived from a retrospective cohort of 600 HCC patients treated at Japanese institutions and is the first to combine tumor extension with the liver functional reserve. It incorporates the tumor size (≤ or > 50% of the entire liver), presence or absence of ascites, serum albumin level (≤ or > 3.0 g/dL) and serum bilirubin level (≤ or > 3.0 mg/dL), in which patients are classified into three stages based on these variables (Ⅰ: not advanced, Ⅱ: moderately advanced, Ⅲ: very advanced). Subsequently, the same group validated the Okuda system in 850 HCC patients[9]. In that study, the median survival was 11.5 mo for the stage Ⅰ patients, 3.0 mo for the stage Ⅱ patients and 0.9 mo for the stage Ⅲ patients. Among the patient cohort, hepatic failure (45% in surgically treated cases, 38.5% in non-surgically treated cases) was the leading cause of death followed by gastrointestinal bleeding. The authors underlined the importance of assessing the hepatic functional reserve.

Although the Okuda system was the first integrated system for classifying HCC patients, there are major concerns with this system. For example, one variable, tumor extension (≤ or > 50% of the entire liver), is too rough, considering recent developments in imaging techniques and the use of adequate surveillance programs. Moreover, this system does not include variables such as the degree of vascular invasion or extent of extrahepatic metastasis, both of which affect patient outcomes. Therefore, the Okuda system often makes way for newer staging systems and functions as the standard for comparison[7].

Cancer of the Liver Italian Program score (Table 2)

The Cancer of the Liver Italian Program (CLIP) score was proposed by an Italian group, the CLIP investigators, in 1998 for the purpose of producing a more sensitive prognostic index than the Okuda

Table 2 Cancer of the Liver Italian Program

Variables	Scores		
	0	1	2
Child-Pugh stage	A	B	C
Tumor morphology	uninodular and extension ≤ 50%	multinodular and extension ≤ 50%	massive or extension > 50%
AFP (ng/dL)	< 400	≥ 400	
Portal vein thrombosis	-	+	

AFP: Alpha-fetoprotein.

staging system[10]. This score is derived from the results of a retrospective cohort of 435 HCC patients treated at 16 Italian institutions. This model incorporates four covariates (Child-Pugh grade, tumor morphology, serum alpha-fetoprotein (AFP) level and portal vein thrombosis), assigning a linear score (0/1/2) to each covariate. Patients are subsequently classified into seven groups according to the sum of these scores (0-6). Overall, the differences in survival based on this score are proper.

Subsequently, the same group externally validated the CLIP score in 196 HCC patients enrolled in a randomized clinical trial and confirmed the greater predictive accuracy of this score compared with the Okuda staging system[11].

Although the CLIP score was developed using an appropriate method and has been externally validated, several limitations have been reported. First, half of the patients (235/435, 54%) in the above study received locoregional treatments, such as PEI or TACE, while only 12 patients (2.8%) underwent surgical resection. Therefore, this score may not be suitable for predicting the survival of HCC patients who undergo surgical resection. Second, the covariate "massive" tumor morphology is subjective, without specific size criteria. Therefore, its objectivity and reliability in predicting outcomes may be compromised[4,7]. Third, in this study, information regarding underlying liver diseases, which affect patient outcomes, was lacking. Fourth, in the validation study conducted by the same group, the differences among the patient populations assigned to the CLIP 2-3 and 4-6 groups were not significant. In fact, the authors grouped patients with a CLIP score of 4-6 into one group[4,11,12].

Barcelona clinic liver cancer classification (Figure 1)

The barcelona clinic liver cancer (BCLC) classification was first proposed by the Barcelona Clinic Liver Cancer group in 1999[13]. This model is derived from the results of a study of the outcomes of radical therapy and/or the natural history of untreated HCC patients[14-16]. It is comprised of four elements (tumor extension, liver functional reserve, physical status and cancer-related symptoms). Tumor extension incorporates the number of tumors, tumor size and presence of portal vein invasion or extrahepatic metastasis. Meanwhile, the liver functional reserve is substituted for the Child-Pugh grade, and the physical status is determined according to the ECOG performance status. Patients are subsequently assigned to five categories (0, A, B, C and D) based on these elements. A BCLC stage of 0 (defined as very early stage disease) comprises patients exhibiting a well-preserved liver function (Child-Pugh A) diagnosed with one asymptomatic nodule measuring less than 2 cm, without vascular invasion or satellites. A BCLC stage of A (defined as early-stage disease) includes patients with a Child-Pugh A or B status diagnosed with one nodule of any size or a maximum of three nodules measuring < 3 cm. A BCLC stage of B (defined as intermediate-stage disease) corresponds to patients with a Child- Pugh grade A or B status diagnosed with multiple nodules without vascular invasion or extrahepatic metastasis. Patients with a Child- Pugh grade of A or B, vascular invasion or extrahepatic metastasis and cancer-related symptoms (PS 1-2) are classified as having BCLC C disease (defined as advanced-stage disease). Finally, patients with a Child-Pugh grade of C, in any tumor stage and cancer-related symptoms (PS > 2) are classified as belonging to the BCLC D disease (defined as terminal stage disease)[1,13].

The notable feature that distinguishes the BCLC system from other staging systems for HCC is the assignment of treatment recommendations for each stage based on the best treatment options currently available[4,13]. That is, for patients with a stage 0 and A status, curative treatment options, such as surgical resection, liver transplantation and ablation, are recommended. Meanwhile, TACE is recommended for patients with a stage B status, sorafenib, multikinase inhibitor, is recommended for patients with a stage C status and best supportive care is recommended for patients with a stage D status.

The BCLC classification was updated by incorporating the category of stage 0 (very early stage) and the use of chemoembolization for stage B (intermediate stage) patients in 2003 and further modified to include sorafenib as a first-line treatment option for stage C (advanced stage) patients in 2008[17,18].

Currently, the BCLC classification is endorsed as the standard system for HCC management by the American Association for the Study of Liver Disease, American Gastroenterology Association, European Association for the Study of Liver and the European Organization for the Research and Treatment of Cancer[3,19]. However, the BCLC classification has some limitations.

First, stage B (intermediate stage) includes a considerable heterogeneous population of HCC patients with varying degree of tumor extension, liver functional reserve and disease etiology, thus resulting in prognostic heterogeneity and preventing the determination of the optimal treatment regimen[20,21]. Second, the variable ECOG PS is somewhat subjective. Hence, the reliability of this system in predicting patient outcomes is compromised. Third, the one-to-

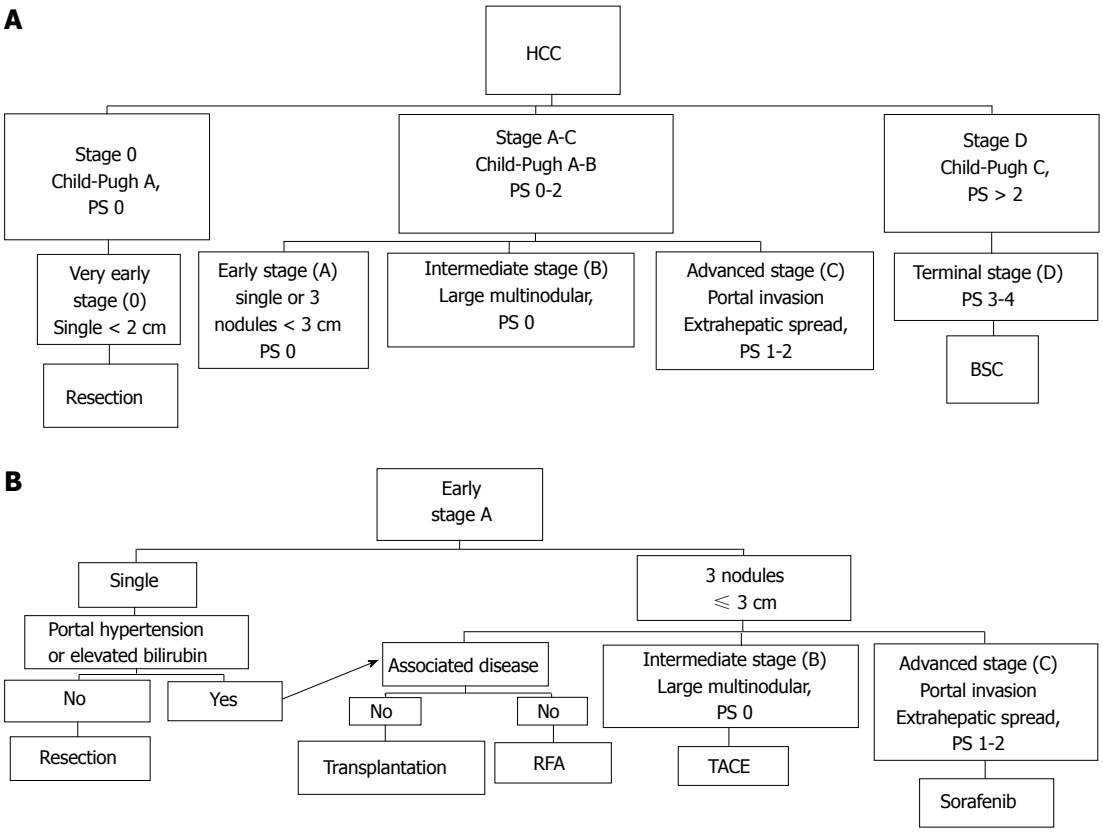

Figure 1 Barcelona clinic liver cancer classification. A: BCLC classification; B: BCLC classification. HCC: Hepatocellular carcinoma; BCLC: Barcelona clinic liver cancer; TACE: Transarterial chemoembolization.

one correspondence treatment recommendations for each stage of the BCLC system may be not suitable for use in actual clinical practice (*i.e.,* resection or liver transplantation after TACE, the combination of TACE with RFA and/or the combination of TACE with sorafenib, TACE for patients with BCLC 0 or A status and resection for patients with BCLC B or C status).

GRETCH system (Table 3)

The GRETCH system was proposed by the French group Goupe d'Etude et de in 1999[22]. This system is derived from the finding of a prospective cohort of 761 HCC patients (516 training cohort, 255 validation cohort) treated at 24 Western medical centers. It incorporates five prognostic factors (the Karnofsky index, serum bilirubin, serum alkaline phosphatase (ALP) and serum AFP levels and ultrasonographic portal obstruction) based on a multivariate Cox model. Patients are classified into three risk groups (A: low risk of death, B: intermediate risk of death, C: high risk of death) according to these factors. The overall survival differs markedly for the three groups, with a one-year survival rate in group A of 72% (training cohort) and 79% (validation cohort), compared to 34% (training cohort) and 31% (validation cohort) in group B and 7% (training cohort) and 4% (validation cohort) in group C.

The strength of this system is that it is based on baseline characteristics that are routinely available at diagnosis and the scores allocated to the respective predictive factors are based on the estimated Cox regression coefficient.

However, half of the patients (401/761, 53%) in this study received no specific therapy, while only 56 patients (7.4%) underwent surgical resection. Therefore, this score may not be suitable for predicting the survival of HCC patients who undergo surgical resection. In addition, evaluating portal obstruction using ultrasound is somewhat out of touch with the current times, considering recent advances in imaging techniques.

Chines University Prognostic Index (Table 4)

The Chines University Prognostic Index (CUPI) was proposed by a Hong-Kong group in 2002[23]. This score is derived from the results of a cohort of 926 HCC patients (713 training set, 213 validation set) treated at a single Hong-Kong hospital. This score is obtained by adding five prognostic factors (serum bilirubin, ascites, serum ALP, serum AFP and asymptomatic disease on presentation) based on a multivariate Cox model to the TNM staging system. Patients are subsequently divided into three groups (low risk, intermediate risk and high risk) according to the sum of the weights of the six prognostic factors. The differences in the three-month survival among different risk groups classified using this system are highly significant (low-risk group: 85.7%, intermediate-risk group: 56.4% and high-risk

Table 3 GRETCH

Weight	0	1	2	3
Karnofsky index (%)	⩾ 80			< 80
Serum bilirubin (μmol/L)	< 50			⩾ 50
Serum ALP	< 2 × ULN		⩾ 2 × ULN	
Serum AFP (μg/L)	< 35		⩾ 35	
Portal obstruction (US)	-		+	

AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; US: Ultrasound.

Table 4 Chines University Prognostic Index

Variable	Weight
TNM stage	
Ⅰ and Ⅱ	-3
Ⅲa and Ⅲb	-1
Ⅳa and Ⅳb	0
Asymptomatic disease on presentation	-4
Presence of ascites	3
AFP ⩾ 500 ng/mL	2
Total bilirubin (μmol/L)	
< 34	0
34-51	3
⩾ 52	4
ALP ⩾ 200 (IU/L)	3

⩽ 1: Low risk; 2-7: Intermediate risk; ⩾ 8: High risk. AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase.

group: 20.2%). Moreover, the authors demonstrated that the CUPI system is more discriminant in predicting survival than the conventional TNM staging system, Okuda system or CLIP score.

In 2011, the group validated the CUPI system in another cohort of 595 HCC patients with predominant HBV infection[24].

Although the CUPI has a strength in that the prognostic factors in this system are readily available in daily clinical practice and are determined based on the estimated Cox regression coefficient, there are various concerns. First, this score was derived from a cohort of HCC patients with predominant HBV infection (79% of the whole cohort), as the authors adequately mentioned. Therefore, this system may be not suitable for application in Western populations with predominant HCV infection or a history of alcohol abuse. Second, the cohort was composed of a large proportion of patients who received only best supportive care (58.4%, *vs* resection 10.4%). Hence, this system is not preferable for assessing patients who undergo curative treatment, such as surgical resection or RFA.

TNM classification (Table 5)

The TNM classification was developed by the American Joint Committee on Cancer (AJCC) and International Union for Cancer Control (UICC) and has been updated regularly since the first edition was published in 1977. The 7th edition has become widespread since 2010[25]. The TNM classification assesses the extent of the primary tumor (T) as well as the presence of lymph node involvement (N) and/or extrahepatic metastasis (M). It also includes the histologic grade (G) and fibrosis score (F), which do not affect staging. The current AJCC/UICC 7th edition is a modification of the following simplified staging system[26].

Table 5 American Joint Committee on Cancer /TNM 7th edition

Primary tumor	Description		
T1	Single tumor without vascular invasion		
T2	Single tumor with vascular invasion or multiple tumors, none > 5 cm		
T3a	Multiple tumors, any > 5 cm		
T3b	Single tumor or multiple tumors of any size involving a major branch of the portal vein or hepatic vein		
T4	Tumors with direct invasion of adjacent organs or perforation of visceral peritoneum		
Stage grouping			
Stage Ⅰ	T1	N0	M0
Stage Ⅱ	T2	N0	M0
Stage Ⅲ A	T3a	N0	M0
Stage Ⅲ B	T3b	N0	M0
Stage Ⅲ C	T4	N0	M0
Stage Ⅳ A	Any T	N1	M0
Stage Ⅳ B	Any T	Any N	M1

Simplified staging

The Simplified Staging system was proposed by Vauthey *et al*[27] in 2002. It is derived from the finding of a cohort of 557 HCC patients who underwent surgical resection at four centers (United States, France and Japan). The authors identified independent prognostic factors (major vascular invasion, microvascular invasion, severe fibrosis/cirrhosis, multiple tumors and a tumor size greater than 5 cm) using a multivariate analysis. Based on these variables, they reclassified the AJCC T classification in use at the time, creating the simplified T classification (sT1: single tumor with no vascular invasion, sT2: single tumor with microvascular invasion or multiple tumors, none measuring < 5 cm, sT3: multiple tumors (any measuring > 5 cm) with major vascular invasion). While the original AJCC T classification failed to stratify patients into distinct prognostic groups, the simplified T classification divides patients into independent prognostic groups (five-year survival rates: stage Ⅰ 55%, stage Ⅱ 37% and stage Ⅲ 16%, $P < 0.001$).

However, both the AJCC/UICC and simplified system are limited to patients who undergo surgical resection and lack factors related to the liver functional reserve[28]. Therefore, these systems may be not suitable for application in patients not indicated for surgical resection or with a reduced liver functional reserve.

Japan Integrated Staging (Table 6)

The Japan Integrated Staging Score (JIS score) was proposed by Kudo *et al*[29] in 2003. It is derived from

Table 6 Japan Integrated Staging Score

T factors	Ⅰ: Single;	Ⅱ: Size < 2 cm;	Ⅲ: No vascular invasion	
T1	Fulfilling 3 factors			
T2	Fulfilling 2 factors			
T3	Fulfilling 1 factors			
T4	Fulfilling 0 factors			
Stage Ⅰ	T1N0M0			
Stage Ⅱ	T2N0M0			
Stage Ⅲ	T3N0M0			
Stage ⅣA	T4N0M0 or any TN1M0			
Stage ⅣB	Any TN0-1M1			
Scores				
Variables	0	1	2	3
Child-Pugh grade	A	B	C	
TNM stage by LCSGJ	Ⅰ	Ⅱ	Ⅲ	Ⅳ

LCSGJ: Liver Cancer Study Group of Japan.

a cohort of 722 HCC patients treated at two Japanese institutions. The authors combined the Child-Pugh grade and the TNM stage based on the criteria of the Liver Cancer Study Group of Japan (LCSGJ), thus creating the JIS score. Patients with a Child-Pugh grade A, B and C status are allocated a score of 0, 1, and 2, respectively. Patients with the TNM stage by LCSGJ of stage Ⅰ, Ⅱ, Ⅲ and Ⅳ are allocated to score of 0, 1, 2 and 3, respectively. Patients are subsequently classified into six groups (0-5) based on the sum of these scores. Statistically significant differences are observed between the survival curves for almost all JIS scores, whereas no differences are observed between the patients with CLIP scores 3, 4, 5 and 6.

Thereafter, the same group externally validated the JIS score in 4525 HCC patients treated at five Japanese institutions in 2004[30]. Their findings showed that the JIS score could be used to correctly identify the patient subgroup among early, intermediate, advanced and end-stage HCC patients. Moreover, the authors demonstrated that the JIS score exhibits a better prognostic ability when using the likelihood ratio test and Akaike Information Criteria (AIC) than CLIP score.

Although the JIS score is readily available and relatively objective, it has not been validated in a Western population.

Estrogen receptor classification

The estrogen receptor (ER) classification was proposed by Villa *et al*[31] in 2003. This system is derived from a cohort of 96 unresectable HCC patients treated at a single Italian institution. Based on the prognostic relevance of identifying of ER transcripts in individuals with HCC, the authors classified patients into two groups according to the presence or absence of the ER in HCC specimens. Consequently, the overall survival rate is significantly higher among patients with the wild-type ER (wt ER) (MST: 36 mo) than among those with the variant-ER (w ER) (MST: 13 mo) ($P < 0.0001$).

Although the ER classification is a simple prognostic model for assessing HCC patients, this system has a flaw in that it requires the use of a liver biopsy. In addition, the evaluation of the ER is not readily available in daily clinical practice.

Table 7 Stage, Liver damage and des-gamma-carboxy prothrombinscore

Parameter/score	0	1	2	3
Liver damage by LCSGJ	A	B	C	
Stage by LCSGJ	Ⅰ	Ⅱ	Ⅲ	ⅣA or ⅣB
DCP (mAU/mL)	< 400	⩾ 400		

LCSGJ: Liver Cancer Study Group of Japan; DCP: Des-gamma-carboxy prothrombin.

Stage, Liver damage and des-gamma-carboxy prothrombin score (Table 7)

The Stage, Liver damage and des-gamma-carboxy prothrombin (DCP) score (SLiDe score) was established by Omagari *et al*[32] in 2004. This score is derived from the analysis of a cohort of 177 HCC patients treated at a single Japanese institution. The authors identified liver damage according to the LCSGJ criteria, the TNM stage according to the LCSGJ criteria and the serum level of DCP as independent prognostic factors using a Cox proportional hazard model. The authors then assigned a linear score (0, 1, 2 and 3) to these three variables to create the SLiDe score. Patients are classified into seven groups (0-6) based on the sum of these scores. The discriminatory value of the survival curves between each group (SLiDe score 0-1, 2, 3 and 4-6) is evident, and the prognostic ability of the SLiDe score is superior to that of the CLIP score and JIS score as judged according to AIC.

In 2009, the same group validated the SLiDe score in another cohort of 207 HCC patients who underwent surgical resection[33]. The authors subsequently showed that there were significant survival differences between the score 0-1, 2-3 and 4-5 groups ($P < 0.005$).

However, the SLiDe score has some shortcomings. First, the original study population ($n = 177$) was relatively small. Second, the variables DCP and ICG R15, which reflect the degree of liver damage according to the LCSGJ criteria, are not routinely examined worldwide.

Tokyo score (Table 8)

The Tokyo score was established by Tateishi *et al*[34] in 2005 for the purpose of providing a more precise prognostic system for patients with early-stage HCC. This score is derived from the results of a cohort of 403 HCC patients who received percutaneous ablation (PEIT or PMCT) at a single Japanese institution. The authors identified the following four independent predictors for survival using a Cox proportional hazard analysis: the serum albumin level (3.5 g/dL and 2.8 g/dL), serum bilirubin level (1 mg/dL and 2 mg/dL), tumor size (2 cm and 5 cm) and tumor number (1-3 *vs* > 3). Scores

Table 8 Tokyo score

	Score		
	0	1	2
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Total bilirubin (mg/dL)	< 1	1-2	> 2
Tumor size (cm)	< 2	2-5	> 5
Tumor number	⩽ 3		> 3

Table 9 BALAD score: Scoring of remnant liver function

	Score			
	0	1	2	
Serum bilirubin (mg/dL)	< 1.0	1.0-2.0	> 2.0	
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8	
	0	1	2	3
Bilirubin-albumin score	A	B	C	
Number of elevated tumor markers	0	1	2	3

A: 0-1 points; B: 2-3 points; C: 4 points.

are assigned to each of the four factors according to the estimated regression coefficient, and the total score is defined as the sum of each subscore. Distinct survival curves are observed for each group based on the Tokyo score, with five-year survival rates of 78.7%, 62.1%, 40%, 27.7% and 14.3% for scores 0, 1, 2, 3 and 4-6, respectively. The authors then validated the Tokyo score in a testing sample consisting of 203 HCC patients who underwent surgical resection at the same institution using the AIC and Harrell's C index and demonstrated that the predictive ability of the Tokyo score is equal to that of the CLIP score and better than that of the BCLC classification.

Although the Tokyo score is useful for predicting the outcomes of HCC patients who are candidates for curative treatment, such as surgical resection and percutaneous ablation, it may be not suitable for use in patients with advanced stages of disease, as the authors adequately mentioned.

BALAD score (Table 9)

The Bilirubin, Albumin, Lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3), AFP and DCP Score (BALAD score) was constructed by Toyoda *et al*[35] in 2006 for the purpose of providing a simple and objective staging system that requires no imaging studies or pathological or clinical evaluations[35]. This score is derived from the findings of a cohort of 2600 HCC patients treated at five Japanese institutions. The authors adopted three tumor markers (AFP-L3 > 15%, AFP > 400 ng/dL, DCP > 100 mAU/mL) as factors reflecting tumor progression. The authors also used two serum markers (serum bilirubin and albumin) as factors indicating the liver functional reserve, according to the Tokyo score[34]. Patients are classified into six categories based on the sum of the scores assigned to these factors. Survival curves determined according to the BALAD score are well distributed, and the discriminative ability of the BALAD score is comparable to that of the CLIP score and JIS score.

Although the BALAD score is a simple and objective tool that requires the use of only a serum sample, without imaging, pathological or clinical assessments, it is not easy to measure the AFP-L3 and DCP values in routine clinical practice worldwide.

Memorial Sloan-Kettering Cancer Center nomogram (Table 10)

The Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram was generated by Cho *et al*[36] in 2008 for the purpose of identifying the optimal staging system for HCC patients who undergo surgical resection[36]. This system is derived from the findings of a cohort of 184 HCC patients treated at a single institution in the United States (MSKCC). The authors identified seven prognostic factors (patient age, serum AFP level, operative blood loss, resection margin status, tumor size, satellite lesions and vascular invasion) for their novel prognostic nomogram. This nomogram demonstrates a superior concordance index of 0.74 (95%CI: 0.68-0.8) compared to that of eight other contemporary staging systems (AJCC/TNM 1997 edition, International Hepato-Pancreto-Biliary Association staging system, AJCC/TNM 2002 edition, Vauthey Simplified Staging, Okuda, BCLC, CLIP and JIS).

However, the study population (n = 184) was relatively small, and this nomogram is not suitable for application in patients treated with RFA, TACE or systemic therapy.

Advanced Liver Cancer Prognostic System (Table 11)

The Advanced Liver Cancer Prognostic System (ALPCS) was constructed by Yau *et al*[37] in 2008 for the purpose of creating an optimal staging system for classifying advanced HCC patients not indicated for surgical resection or locoregional therapy. This system is derived from the analysis of a cohort of 1470 advanced HCC patients (1109 training set, 361 validation set) treated at a single center in Hong Kong. The authors identified 11 prognostic factors (ascites, abdominal pain, weight loss, Child-Pugh grade, ALP, serum total bilirubin, serum AFP, serum urea, tumor size, portal thrombosis and lung metastasis) using a multivariate Cox model. A point is given for each prognostic factor determined according to the relative magnitude of the regression coefficient of the final Cox model. Patients are subsequently divided into three groups (score ⩽ 8: good prognostic group, 9-15: intermediate prognostic group, ⩾ 16: poor prognostic group) based on the sum of the scores assigned to each factor (range: 0-39). Survival curves for each prognostic group created according to this system show clear differences, with a median OS of 7.9, 3.2 and 1.4 months for the good, intermediate and poor prognostic groups, respectively (P < 0.0001). The median OS

Table 10 Memorial Sloan-Kettering Cancer Center nomogram: Prognostic factors

Age
Estimated blood loss
Margin
Satelites
Vascular invasion
Size
Log (alpha-fetoprotein)

and three-month survival rates in the validation set (n = 320) are similar to those obtained for the training set, with a median OS of 7.5, 3.2 and 1.2 months for the good, intermediate and poor prognostic groups, respectively (P < 0.0001). Moreover, the authors demonstrated that the discriminatory ability of the ALPCS (AUC 0.77) is significantly better than that of the Okuda system (AUC 0.66) and CLIP score (AUC 0.71).

However, the ALPCS system was constructed based on the results for a cohort of HCC patients with predominant HBV infection (73% of the whole cohort). Therefore, this system needs to be validated in a Western population with predominant HCV infection and/or a history of alcohol abuse. In addition, many prognostic factors are included in this system (n = 11), making calculating the total score somewhat complicated in daily clinical practice.

China Integrated Score (Table 12)

The China Integrated Score (CIS) was established by Zhang *et al*[38] in 2010. This score is derived from a cohort of 220 patients (166 training set, 54 validation set) with unresectable HCC treated at a single institution in China. The authors identified three prognostic factors (TNM stage, serum AFP and Child-Pugh grade) using a Cox proportional hazard regression model. Patients are classified into six groups (0-5) based on the sum of the scores assigned to the three covariates. The survival curves for a prospective validation cohort of 54 HCC patients were found to be clearly distributed among the groups, with a median survival rate of 9.0, 2.3, 2.1 and 0.6 mo in the patients classified with CIS stages 2, 3, 4 and 5, respectively. The discriminatory ability of the CIS is comparable to that of the CLIP score. According to this system, the authors subsequently proposed a set of guidelines for selecting the optimal treatment in patients with unresectable HCC based on this system.

However, the study population (n = 220) was relatively small. Therefore, the CIS needs to be externally validated in a large scale, prospective study.

Taipei Integrated Score System (Table 13)

The Taipei Integrated Score System was proposed by Hsu *et al*[39] in 2010. This system is derived from the investigation of a cohort of 2030 HCC patients undergoing different treatment modalities at a single institution in Taiwan. The authors adopted the calculated total tumor volume (TTV) as a surrogate marker of the tumor burden and combined the TTV with four cirrhosis associated models (Child-Pugh grade, MELD, MELDNa and MELD-Na) to create the TTV-based staging system. The TTV was categorized into four groups (< 50 cm^3, 50-250 cm^3, 250-500 cm^3 and > 500 cm^3), and single-digit values were assigned to each TTV group (0: < 50 cm^3, 1: 50-250 cm^3, 2: 250-500 cm^3 and 3: > 500 cm^3). A total of 12 new staging models were created and patients were classified into seven groups (0-6) based on these methods. Among the 12 TTV-based staging systems, the TTV-Child-Pugh grade-AFP combination model provides the lowest AIC value. Moreover, the TTV-Child-Pugh grade-AFP model shows superior prognostic value compared with the four current staging systems (CLIP, BCLC, JIS and Tokyo). In particular, the TTV-Child-Pugh grade-AFP model has the smallest AIC value among patients receiving non-curative treatment.

Table 11 Advanced liver cancer prognostic system

Characteristics		Points
Ascites	Yes	2
	No	0
Abdominal pain	Yes	2
	No	0
Weight loss	Yes	2
	No	0
Child-Pugh grade	A	0
	B	2
	C	5
ALP (IU/L)	> 200	3
	≤ 200	0
Total bilirubin (mmol/L)	> 50	3
	33-50	1
	≤ 33	0
Urea (mmol/L)	> 8.9	2
	≤ 8.9	0
Portal vein thrombosis	Yes	3
	No	0
Tumor size	Diffuse	4
	> 5 cm	3
	≤ 5 cm	0
Lung metastases	Yes	3
	No	0
AFP (ng/mL)	> 400	4
	≤ 400	0
Prognosis	Score	3-mo survival rate
Good	0-2	> 0.81
	3-6	0.72-0.8
	7-8	0.66-0.69
Intermediate	9	0.63
	10-12	0.51-0.59
	13-14	0.42-0.47
	15	0.38
Poor	16	0.33
	17-19	0.21-0.29
	20-22	0.1-0.17
	≥ 23	< 0.1

AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase.

Table 12 China Integrated Score

Variables	Scores				
	0	1	2		
TNM stage	≤ Ⅲ	ⅣA	ⅣB		
Child-Pugh grade	A	B	C		
AFP (mg/L)	≤ 400	> 400			
CIS score	0	1-2	3	4	5
	PEI or TACE	Herbs + TACE	TACE with herbs RCT	Chemotherapy	Symptomatic

CIS: China Integrated Score; TACE: Transarterial chemoembolization; AFP: Alpha-fetoprotein; PEI: Percutaneous ethanol injection; RCT: Randomized controlled trials.

Table 13 Taipei Integrated System

Variables	Scores			
	0	1	2	3
Total tumor volume (cm^3)	< 50	50-250	250-500	> 500
Child-Pugh grade	A	B	C	
AFP (ng/mL)	≤ 400	> 400		

AFP: Alpha-fetoprotein.

Although the TTV-based staging system is a useful and reliable system based on the findings of a large cohort of HCC patients with early to advanced stage disease undergoing various treatment modalities, this system is associated with several concerns. First, the TTV is estimated based on the assumption that all tumors are spherical. Therefore, the TTV value may not be accurate in cases involving tumors that are infiltrative or numberless. Second, this system was constructed based on the results for a cohort of HCC patients with predominant HBV infection (55% of the whole cohort) and must therefore be externally validated in Western population.

Eastern staging system (Table 14)

The Eastern staging system was established by Yang *et al*[40] in 2011. This system is derived from the analysis of a cohort of 958 HCC patients with predominant HBV infection (91.8%) who underwent surgical resection at a single institution in China. The authors identified 10 independent prognostic factors, including macroscopic vascular invasion, multiple tumors, the PS1-2 status, microscopic vascular invasion, extrahepatic spread, a maximum tumor size of > 5 cm, a serum albumin level of < 35 g/L, a serum AST level of > 40 U/L, a serum total bilirubin level of > 17 μmol/L and the presence of cirrhosis, using a Cox proportional hazard regression analysis. Based on these variables, the authors established a new staging system for classifying resectable HCC patients, named the Eastern staging system, in which the patients are classified into five groups (stage 1-5) according to the sum of the score (0-10) allocated to each prognostic factor. The Eastern staging system exhibits significant differences in the probability of survival of the patients in different stages ($P < 0.001$). The Eastern staging system provides the highest likelihood ratio according to the χ^2 and linear trend χ^2 tests (543.51 and 414.97) among six other staging systems (Okuda, CLIP, BCLC, CUPI, AJCC/TNM and JIS), indicating superior homogeneity and monotonicity of the gradients. Moreover, the AUC of the Eastern staging is higher at each time point than that of the other six staging systems (1, 3 and 5 years: 0.846, 0.811 and 0.815, respectively).

Table 14 Eastern stage

Variables	Score	
	0	1
Macroscopic vascular invasion	-	+
Tumor number	Solitary	Multiple
PS	0	1-2
Microscopic vascular invasion	-	+
Extrahepatic spread	-	+
Maximum tumor size (cm)	≤ 5	> 5
Albumin (g/L)	≥ 35	< 35
AST (U/L)	≤ 40	> 40
Total bilirubin (μmol/L)	≤ 17	> 17
Presence of cirrhosis	-	+
	Cumulative score	
Stage Ⅰ	0-1	
Stage Ⅱ	2-3	
Stage Ⅲ	4-5	
Stage Ⅳ	6-7	
Stage Ⅴ	8-10	

AST: Aspartate transaminase.

However, the Eastern staging system is associated with some limitations. First, it was derived from a cohort of HCC patients with predominant HBV infection, as the authors adequately mentioned. Hence, this system must be externally validated in a Western population. Second, the weight for survival of each prognostic factor was not taken into account when identifying the prognostic factors.

Portal vein tumor thrombus classification (Table 15)

The portal vein tumor thrombus (PVTT) classification was proposed by Shi *et al*[41] in 2011. This system is derived from the investigation of a retrospective cohort of 441 HCC patients with macroscopic PVTT treated with partial hepatectomy at a single institution of China. The authors proposed the PVTT classification based on the extent of tumor thrombosis in the portal vein, as follows: Type Ⅰ 0 Tumor thrombus formation on microscopy, Type Ⅰ tumor thrombosis involving segmental branches of the portal vein or above, Type Ⅱ tumor thrombosis involving the right/left portal vein, Type Ⅲ tumor thrombosis involving the main portal vein trunk and Type Ⅳ tumor thrombosis involving the superior mesenteric vein. The one-, two- and three-year survival rates for Types Ⅰ to Ⅳ PVTT are 54.8%, 33.9%and 26.7%, 36.4%, 24.9% and 16.9%, 25. 9%,

Table 15 Portal vein tumor thrombus classification

Types
Type Ⅰ 0: Tumor thrombus formation found under microscopy
Type Ⅰ: Tumor thrombi involving segmental branches of portal vein or above
Type Ⅱ: Tumor thrombi involving right/left portal vein
Type Ⅲ: Tumor thrombi involving the main portal vein trunk
Type Ⅳ: Tumor thrombi involving the superior mesenteric artery

Table 16 A prognostic model for hepatocellular carcinoma patients within the Milan criteria undergoing non-transplant therapies

Variables	Scores		
	0	1	2
Total bilirubin (mg/dL)	< 1.5	≥ 1.5	
AFP (ng/mL)	< 100	≥ 100	
Ascites	-	Mild	Moderate to severe

AFP: Alpha-fetoprotein.

12.9% and 3.7%, 11.1%, 0% and 0%, respectively (P < 0.0001). The discriminatory ability of the PVTT classification is superior to that of the AJCC/TNM staging system, CLIP score and JIS score.

Although the PVTT classification appears to be useful for predicting the outcomes of HCC patients with surgically treated macroscopic PVTT, it has some limitations. First, it was derived from a cohort of HCC patients with predominant HBV infection (87.5% of the whole cohort) and needs to therefore be externally validated in a Western population. Second, the use of surgical resection with or without portal thrombectomy for HCC associated with PVTT is not a global standard. In fact, in the BCLC classification, sorafenib is recommended as the first-line treatment for HCC patients with PVTT (Stage C). Therefore, this system may be not suitable for use in all HCC patients with PVTT.

Staging systems proposed since 2012

Several staging systems have been newly proposed since 2012. However, many of these systems have not been externally validated.

Prognostic model within the Milan criteria for patients undergoing non-transplant therapy (Table 16)

Lee *et al*[42] proposed a prognostic model based on the serum bilirubin level, AFP level and severity of ascites in patients meeting the Milan criteria treated with non-transplant therapy in 2012[42]. This system is derived from the findings of a cohort of 1106 HCC patients (49% HBV infection, 553 deviation set, 553 validation cohort) receiving treatment at a single institution in Taiwan. The authors constructed a new system based on three independent prognostic factors identified in a multivariate Cox model of the deviation set. Subsequently, the predictive accuracy was confirmed in the validation set, irrespective of the treatment strategy (curative or non-curative).

However, evaluations of the amount of ascites are subjective and affected by the use of diuretics, as the authors adequately mentioned. Therefore, objective assessments of ascites are required.

Table 17 Model to Estimate Survival in Ambulatory hepatocellular carcinoma patients score

MESIAH score
= 0.232 * (age in decades)
+ 0.099 * (MELD)
- 0.391 * (serum albumin level)
+ 0.290 * (tumor size)
+ 0.153 * (tumor number)
+ 1.122 * (vascular invasion)
+ 1.130 * (extrahepatic metastasis)
+ 0.082 * (serum AFP level)
+1

AFP: Alpha-fetoprotein. MESIAH: Model to Estimate Survival in Ambulatory HCC patients score; HCC: Hepatocellular carcinoma.

MESIAH score (Table 17)

The Model to Estimate Survival in Ambulatory HCC patients score (MESIAH score) was developed by Yang *et al*[43], from the Mayo group, in 2012. This score is derived from a cohort of 477 HCC patients (derivation cohort) treated at the Mayo Clinic and 904 HCC patients (validation cohort) treated at a Korean institution. The authors identified independent predictors for survival in a multivariate Cox model (age, MELD score, serum albumin level, tumor size, tumor number, vascular invasion and extrahepatic metastasis), thus creating a new risk score. Following internal validation, the prognostic value of the MESIAH score was confirmed in the validation cohort, with a concordance statistics of 0.82, which is higher than that for the CLIP score (0.75) and JIS score (0.78). The derivation cohort differed from the validation cohort with regard to the underlying liver disease (derivation cohort: HBV 18%, HCV 81%, validation cohort: HBV 75%) and treatment modality (derivation cohort: transplantation 31%, resection 17%, TACE 25%, validation cohort: resection 13%, TACE 57%). Conversely, however, it can be said that the predictive accuracy of MESIAH is highly stable, irrespective of the underlying liver disease and/or treatment modality.

More recently, the same group validated this score in another cohort of 1969 HCC patients with predominant HBV infection (74.6%) treated at a Korean institution[44]. The discriminatory ability of the MESIAH score, as evidenced by the C-statistics, LRχ2 value and AIC, is better than that of the BCLC, CLIP, JIS and Tokyo.

However, calculating the MESIAH score is somewhat complicated in daily clinical practice.

Considering the advantages of superior predictive accuracy and objectivity of the prognostic factors,

Table 18 Alpha-fetoprotein staging

AFP (ng/mL)	Stage
< 10	N (normal)
10-150	A
150-500	B
> 500	C

AFP: Alpha-fetoprotein.

Table 19 Hepatoma arterial-embolisation score

Variables	Points
Albumin < 36 g/dL	1
AFP > 400 ng/mL	1
Total bilirubin > 17 μmol/L	1
Maximum tumor diameter > 7 cm	1

AFP: Alpha-fetoprotein.

independent of the underlying liver disease and treatment modality, the MESIAH score is one of the most promising staging systems for evaluating HCC patients.

AFP staging (Table 18)

The AFP staging was proposed by Burnet *et al*[45] in 2013 against a background in which a substantial proportion of HCC patients in Kentucky have no underlying liver disease[45]. This score is derived from the findings of a cohort of 518 HCC patients (272 cirrhotic, 246 non-cirrhotic) treated at a single institution in the United States. The authors defined the AFP stage based on the report by Muscari *et al*[46], as follows: stage N (AFP < 10 ng/mL), stage A (10 < AFP < 150 ng/mL), stage B (150 < AFP < 500 ng/mL) and stage C (AFP ⩾ 500 ng/mL). Survival curves determined according to the AFP stage for each prognostic group show clear survival differences (P < 0.0001), similar to the BCLC classification. In particular, in non-cirrhotic patients, the AFP staging system has a lower P value than the BCLC classification.

However, this study is associated with some limitations. First, there is no information regarding the treatment modality, which affects patient outcomes. Second, the survival differences among patient populations assigned to AFP stage B and C are not significant. Third, although the authors stated that the AFP stage is more suitable for assessing non-cirrhotic HCC patients, no comparative analyses with the BCLC classification have been carried out.

Hepatoma arterial-embolisation score (Table 19)

The hepatoma arterial-embolisation (HAP) score was developed by Kadalayil *et al*[47] in 2013. This score is derived from a cohort of 281 HCC patients (114 training set, 167 validation set) who received TACE at three institutions in England. The authors identified four prognostic factors (a serum albumin levels of < 36 g/dL, serum AFP level of > 400 ng/mL, serum bilirubin level of > 17 μmol/L and maximum tumor diameter of > 7 cm) using a multivariate Cox model. Patients are classified into four groups (HAP A-D) based on the sum of the scores assigned to the prognostic factors. The survival curves for both the training and validation sets stratified according to the HAP score were clearly distributed (P < 0.001), and the authors demonstrated that the HAP score provides superior predictive value compared to the Okuda, MELD, BCLC and Child-Pugh grade based on the AUC.

However, the HAP score has not been externally validated.

5-gene score

The 5-gene score was proposed by Nault *et al*[48] in 2013. This score is derived from a cohort of HCC patients who underwent surgical resection at two French institutions and several institutions in the United States, Italy, Spain, Japan and China. The authors constructed the 5-gene score based on findings showing that the expression patterns of five genes (*TAF9*, *RAMP3*, *HN1*, *KRT19* and *RAN*) had strong prognostic relevance. This score was found to be significantly associated with the disease-specific survival and rate of early tumor recurrence in both the training cohort (n = 189) and validation cohort (n = 125). The authors further validated the 5-gene score in HCC patients with predominant HCV infection in Europe and the United States and HCC patients with predominant HBV infection in Asia. However, this score is not readily available in daily clinical practice.

Hong Kong Liver Cancer classification (Figure 2)

The Hong Kong Liver Cancer (HKLC) classification was developed by a Hong Kong group in 2014[49]. This system is derived from the results of a large cohort of 3856 HCC patients (1968 training set, 1888 test set) with predominant HBV infection treated at single institution in Hong Kong. Four established prognostic factors (ECOG PS, Child-Pugh grade, liver tumor status and presence of extrahepatic vascular invasion or metastasis) were selected when building the system using the training set according to a multivariate Cox regression model. Patients are classified in five main stages and nine substages (stages Ⅰ-Ⅴb) based on these prognostic factors. The constructed staging system and treatment guidelines were subsequently assessed in the test set for internal validation. This classification is based on five main stages with distinct survival outcomes, which were very similar between the training set and the test set. This classification exhibits better prognostic value than the BCLC classification, with an AUC at one year of 0.851, three years of 0.8 and five years of 0.83 for the HKLC classification, compared to an AUC at one year of 0.804, three years of 0.8 and five years of 0.795 for the BCLC classification. In the

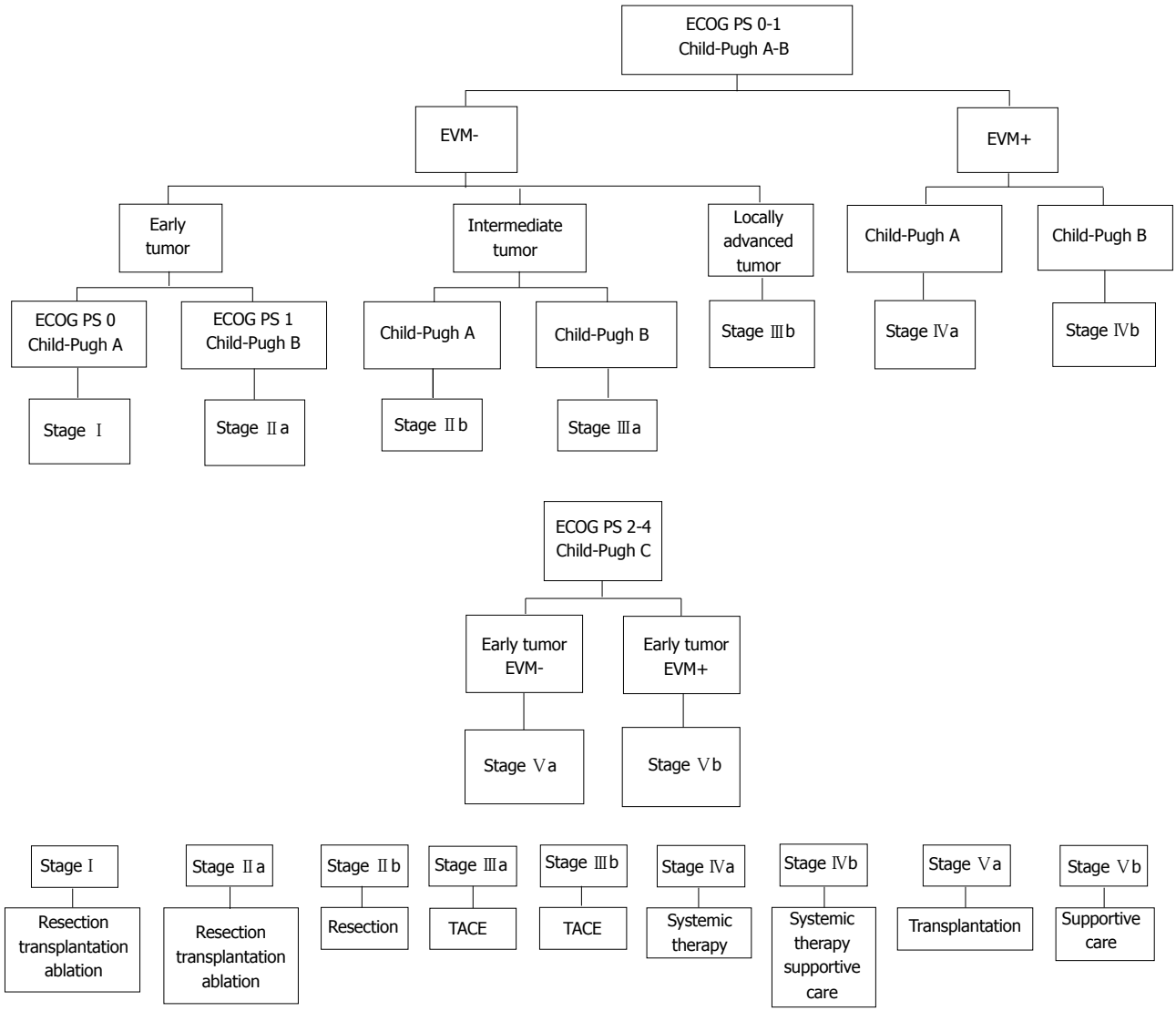

Figure 2 Hong Kong Liver Cancer classification. EVM: Extrahepatic vascular invasion/metastasis; ECOG: Eastern cooperative oncology group; TACE: Transarterial chemoembolization.

authors' analysis, the C-index for the HKLC was 0.739, which is higher than that for the BCLC classification (0.703). Notably, the HKLC classification is able to better stratify patients in the BCLC B and C stages into distinct groups, with better survival outcomes based on more aggressive treatment recommendations than that observed in the BCLC treatment algorithm.

However, the HKLC classification is associated with several limitations. First, it was derived from a cohort of HCC patients with predominant HBV infection (80% of the whole cohort) and should therefore be validated in a Western population as well as patients with different disease etiologies, as the authors adequately described. Second, the authors subdivided the BCLC classification into five groups (A1-2, A3-4, B, C and D) when assessing the AUC values for the HKLC and BCLC classifications, which is not appropriate considering the current categories of the BCLC classification (0, A, B, C and D)[50].

Despite these limitations, the HKLC system appears to have a greater impact on the current BCLC classification, addressing the problems with the heterogeneity of the BCLC B and C stages and rigidity of treatment allocation. Regarding the former problem, it is interesting that the HKLC classification is compared with the subclassification of the BCLC B stage proposed by Bolondi *et al*[20]. Regarding the latter problem, the expanded treatment guidelines of the HKLC classification, such as surgical resection for BCLC B patients or TACE for BCLC C patients, should be verified in a large-scale prospective study in addition to HCC patients with etiologies other than HBV infection.

External validation and comparison of currently available staging systems

As mentioned above, a number of staging systems and/or scoring systems for HCC have been proposed and established. Several studies have also externally validated and compared the prognostic value of various staging systems.

AJCC/TNM 7th edition

Kee *et al*[51] demonstrated that the 7th edition of the TNM staging system provides a superior discriminatory value than 6th edition of the TNM system based on the findings of a cohort of 8828 HCC patients treated at a single institution in Taiwan[51]. Chun *et al*[52] also showed that the 7th edition of the TNM system has greater prognostic power than the 6th edition of the TNM system based on an analysis of a cohort of 877 HCC patients with predominant HBV infection treated at a single Korean institution[52], and Zhou *et al*[53] showed that the 7th edition of the TNM system was the best prognostic model for HCC patients without AFP elevation who undergo surgical resection[53].

However, the prognostic ability of the 7th edition of the TNM system is poorer than that of the BCLC classification, particularly in patients with advanced stages of the disease[54]. Studies from China have also reported the predictive inaccuracy of the 7th edition of the TNM system, although the patient populations were limited to subjects undergoing surgical resection[55]. Due to its inherent lacks of factors related to the liver functional reserve, the prognostic relevance of the TNM staging system appears to be limited to HCC patients with early-stage tumors and a preserved liver functional reserve.

CLIP score

Because the CLIP score was originally derived from a cohort of HCC patients who primarily presented with advanced stage tumors, it is generally accepted that the CLIP score is suitable for use in HCC patients with advanced tumors or those receiving non-surgical treatments.

In fact, investigators from Japan, Canada, Italy, France, Taiwan, the United States and Germany recently demonstrated that the CLIP score provides better prognostic value than other staging systems in HCC patients with advanced stage tumors[56-63]. In a cohort of HCC patients who received specific treatment modalities, including TACE or radioembolization, systemic chemotherapy and BSC, the CLIP score proved to be the best prognostic model[64-66]. However, studies from Japan and Taiwan have shown that the CLIP score provides a superior predictive value compared to other staging systems, even in HCC patients undergoing surgical resection[67,68]. Finally, a large-scale study from Taiwan demonstrated that the CLIP score is the best prognostic model in patients with early to advanced stages of disease, irrespective of the use of curative or non-curative treatment[69]. These results indicate that the predictive accuracy of the CLIP score is highly stable, independent of the tumor stage, treatment modality, underlying liver disease and geographic differences.

BCLC classification

As expected, several studies from Italy and China have shown that the BCLC classification is the best prognostic model in HCC patients who receive radical therapy, including surgical resection or percutaneous ablation[70-74]. In contrast, investigators from Italy, the United States, Spain, South Korea and Egypt demonstrated that the BCLC classification provides the best prognostic value in HCC patients with early to advanced stage tumors treated with various modalities[75-79]. These results indicate that the predictive accuracy of the BCLC classification is highly stable, independent of the tumor stage, treatment modality, underlying liver disease and geographic differences.

With regard to treatment allocation, a large-scale trial from Taiwan (n = 3892) showed that the treatment schedules determined according to the BCLC classification are both reasonable and beneficial for survival in patients with HCC[80].

CUPI

Studies from Taiwan and China have demonstrated that the CUPI is the best prognostic model in advanced HCC patients with portal vein invasion or extrahepatic metastasis[63,81].

However, this score has not been validated in either a Western population or in patients with etiologies other than HBV infection.

JIS

A study from Japan showed that the JIS score provides the best prognostic value in HCC patients treated with surgical resection[82]. Other studies from Japan have also demonstrated the JIS score to be the best prognostic model in HCC patients who receive various treatment modalities[83,84]. However, the JIS score has not been validated in countries outside of Japan.

Tokyo

Investigators from Taiwan reported that the Tokyo score was the most informative tool in a large cohort (n = 2010) of HCC patients with predominant HBV infection (67%) who underwent various treatment regimens[66]. However, the Tokyo score has not been validated in a Western population.

ALCPS

A study from China demonstrated the ALCPS system to be the best prognostic model in advanced HCC patients with predominant HBV infection (88%)[85]. However, this score has not yet been validated in a Western population.

Staging systems must to be validated in both Western and Asia-Pacific patient populations, irrespective of the underlying liver disease and etiology, before they can be considered to be globally applicable, as there are significant regional and institutional differences in HCC in terms of etiology, underlying liver disease and feasible treatment modality[4]. In this context, among many staging systems BCLC and CLIP can be currently globally applicable staging systems for HCC patients.

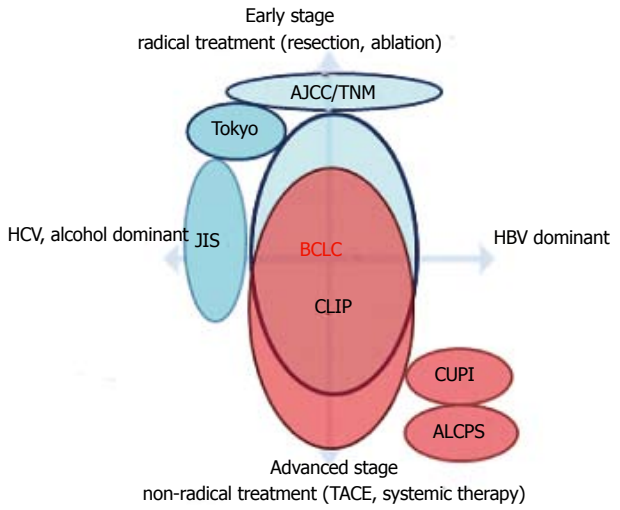

Figure 3 A positioning map of existing validated staging systems. AJCC: The American Joint Committee on Cancer; BCLC: The Barcelona Clinic Liver Cancer; CLIP: The Cancer of the Liver Italian Program; JIS: The Japan Integrated Staging Score; CUPI: The Chines University Prognostic Index; ALCPS: The Advanced Liver Cancer Prognostic System; HBV: Hepatitis B virus.

A positioning map of existing validated staging systems is shown in Figure 3.

New attempts

In addition to creating new models, investigators have made attempts to modify and/or add other variables, such as biomarkers or the general status, into existing prognostic systems.

Modifying currently available staging systems: Huo *et al*[86] proposed the MELD-based model in 2007. In this model, the Child-Pugh grade, which is used in the CLIP, BCLC and JIS scores to assess the liver functional reserve, is replaced with the MELD score, and the authors subsequently created MELD-based modified CLIP, BCLC and JIS scores. These scores have better predictive value than the original scores. Ling *et al*[87] also incorporated the MELD score into the TNM system for use in patients undergoing surgical resection, thus creating the MELD-based TNM staging system and demonstrated that the MELD-based TNM stage provides better prognostic stratification[87].

Meanwhile, Lin *et al*[88] subdivided the CLIP score into 36 subgroups[88]. The authors showed that different prognostic weighting of four predictive factors of the CLIP score (PVT followed by the Child-Pugh grade, AFP and tumor morphology) resulted in heterogeneity of survival within the same score group.

Furthermore, Santambrogio *et al*[89] proposed a simplified BCLC staging system (s-BCLC) for assessing resectable HCC patients. This score is defined by only two groups (AA: BCLC A1 + A2 with a serum AFP level of $\leqslant$ 20 ng/mL, AB: BCLC A1 + A2 with a serum AFP level of > 20 ng/mL or A3, A4). The authors demonstrated that the s-BCLC is more suitable for prognostic stratification in HCC patients who undergo surgical resection than the original BCLC or other staging systems.

Regarding the heterogeneity of patients in BCLC stage B, Bolondi *et al*[20] proposed a subclassification of stage B (B1-B4) in 2012, in association with different first-line and alternative treatment options (Table 20). Notably, the authors adopted the up-to-7 criterion in order to distinguish major from minor tumor extension[90]. Recently, this subclassification was externally validated in a cohort of HCC patients in both South Korea and Taiwan[91,92].

Adding biomarkers to existing staging systems: Kitai *et al*[93] combined the JIS score and three tumor markers (AFP, AFP-L3 and DCP), to create a new staging system, the Biomarker combined JIS (bm-JIS), in 2008. This system is derived from a cohort of 1824 HCC patients treated at five Japanese institutions. The authors showed that the bm-JIS score has better stratification value than the conventional JIS score. The group also externally validated the bm-JIS score in 1173 HCC patients treated at five Japanese institutions[94].

Kaseb *et al*[95] proposed the VEGF-CLIP (V-CLIP) score in 2011 based on findings showing that the VEGF, the major mediator of angiogenesis in the setting of HCC, is associated with the overall survival of HCC patients. The authors added the VEGF (cutoff point: 450 pg/mL) to the CLIP score, thus creating the V-CLIP score. The V-CLIP score stratifies patients into homogenous prognostic groups (P = 0.005) and provides superior predictive accuracy compared to the original CLIP score (P = 0.005). The same group proposed the insulin-like growth factor-1 (IGF-1) CLIP (I-CLIP) score in 2011 based on findings demonstrating that the IGF-1 value, which reflects the synthetic function of the liver, is an independent prognostic factor for overall survival of HCC patients[96]. The authors subsequently integrated the dichotomized IGF-1 level (cutoff point: 26 ng/mL) into the CLIP score, thereby creating the I-CLIP score. The I-CLIP score classifies patients into independent prognostic groups (P < 0.0001) and displays a better prognostic ability than the original CLIP score (P < 0.0001). Based on these results, Kaseb *et al*[97] established the IGF-1, VEGF-BCLC (IV-BCLC) score in 2011 in which they integrated the IGF-1 value (cutoff point 26 ng/mL) and VEGF value (cutoff point 450 pg/mL) into the BCLC score, to create the IV-BCLC score. The authors demonstrated that IV-BCLC score is more accurate in predicting overall survival and provides better prognostic stratification than the original BCLC score (P < 0.0001).

More recently, Kinoshita *et al*[98,99] reported that the addition of the serum CRP level to previously validated staging systems (CLIP, BCLC, JIS, BCLC, Tokyo and TNM according to LCSGJ) improves the prognostic value of each staging system, based on

Table 20 Barcelona Clinic Liver Cancer B subclassification

Sub-Stage	B1	B2	B3	B4
Child-Pugh score	5-6-7	5-6	7	8-9 (with severe/ refractory ascites and/or jaundice)
Beyond Milan and within Ut-7	In	Out	Out	Any
ECOG (tumor related) PS	0	0	0	0-1
Portal vein thrombosis	-	-	-	-
1st option	TACE	TACE or	Research	BSC
Alternative	Liver transplantaion TACE + ablation	TARE sorafenib	trials TACE sorafenib	Liver transplantaion (only if Up-to-7 IN and PS0)

TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; BSC: Best supportive care.

results showing an elevated serum CRP level to be independently associated with a poor prognosis in HCC patients.

Adding the general status to existing staging systems: Tournoux-Facon *et al*[100] reported that the addition of the WHO PS to the CLIP score improves the discriminatory ability compared to that of the original CLIP and BCLC scores in patients treated in the palliative setting (BoBar). The same group also demonstrated that incorporating quality of life data improves the prognostic value of the CLIP, BCLC, GRETCH and BoBar scores in palliative HCC patients[101].

Furthermore, Hsu *et al*[102] showed that the modifying the BCLC system according to the ECOG PS enhances the prognostic ability in HCC patients in early to advanced stages of the disease.

Problems with currently available staging systems and future perspectives

As mentioned above, many staging systems and scoring systems have been established and refined. However, there is currently no globally accepted system for assessing HCC patients, due to heterogeneity of the extent of tumor extension, underlying liver disease and liver functional reserve. There are several problems regarding currently available staging systems.

First, none of these systems take into account the location of the tumor or its proximity to major vessels, which affect both treatment selection and tumor progression[7].

Second, none of the above systems incorporate the etiology (HBV infection, HCV infection, alcoholism and NASH) or underlying liver disease (LC, hepatitis and a normal liver). Generally, the outcomes of HCC patients differ according to the etiology of the liver disease. Several studies have shown that HCC patients with HCV infection or alcoholic liver disease exhibit poorer outcomes than those with HBV infection[7,103,104]. This is because HCC patients with HBV infection generally have a better liver functional reserve than those with HCV infection or alcoholic liver disease[50]. An increasing number of patients develop HCC based on the presence of nonalcoholic fatty liver disease (NAFLD) or NASH, both of which affect the liver functional reserve and patient outcomes. In fact, Reddy *et al*[105] demonstrated that HCC patients with NASH undergoing surgical resection display a better liver functional reserve and survival outcomes than those with HCV infection and/or alcoholic liver disease[105]. More recently, Kaseb *et al*[106] showed that currently available staging systems (Okuda, CLIP, BCLC, CUPI and TNM 6th edition) are significantly less predictive of overall survival in HCC patients without cirrhosis or hepatitis, advocating that staging systems should be modified to include factors related to viral hepatitis and cirrhosis in addition to demographics and geographic location.

Third, many staging systems lack optimal treatment allocation, with the exception of BCLC and HKLC. There is also controversy regarding current BCLC treatment recommendations. First, this system does not provide recommendations for second-line therapy or combined treatment, such as resection or liver transplantation after TACE, the combination of TACE with RFA and/or the combination of TACE with sorafenib. Second, it is rigid. In a study from South Korea, many patients with a BCLC 0 (62.9%) or BCLC A (54%) status underwent TACE rather than radical therapies, such as surgical resection or percutaneous ablation, as proposed by the BCLC classification. Moreover, patients with BCLC C stage disease underwent TACE (35.7% of patients) or HAIC (24.6% of patients) rather than receive treatment with sorafenib, which is inconsistent with the recommendations in the BCLC classification[78]. More recently, a multicenter Italian study demonstrated that the survival rate of BCLC B patients undergoing TACE (MST: 27 mo) was significantly shorter than that of BCLC B patients who underwent surgical resection (MST: 37 mo) and percutaneous ablation (MST: 36 mo) ($P < 0.001$), indicating that patients with a BCLC B status are often suitable candidates for more aggressive therapies than TACE based on proper patient selection[107]. In addition, a multicenter study from Italy showed that liver transplantation could result in survival benefit for HCC patients with BCLC D status[108]. These results also suggest the need for careful multidisciplinary evaluations of optimal treatment modalities as recommended by the BCLC classification.

In conclusion, although many staging and/or scoring systems have been proposed, there is currently no globally accepted system for assessing HCC patients due to the extreme heterogeneity of the disease. Clinicians involved in treating HCC patients

should use currently available staging systems or treatment algorithms carefully while understanding their features and limitations. Growing evidence regarding understanding of tumor biology as well as advancements in imaging techniques and treatment modalities will result in the development of better staging systems that refine the process of stratification, survival prediction and treatment allocation in order to optimize the management of HCC patients.

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REVIEW

Gut-liver axis in liver cirrhosis: How to manage leaky gut and endotoxemia

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Abstract

A "leaky gut" may be the cutting edge for the passage of toxins, antigens or bacteria into the body, and may play a pathogenic role in advanced liver cirrhosis and its complications. Plasma endotoxin levels have been admitted as a surrogate marker of bacterial translocation and close relations of endotoxemia to hyperdynamic circulation, portal hypertension, renal, cardiac, pulmonary and coagulation disturbances have been reported. Bacterial overgrowth, increased intestinal permeability, failure to inactivate endotoxin, activated innate immunity are all likely to play a role in the pathological states of bacterial translocation. Therapeutic approach by management of the gut-liver axis by antibiotics, probiotics, synbiotics, prebiotics and their combinations may improve the clinical course of cirrhotic patients. Special concern should be paid on anti-endotoxin treatment. Adequate management of the gut-liver axis may be effective for prevention of liver cirrhosis itself by inhibiting the progression of fibrosis.

Key words: Gut-liver axis; Liver cirrhosis; Pathogenesis; Complications; Endotoxemia; Bacterial translocation; Leaky gut; Toll-like receptors; Selective intestinal decontamination; Probiotics

Core tip: A "leaky gut" may be the cutting edge for the passage of toxins, antigens or bacteria into the body, and may play a pathogenic role in advanced liver cirrhosis and its complications. More attention should be paid to the role of intestinal bacteria and bacterial products in the field of Hepatology. Here, I would like to overview the history of endotoxin assay in the blood, clinical significance of endotoxemia in liver cirrhosis and then shift to the topic of gut and liver in general. Understanding of the gut-liver axis, leaky gut and endotoxemia in cirrhosis may give us new ideas.

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INTRODUCTION

Bacterial infections account for significant morbidity

and mortality in patients with liver cirrhosis[1]. Infections increase mortality 4-fold in cirrhotic patients[2]. Although urinary, respiratory, ascitic fluid infections and bacteremia are common infectious complications, spontaneous bacterial peritonitis (SBP) occurs most frequently. A vast majority of such infections are due to enteric gram-negative bacteria, mainly *Enterobacteriaceae*[3]. Passage of viable bacteria from the intestinal lumen through the intestinal wall and to mesenteric lymph nodes (MLNs) and other sites, defined as bacterial translocation (BT), explains the development of SBP[4]. The concept of BT was later broadened to microbial products or their fragments, such as endotoxin [lipopolysaccharide (LPS)], peptidoglycan, lipopeptides and bacterial DNA. The liver receives portal blood containing these microbial products and acts as the initial site of their filtration and detoxication. These defense mechanisms are impaired in the cirrhotic liver, which finally results in spillover of these products and secretion of various inflammatory mediators.

LPS is a major component of the gram-negative bacterial wall. Its detection in the blood has a long history and is still not complete with several methodological difficulties. However, the endotoxemia detected by the Limulus-lysate test or its modifications has been well correlated to the severity, complications and mortality of liver cirrhosis. Control of endotoxemia is still considered to be the mainstay of therapy for advanced liver cirrhosis.

Here, I would like to overview the history of endotoxin assay in the blood, clinical significance of endotoxemia in liver cirrhosis and then shift to the topic of gut and liver in general. Understanding of the gut-liver axis, leaky gut and endotoxemia in cirrhosis may give us new ideas for hepatology tomorrow.

DETECTION OF ENDOTOXEMIA IN PATIENTS WITH LIVER CIRRHOSIS

Levels of bacterial LPS are increased in the portal and/or systemic circulation in liver cirrhosis. Endotoxemia was first demonstrated by the Limulus amebocyte lysate (LAL) test and later by several quantitative assay, such as the chromogenic Limulus assay and the turbidimetric endotoxin assay. Although endotoxemia is now considered to be a common feature of liver cirrhosis, there has been much debate and still disagreement about plasma endotoxin levels.

Early study using LAL test revealed that systemic endotoxemia in liver cirrhosis occurred with a frequency of 15 of 31 compared with 2 of 21 venous samples and 9 of 21 portal venous samples from patients without liver disease[5]. Tarao *et al*[6] reported that death occurred within 6 mo in 47.8% of the patients with a positive endotoxin test, whereas only 16.7% of those with a negative test died in the same period. Clemente *et al*[7] showed that a positive LAL test was almost exclusively associated with a progressive functional renal failure (8 of 10 patients) and all but one of them died. Endotoxemia was also associated with hemorrhage due to acute erosions of the gastric mucosa (6 of 7 patients)[7]. On the contrary, Gaeta *et al*[8] found no significant difference in the frequency of endotoxemia between patients with impaired and unimpaired renal blood flow. Moreover, no relation was found between endotoxin plasma levels and renal blood flow in their study. Bode *et al*[9] reported that the prevalence of endotoxemia was not significantly higher in cirrhotics with ascites or esophageal varices when compared to the subgroup without ascites or esophageal varices. They additionally found endotoxemia more frequently in patients with alcoholic cirrhosis (67.3%) than in patients with non-alcoholic cirrhosis (45.5%)[9].

In general, nonspecific gelation has been reported by this LAL test, and interpretation of the results has often caused confusion[10]. Finally, Fulenwider *et al*[11] reported that they could not detect any endotoxin by the LAL test in peripheral plasma, portal plasma and ascites. They concluded that the ubiquity of endotoxin, with the attendant opportunities for specimen contamination, is the most likely explanation for the high prevalence of endotoxin in the plasma and ascites of cirrhotic patients.

In 1978, Iwanaga *et al*[12] developed a quantitative endotoxin assay using a synthetic chromogenic peptide as a substrate for the endotoxin-sensitive Limulus enzyme. This assay has been shown to give reliable results for minute amount of endotoxin in water. However, measurements of endotoxin in blood present some difficulties. Major problems in plasma endotoxin assay are (1) disagreement about the best way of preparing standard curves[13-15]; (2) lack of an optimal method for eliminating plasma inhibitors for endotoxin assay[15-17]; and (3)necessity of endotoxin-specific chromogenic substrate[18]. We have insisted that a standard curve should be prepared for each individual plasma sample in the endotoxin determination, because ideal 100% recovery of endotoxin could not be validated in any trial of plasma pretreatment[15]. The internal standard is especially necessary in the perchloric acid treatment, where strict adjustment of pH in samples is difficult before the chromogenic assay[19]. Our study demonstrated hidden extra portion of endotoxin in plasma of patients with chronic liver diseases, using new way of plasma pretreatment either by Tween 80 in the dilution and heating method or by triethylamine in the perchloric acid method[20]. The chromogenic substrate widely used in the world is considered to react not only endotoxin but also other substances such as (1,3)-β-D-glucan, component of cell wall of fungus[21]. Endotoxin-specific chromogenic substrate was produced by removing G-factor from the lysate[21].

Although most studies on plasma endotoxin

levels ignored these points, using simply the dilution and heating method and non-specific chromogenic substrate, results obtained were generally useful for evaluation of the clinical significance in liver cirrhosis. Lumsden *et al*[22] found that endotoxin levels in the portal venous blood was significantly higher than that in the peripheral venous blood, although there was a wide variability. However, neither hepatic nor peripheral venous endotoxin levels correlated significantly with a variety of clinical, biochemical or radiological parameters[22]. Tachiyama *et al*[23] also confirmed that endotoxin levels in the portal blood was higher than that in the peripheral venous blood by their Limulus gelation turbidimetric LAL assay. Moreover, they found that portal endotoxin levels in patients with cirrhosis was higher than those without cirrhosis[23]. This finding suggests an enhanced intestinal production and/or absorption of endotoxin in liver cirrhosis, which later developed the discussion on bacterial overgrowth and leaky gut in liver cirrhosis. Bigatello *et al*[24] detected endotoxemia in 36 of 39 cirrhotic patients and in none of healthy volunteers by their chromogenic LAL test. They found that systemic endotoxemia was higher in patients with hepatic encephalopathy after esophagogastric hemorrhage than in well-compensated cirrhotics. It was higher in patients with deep coma than in those with light coma and also higher in those who died than in those who survived. They concluded endotoxemia without sepsis is a constant finding in cirrhosis and increasing levels of endotoxemia are associated with hepatic failure, encephalopathy, and death[24].

We found higher plasma endotoxin level in patients with alcoholic cirrhosis than in patients with non-alcoholic cirrhosis with an improved chromogenic substrate assay, using individual standard curves for each plasma sample[25]. On admission endotoxin concentrations in alcoholics with fatty liver were similarly elevated as observed in alcoholic cirrhosis. In 6 out of 12 patients with fatty liver or alcoholic hepatitis, in whom a second sample of plasma was investigated after 6 to 8 d, endotoxemia was no longer detectable. The results indicate that, irrespective of the stage of liver disease, alcohol abuse favors the development of endotoxemia[25]. In the measurement of plasma endotoxin by the dilution and heating method, we frequently experienced that plasma endotoxin level in patients with advanced liver cirrhosis was unexpectedly low and speculated that some part of plasma endotoxin might be lost in the procedure of dilution and heating. To overcome this situation, we added a detergent Tween 80 after heating plasma and discovered much hidden endotoxin in the sample[20]. Significantly higher plasma endotoxin levels in cirrhotics with upper gastrointestinal (GI) bleeding compared with those without upper GI bleeding was detected by this Tween 80 method[26].

To attain endotoxin-specific chromogenic assay, we further improved our method using triethylamine in the perchloric acid method and endotoxin-specific substrate Endospecy (Seikagaku Kogyo Co., Tokyo, Japan) with kinetic analysis[19,20]. A final pH of the assay sample was always adjusted to 7 by careful titration of triethylamine[19]. The results of our measurement of plasma endotoxin in 90 patients with liver cirrhosis and 11 patients with chronic hepatitis with this method were summarized as follows: (1) there was an increase of plasma endotoxin with the progression of chronic liver disease; (2) in patients with bleeding from esophageal varices, plasma endotoxin increased for 3 d after the bleeding and thereafter decreased; and (3) endotoxin level increased as the progression of Child-Pugh grades and was negatively related to prothrombin time[18].

RELATIONSHIP OF ENDOTOXEMIA TO PATHOGENESIS OF CIRRHOSIS AND ITS COMPLICATIONS

Hyperdynamic circulation

Hyperdynamic circulation characterized by hypotension, low systemic vascular resistance, high cardiac output and a reduced sensitivity to vasoconstrictors are features of cirrhosis. These cardiovascular changes might be the result of increased synthesis of a vasodilator[27]. Nitric oxide derived from vascular endothelium is a potent vasodilator that plays a key role in the homeostasis of blood pressure[28]. Cirrhotic patients showed significant increases in serum nitrite/nitrate which was significantly correlated with endotoxemia[28]. Oral administration of colistin to 15 cirrhotic patients reduced significantly plasma endotoxin levels and serum nitrite/nitrate levels[28]. A lower systemic vascular resistance and a higher cardiac output were found in cirrhotics with endotoxemia than in those without endotoxemia[29]. These studies support that endotoxemia may be responsible, at least in part, for the hyperdynamic circulation found in patients with liver cirrhosis. On the contrary, Campillo *et al*[30] showed that serum nitrate levels did not correlate with endotoxemia and that cardiac index did not correlate with serum nitrate levels, urine nitrate excretion and endotoxemia. Plasma interleukin (IL)-6 levels were correlated negatively with systemic vascular resistance in patients with cirrhosis, but no correlation was observed between plasma endotoxin levels and plasma IL-6 levels[31]. Finaly, Bhimani *et al*[32] concluded from their experiments that an endotoxin-induced increase in mesenteric iNOS activity and a decrease in hepatic cNOS activity may account for, respectively, the hyperdynamic visceral circulation and the increased intrahepatic resistance of cirrhosis. Although the role of endotoxin on the hyperdynamic circulation still remains controversial, endotoxemia, possibly from gut-derived bacterial translocation, causes induction of

nitric oxide (NO) synthase (NOS) leading to increased vascular NO production, which is the primary stimulus for the development of vasodilatation in cirrhosis and its accompanying clinical manifestations[33].

Portal hypertension

In cirrhosis, portal hypertension can promote bacterial translocation and increase serum endotoxin levels. Vice versa, endotoxin aggravates portal hypertension by induction of systemic and splanchnic vasodilation, and by triggering hepatic inflammatory response *via* tumor necrosis factor α (TNF-α)[34]. Endotoxin levels correlated with hemodynamic derangement in cirrhotic severe portal hypertension, and with levels of soluble TNF-α receptors in patients with alcoholic liver cirrhosis receiving elective transjugular intrahepatic portosystemic shunt[34]. Thromboxane (TX) A2 has been suggested to play a significant role in the development of portal hypertension in fibrosis, and Kupffer cell (KC) derived TXA2 has been shown to mediate the hyperresponsiveness of the portal circulation to the vasoconstrictive actions of endothelin-1 during endotoxemia[35]. The double stresses of early fibrosis additively activate KC and release increased amount of TXA2 in response to ET-1, which leads to the increased portal resistance and ultimately hepatic microcirculatory dysfunction[35]. Steib *et al*[36] concluded from their experiment in bile-duct ligated rats that upregulation of Toll-like receptors (TLR)4 and MyD88 expression in fibrotic livers confers hypersensitivity to LPS. This may lead to escalation of portal hypertension by production of TX and Cys-leucotriene after LPS-induced KC activation.

Hepatic encephalopathy

Except for the early human study by Bigatello *et al*[24], the role of endotoxin on hepatic encephalopathy had not been investigated until recently. Wright *et al*[37] showed that the injection of endotoxin into cirrhotic rats induced pre-coma and exacerbates cytotoxic edema because of the synergistic effect of hyperammonemia and the induced inflammatory response. Bajaj *et al*[38] further extended the problem to microbiome in the intestine and concluded that cirrhosis with hepatic encephalopathy is associated with significant alterations in the stool microbiome compared with healthy individuals. Specific bacterial families (*Alcaligeneceae, Porphyromonadaceae, Enterobacteriaceae*) are strongly associated with cognition and inflammation in hepatic encephalopathy. The central role of ammonia in the pathogenesis of hepatic encephalopathy is incontrovertible. However, there is a robust evidence indicating the importance of inflammation in exacerbating the neurological effects of hepatic encephalopathy[39]. Sterile inflammation by circulating endotoxin from the gut (bacterial translocation) inducing immune dysfunction may have some effect *via* the release of pro-inflammatory mediators which directly signal to the brain[39].

Renal disturbance

Endotoxin is a well-known renal vasoconstrictor. Deleterious effect of endotoxin on kidney has been confirmed in various animals, *i.e.*, dogs[40], mouse[41] and bile-duct ligated rats[42]. Uchihara *et al*[43] found that plasma endothelin levels were significantly higher in patients with endotoxemia than in those without and were negatively correlated to creatinine clearance in cirrhotics. They concluded that plasma endothelin closely related to endotoxemia, may play a contributory role in kidney dysfunction in patients with cirrhosis[43]. As stated above, there has been a disagreement about the relation of endotoxemia to renal disturbance in cirrhosis. Close correlation reported in the early period using LAL test[7,44,45] was not always validated in the later period using quantitative LAL test[30]. However, experimental evidences together with beneficial effect of non-absorbable antibiotics support the pathogenetic roles of endotoxin in the renal disturbance.

Shah *et al*[46] demonstrated in their bile-duct ligated rats that kidneys in cirrhosis show an increased expression of TLR4, NFκB, and the pro-inflammatory cytokine TNF-α, which makes them susceptible to a further inflammatory insult. This increased susceptibility to LPS can be prevented with selective decontamination by norfloxacin[46]. The effect of selective decontamination for renal disturbance to patients with liver cirrhosis was first reported with paromomycin sulfate[47] and recently by rifaximin[48].

SBP is the most dangerous infectious complication arising in patients with cirrhosis and ascites. It is associated with high serum and ascitic fluid levels of proinflammatory cytokines. These patients are predisposed to the development of renal impairment, type 1 hepatorenal syndrome[49]. Albumin infusion improves renal function in acutely decompensated cirrhotic patients with acute kidney injury by impacting on renal blood flow autoregulation. This is possibly achieved through endothelial stabilization and a reduction in the sympathetic tone, endotoxemia and oxidative stress[50].

Cirrhotic cardiomyopathy

Liver cirrhosis is associated with several cardiovascular abnormalities. Despite an increased baseline cardiac output, cirrhotic patients have a suboptimal ventricular response to stress. This phenomenon is called cirrhotic cardiomyopathy. The pathogenesis of this syndrome is multifactorial and includes diminished β-adrenergic receptor signal transduction, cardiomyocyte cellular plasma membrane dysfunction, and increased activity or levels of cardiodepressant substances such as cytokines, endogenous cannabinoids, and nitric oxide[51]. Patients with severe cirrhotic cardiomyopathy have higher lipopolysaccharide binding protein (LBP) levels, which are significantly correlated with the degree of diastolic dysfunction. This findings support a potential role of bacterial endotoxemia on the aggravation of cardiomyopathy in cirrhotic patients[52].

The development of severe renal failure type 1 HRS seems to be related to a cardiac systolic dysfunction[53]. In addition to the above myocardial dysfunction, the release of endotoxins and biologically active substances such as inflammatory cytokines, nitric oxide, carbon monoxide related to bacterial infection may further impair cardiac function in patients with advanced cirrhosis[54].

Hepatopulmonary syndrome

Hepatopulmonary syndrome (HPS) is an important cause of dyspnea and hypoxia in 10%-30% of patients with cirrhosis[55]. It is due to vasodilation and angiogenesis in the pulmonary vascular bed, which leads to ventilation-perfusion mismatching, diffusion limitation to oxygen exchange, and arteriovenous shunting[55].

In experimental studies, Zhang *et al*[56] demonstrated that progression and severity of HPS as indicated by both increased pulmonary capillaries and histological changes are closely associated with endotoxin levels in the cirrhotic rat model. They thought that overproduction of TNF-α due to endotoxin stimulation of KCs *via* mitogen-activated protein kinase signal transduction pathway may be a major mechanism mediating the pathologic alterations of HPS[57]. There was a case report[58] that showed a beneficial effect of oral norfloxacin for hypoxia in a patient with this syndrome. The authors speculated that norfloxacin reduced endotoxemia and concomitant nitric oxide production in patients with cirrhosis. However, a following pilot study of intestinal decontamination with norfloxacin in patients with HPS, in an attempt to reduce endotoxemia, failed to produce any improvement in gas exchange[55].

Coagulation and platelet abnormalities

Patients with advanced liver cirrhosis paradoxically have both risks of bleeding and thrombosis. They should face fragile balance between hypercoagulability and hypocoagulability related to reduced synthesis of clotting factors, accelerated fibrinolysis, platelet dysfunction and low-grade intravascular clotting. Hyperfibrinolysis is not a primary phenomenon but occurs as a consequence of clotting activation and that endotoxemia might play a pathophysiological role[59]. Cirrhotic patients are at increased risk for thrombotic events, particularly in the portal venous system[60]. In cirrhotics, plasma levels of von Willebrand factor (vWF) antigen and endotoxemia progressively increased from Child Pugh's classification A to class C[61]. vWF is a marker of endothelial perturbation and endotoxin releases vWF from endothelial cells *in vitro*[61]. Endothelial procoagulant activation induced by low-grade endotoxemia may represent a trigger for systemic clotting activation in liver cirrhosis patients[62]. Violi *et al*[63] reported that endotoxemia was directly correlated with F1 + 2[59] and D-dimer. These studies show that an ongoing prothrombotic state is present in the portal circulation of cirrhotic patients and may play a pivotal role in the thrombotic episodes[63]. They further confirmed that monocyte expression of tissue factor (TF) was significantly correlated with plasma levels of F1 + 2 and with endotoxemia[64]. TF mRNA expression was detected only in three patients with endotoxemia[64].

Decreased plasma ADAMTS13 activity results in the accumulation of unusually large vWF multimer (UL-VWFM) and the formation of platelet thrombi[65]. Uemura *et al*[65] showed that ADAMTS13 activity decreased with increasing severity of liver disease (controls means 100%, chronic hepatitis 87%, Child A cirrhosis 79%, Child B cirrhosis 63%, and Child C cirrhosis 31%), and showed severe deficiency (< 3% of controls) in five end-stage cirrhotics. This ADAMTS13 activity may be a useful prognostic marker that is equal or superior to the Child-Turcotte-Pugh score and the Model for End-Stage Liver Disease score to predict not only the short-term prognosis but also the long-term survival of the cirrhotic patients[66]. As we found that endotoxemia was inversely correlated with ADAMTS13 activity and was higher in patients with UL-VWFM than those without in patients with alcoholic hepatitis[67], this relation should be estimated in liver cirrhosis.

GUT-LIVER AXIS IN HEALTH AND LIVER CIRRHOSIS

The gut and the liver are the key organs in nutrient absorption and metabolism. Bile acids, drugs, and toxins undergo extensive enterohepatic circulation. Bile acids play a major role in several hepatic and intestinal diseases. Endotoxins deriving from intestinal Gram-negative bacteria are important in the pathogenesis of liver and systemic diseases[68]. Gut flora and bacterial translocation play important roles in the pathogenesis of chronic liver disease, including cirrhosis and its complications[69]. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections and major complications[69].

Bacterial translocation

BT or microbial translocation is defined as the migration of viable microorganisms or bacterial products (*i.e.*, bacterial LPS, peptidoglycan, and lipopeptides) from the intestinal lumen to the mesenteric lymph nodes and other extraintestinal sites[70]. Passage of viable bacteria from the intestinal lumen through the intestinal wall and its translocation to mesenteric lymph nodes and other sites is the accepted pathogenic mechanism for the development of spontaneous infections, such as SBP or bacteremia[4]. Bacterial products, such as endotoxin, or bacterial DNA can translocate to extra-intestinal sites

and promote an immunological response similar to that produced by viable bacteria. Pathological BT is a contributing factor in the development of complications in cirrhosis, not only in infections, but by exerting a profound inflammatory state and exacerbating the hemodynamic derangement[4,71].

Small intestinal bacterial overgrowth

Small intestinal bacterial overgrowth (SIBO), defined as $\geqslant 10^5$ total colony-forming units per milliliter of proximal jejunal aspirations, was present in 59% of cirrhotic patients and is associated with systemic endotoxemia[72]. SIBO related with a slowed intestinal transit, low acid gastric secretion, intestinal immunological factors and pancreatic and biliary secretions, is important factor promoting BT[4]. Cirrhotic rats with intestinal bacterial overgrowth had a significantly higher rate of translocation and slower intestinal transit than those without it[73].

SIBO, determined by the breath hydrogen test, is common in patients with cirrhosis, especially in those with advanced liver dysfunction and in those with a history of SBP[4,74,75]. In a study that estimated SIBO by more reliable quantitative cultures of jejunal aspirates, the occurrence of SBP did not correlate with the presence of SIBO[4]. Sánchez *et al*[76] reported that the increase of intestinal aerobic bacteria in experimental cirrhosis is associated with translocation and is supposed to play an important role in the development of BT. Impaired motility may be implicated in the pathogenesis of intestinal bacterial overgrowth[76]. Gut flora imbalances, higher levels of *Enterobacteriaceae* result in significant changes in BT and liver function in cirrhotic rats[77].

Dietary habits, by increasing the percentage of intestinal Gram-negative endotoxin producers, may accelerate liver fibrogenesis, introducing dysbiosis as a cofactor contributing to chronic liver injury in nonalcoholic fatty liver disease[78]. Liver cirrhosis disturbs intestinal microbiota and innate immunity-related genes, which contributes to endotoxemia and bacterial translocation. These had not completely recovered in cirrhotic rats until 1 mo after orthotopic liver transplantation[79].

Increased intestinal permeability

The gut epithelium plays an important role in the immune homeostasis in the gut as the first barrier against the bacterial translocation[80,81]. Because gut barrier system by intestinal epithelial cells prevent translocation of large amounts of bacteria and bacterial products, very small amount of them can reach the liver in a healthy state[82]. The intestinal barrier is formed mainly by intestinal epithelial cells and their mucinous components[4]. In addition, intercellular junctions such as tight junctions and gap junctions allow selective passage of substances[4]. Structural and functional changes in the intestinal mucosa that increase intestinal permeability of bacteria and its products have been found in patients with liver cirrhosis[4]. This intestinal barrier dysfunction may be an important pathogenetic factor for several complications of liver cirrhosis[83]. Characteristics of cirrhosis itself, including portal hypertension, alterations in the intestinal microbiota, inflammation and oxidative stress can affect barrier function of both small and large intestine and may contribute to the development of complications[71]. Although gut barrier function did not show a significant relationship with endotoxemia, increased intestinal permeability may be a significant finding that at least in part is associated with the pathophysiology of viral liver cirrhosis[84].

There is a long-standing debate about the presence and role of increased intestinal permeability in patients with cirrhosis[85]. Some authors have shown an association between increased intestinal permeability and severity of liver cirrhosis assessed by the Child-Pugh classification[85-87], but others have failed to reproduce these results[88-90]. Methodological problems should be taken into account when interpreting these conflicting results[91]. Some authors used sugars[86,87,92], and others used isotope probes[85,88-90], the latter considered to be the gold standard as the probes are not synthesized or digested in the human body[85]. However, the assessment of the mucosal intestinal permeability by urinary excretion of orally administered non-metabolizable sugars gave us some information on the discrimination between transcellular and paracellular fluxes[93] .

Monosaccharides, such as mannitol, are absorbed through the transcellular pathway and reflect the extent of absorption of small molecules. Disaccharides, such as lactulose, are absorbed through the paracellular junction complex (the tight junctions) and extrusion zones of the intervillous spaces, which corresponds to the permeability of larger molecules[92,94]. Mannitol absorption as assessed by urinary excretion can be considered as an indicator of the mucosal absorptive area, and lactulose absorption as a measure of the integrity of intestinal mucosal tight junctions[94,95].

The lactulose/mannitol (L/M) ratio (LMR) thus comprises an index to appraise intestinal permeability and its increase has been traditionally used as a marker of hyperpermeability[96]; this ratio has been reported to be elevated in patients with liver cirrhosis[92] and to be markedly elevated in advanced stage[86,87]. This increase in intestinal permeability determined by LMR has been reported in several previous studies[96,97]. Alcoholics with liver disease also had marked and statistically significant increases in lactulose excretion in addition to increased LMR[96]. The abnormally elevated LMR in alcoholics with liver disease is not simply due to decreased mannitol excretion but represents increased gut permeability[96]. Pascual *et al*[87] found a significantly higher lactulose excretion (%L) with a comparable mannitol excretion

(%M) in patients with liver cirrhosis as compared to controls.

Parlesak *et al*[98] reported that permeability of polyethylene glycol (PEG) with high molecular mass (PEG 1500 and PEG 4000) was increased in patients with alcoholic liver diseases. They discussed PEG is an appropriate probe for the assessment of endotoxin translocation on the basis of its homogeneous chemical properties, appropriately adaptable molecular mass and linear, chain-like shape mimicking the structure of endotoxin[98]. These demands cannot be met by other commonly used permeability marker compounds described above[99]. Lee *et al*[99] reported that intestinal permeability determined by PEG 400 and 3500 was significantly high in cirrhotics with ascites. Kim *et al*[100] reported that the intestinal permeability index, the percentage of permeability of PEG 3350 to that of PEG 400, was increased on admission for active GI bleeding in patients with liver cirrhosis and infections.

Recently, Assimakopoulos *et al*[101] showed that human liver cirrhosis induces significant alterations in tight junctions of enterocytes. They found a significantly reduced expression of the tight junction proteins occludin and claudin-1 in duodenal biopsies of the total patient group compared with healthy controls and this correlated inversely with endotoxemia. In addition, the cirrhotic patients with ascites showed a significantly reduced expression of occluding and claudin-1 compared with those without ascites. These changes might represent an important cellular mechanism for intestinal barrier dysfunction and hyperpermeability in patients with liver cirrhosis[101]. They further showed that human liver cirrhosis is associated with decreased intestinal mucosal proliferation and proliferation/apoptosis ratio even at early stages of cirrhosis and increased intestinal oxidative stress in advanced liver disease[102].

Disturbance of the liver-bile acid-microbiome axis[103]

Bile acids also play a role in the prevention of BT by inhibiting bacterial overgrowth, exerting a trophic effect on intestinal mucosa and neutralizing endotoxin[4,104]. Therefore, bile acids prevent BT and avoid the passage of bacterial products from the lumen of intestine[4,105,106]. During progression of cirrhosis, BT leads to inflammation, which suppresses synthesis of total bile acids in the liver via inhibition of CYP7A1 and induces a shift toward chenodeoxycholic acid production through the alternate pathway[103]. Decrease in bile acids entering the intestines appears to favor overgrowth of pathogenic and pro-inflammatory members of the microbiome including *Porphyromonadaceae* and *Enterobacteriaceae*[103]. Decreasing bile acid concentration in the colon in cirrhosis is also associated with decreases in Clostridium cluster XIVa, which includes bile acid 7α-dehydroxylating bacteria which produce deoxycholic acid[103]. Lorenzo-Zúñiga *et al*[106] found that conjugated bile acid administration reduced bacterial content to normal levels and improves bacterial translocation and endotoxemia in cirrhotic rats. Further studies should evaluate the potential benefits of bile acids in humans[70].

Toll-like receptors and liver disease

Translocated microbial products activate KCs in the liver through pattern recognition receptors, such as toll-like receptors (TLRs) and NOD-like receptors[82]. TLRs, recognize pathogen-derived molecules, *i.e.*, structural components unique to bacteria, fungi, and virus- and activate innate immune responses including cytokine production in the liver[82,107,108]. It should be noted that hepatic non-immune cells, such as hepatic stellate cells (HSCs) and endothelial cells, also respond to bacterial products through TLRs[82].

Currently, more than 10 members of the TLR family have been identified. TLR4 was the first identified isoform that responds primarily to LPS [82]. LPS binds to TLR4 with co-receptor CD14 and MD-2. TLR2 heterodimerizes with TLR1 or TLR6 to recognize lipoprotein and peptidoglycan derived from Gram-positive bacteria. Bacterial flagellin is recognized by TLR5. Intracellular TLR3 and TLR9 are activated by microbe-derived nucleic acids including double stranded RNA and CpG motif containing unmethylated DNA, respectively[108].

After the binding of corresponding ligands, TLRs activate MyD88-dependent and MyD88-independent signaling pathways, which are related to the production of inflammatory mediators, anti-microbial peptides and induction of acquired immunity to eradicate invading microorganisms. The downstream signaling has now been extensively studied, which should be referred to excellent reviews[82, 108] .

It is postulated that TLR4 and gut microflora-derived LPS contribute to the progression of liver fibrosis[108]. Alcohol induces LBP and TLR4, and increases responsiveness to gut-derived endotoxin. Binding of LPS to CD14/TLR4 on KCs activates production of cytokines and oxidants, which leads to T cell recruitment, HSC activation and collagen production in the liver of patients with alcoholic steatohepatitis[107] . The study of TLR4 to fibrosis progression was further extended to viral hepatitis C. A large patient cohort demonstrating that the TLR4 single nucleotide polymorphism (SNP) is one of seven SNPs that may predict the risk of liver cirrhosis in patients with chronic hepatitis C infection[109].

Recent studies suggested that TLR4 in hepatic HSCs also responds to LPS to activate Jun N-terminal kinases and NFκB[110]. Seki *et al*[111] demonstrated that TLR4 signaling in HSCs, but not in KCs, is crucial for the development of liver fibrosis, from the experiment by two different types of TLR4 BM chimeric mice; one group contains TLR4 mutant KCs and TLR4 intact HSCs and hepatocytes, while the other type contains TLR4 intact KCs and TLR4 mutant HSCs and hepatocytes.

Notably, aberrant activation of innate immune signaling due to enhanced BT may trigger "harmful inflammation" that contributes to sepsis, chronic inflammation, autoimmune diseases, tissue and organ injuries, fibrosis and carcinogenesis[112].

Failure to inactivate endotoxin in the blood

It has been proposed that LPS from the portal blood initially is taken up by KCs and then by hepatocytes in the liver[113]. LPS is removed *via* several mechanisms, including molecules that bind LPS and prevent it from activating TLR4, enzymes that degrade the lipid A moiety to decrease its activity, and inactivation of LPS following uptake into the liver and spleen[114]. Another mechanism for LPS neutralization is by serum lipoproteins, high-density lipoprotein (HDL), LDL, VLDL, and chylomicrons, apolipoproteins apoE and apoA-I[115-118]. All of these mechanisms can chaperone endotoxin to hepatocytes, KCs, or sinusoidal endothelial cells, resulting in clearance of LPS without significant inflammatory cell activation[118].

Due to its lipophilic structure, LPS also adheres to plasma lipoproteins, particularly HDL[119]. HDL particles are multifunctional lipoprotein complexes that transport lipids and have several anti-inflammatory properties. Patients with alcoholic cirrhosis had a significantly decreased whole blood endotoxin-binding capacity together with decreased HDL plasma concentrations, which might result in an increase of the portion of unbound and, possibly more toxic, endotoxin[120]. LBP is an acute phase protein induced by LPS, IL-6, and IL-1β. Interestingly enough, this protein has a bi-directional action on inflammation (pro-inflammatory and anti-inflammatory) induced by LPS. It usually catalyzes the transfer of LPS to CD14, and thus enhances the LPS-induced activation of monocytes, macrophages, and other immune cells. However, in the blood rich in HDL, LBP transfers LPS to HDL with an aid of apolipoprotein (Apo) A1[121,122]. Two experimental studies have shown that HDL administration reduced the effects of LPS on tumor necrosis factor-α production[123,124] and systemic hemodynamics, restoring liver endothelial nitric oxide synthase activity and decreasing portal pressure[123]. Incubation of whole blood with reconstituted HDL prevents LPS-induced tumor necrosis factor-α and interleukin-6 overproduction by monocytes of patients with cirrhosis[125].

Until now endotoxin binding and inactivating capacity of albumin have been relatively ignored[18]. In a trial of stabilizing standard endotoxin by an addition of albumin, we happened to notice that albumin inhibits endotoxin activity in the chromogenic assay system[25]. This observations have led us to hypothesize that albumin may act as a protective protein against endotoxemia. In our preliminary study, we first noted that 250 pg/mL endotoxin lost most of their activity in the presence of albumin at physiological concentrations. Another interesting result was that albumin inhibits LPS-stimulated IL-1 secretion in the macrophage culture system[25]. We further noted that the capacity of albumin to bind exogenous ^{3}H-labelled endotoxin decreased in plasma of cirrhotics[18]. In patients with Child A and Child B cirrhosis, the plasma endotoxin inactivating rate was positively correlated to the endotoxin binding capacity of plasma albumin[126]. Albumin has a protective effect against encephalopathy in advanced cirrhosis[127]. Substances which require albumin binding-such as bilirubin, free fatty acids and organic anions[128] markedly increase in the blood in this situation. The increase of these substances may limit endotoxin binding capacity of albumin. In Child C cirrhotics in whom albumin shows very low endotoxin binding capacity, additional microbial loads by acute infection may result in overwhelming endotoxemia and serious clinical results[18] Mechanisms of LPS clearance in the blood and LPS-induced inflammation are summarized in Figure 1.

How to evaluate BT clinically

As described above, plasma endotoxin assay has been most widely used. Despite the development of various new assay technique based on LAL test, there is no standard accepted method for clinical use. We have established chromogenic LAL test with kinetic analysis and compared various methods of plasma pretreatment, including the dilution and heating method and the perchloric acid method under the strict control by internal endotoxin standard. We have compared endotoxin-specific chromogenic substrate with the conventional chromogenic substrate and could not find out any superiority of the endotoxin-specific test for evaluation of BT. Both tests were well correlated to clinical course and considered to be useful markers. It should be noted that (1-3)-β-D-glucan powerfully co-stimulate cytokine production (IL-6/IL-8) induced by ligands for TLR1/2, TLR2/6, TLR4, and TLR5[129]. Plasma glucan in patients with bacterial infections, and the low levels of glucan found in normal individuals, may be attributable to movement of glucan from the GI tract into the blood and not necessarily to the presence of a pathogen[130]. Although most quantitative LAL test reacts (1-3)-β-D-glucan and is not endotoxin-specific, both endotoxin from Gram-negative bacteria and (1-3)-β-D-glucan from fungus are microbial products which translocate from the intestine. They strongly co-stimulate innate immune system and induce the production of inflammatory mediators. The drawbacks of the tests are complexity of the measurement and difficulty in standardization. I could not recommend other endotoxin assay method with very low sensitivity. It was designed to detect endotoxemia with bacteremia but not suitable for detection of spillover endotoxemia in liver diseases. Endotoxin activity assay[131] using a novel chemiluminescent assay will be evaluated for this purpose. I list up current detection methods of

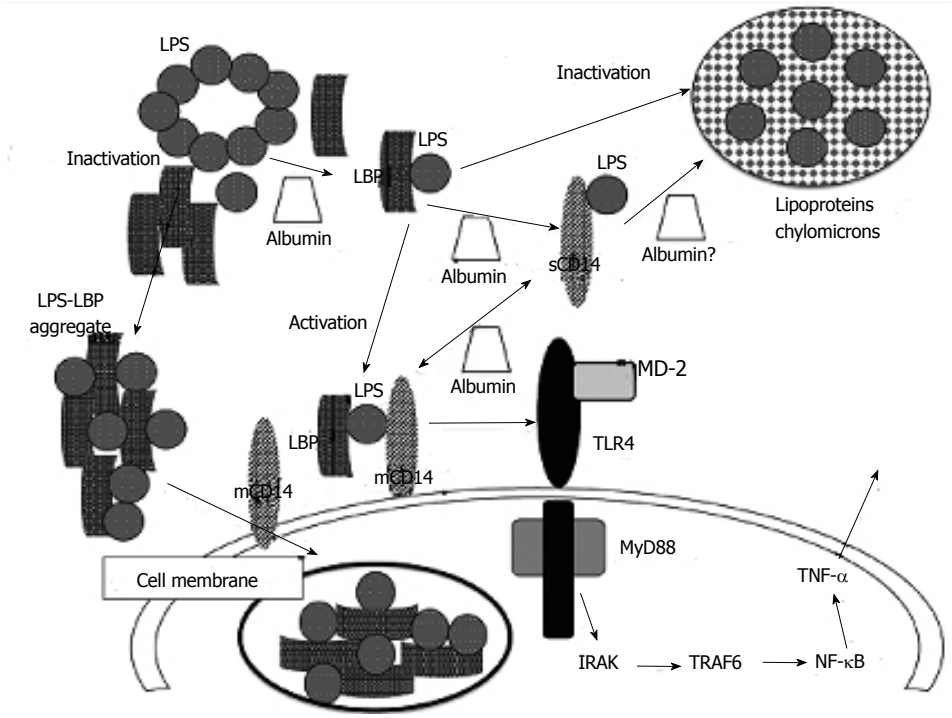

Figure 1 Mechanism of lipopolysaccharide clearance in the blood and LPS-toll like receptors-MyD88 signal transduction. LBP enhances cell responses to LPS by accelerating the binding of LPS to CD14. LBP can also inhibit cell responses to LPS; It transfers LPS to plasma lipoproteins and it combines with LPS aggregates to form large LPS-LBP complexes that are internalized[176]. sCD14 can remove, or divert, LPS from mCD14 and transfer it to plasma lipoproteins, where LPS is inactivated[176]. Albumin is essential during the interaction of LBP with LPS aggregate to produce a LBP: LPS aggregate and the efficient transfer of LPS from the aggregate to a molecule of sCD14[177]. Albumin stabilizes LPS: CD14 complexes for cell activation. Mechanism of inhibitory effect of albumin on LPS is still unknown. It may directly inactivate minute amount of LPS and may also enhance LPS transport to lipoproteins. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; TNF-α: Tumor necrosis factor α; TLR4: Toll-like receptors 4; NF-κB: Nuclear factor kappa B; TRAF6: TNF receptor-associated factor.

endotoxemia with their pros and cons in Table 1.

LBP can be evaluated as another useful surrogate marker of BT, although both endotoxin and LBP reflect only translocation of *Gram-negative bacilli*[4]. Elevated LBP levels in cirrhosis were related to pro-inflammatory state and haemodynamic derangement, which were shown to be ameliorated by intestinal decontamination with norfloxacin[132]. A prospective study in non-infected cirrhotics with ascites showed that increased serum LBP was the only factor independently associated with first severe bacterial infection in a multivariate analysis[133]. Detection of serum peptidoglycan, a polymer consisting of sugars and amino acids that forms cell wall of gram-positive bacteria, has been considered as a marker of BT in an experimental model of hemorrhagic shock[4,134].

Recently, detection of bacterial DNA (bactDNA) by polymerase chain reaction has been proposed as a surrogate marker for BT. It has been simultaneously detected in blood and ascites in 9 of 28 cirrhotics with culture-negative ascites[135]. Detection of bactDNA in biological fluids in experimental cirrhosis and ascites is associated with its simultaneous presence in MLNs[136]. Bellot *et al*[137] reported that bactDNA (+) patients had significantly lower mean arterial pressure and systemic vascular resistance. The increase in hepatic venous pressure gradient after the test meal significantly correlated with serum bactDNA concentration. Uniformity of analytical methods is needed to ascertain its real value in clinical setting[4] .

THERAPEUTIC APPROACH TO LIVER CIRRHOSIS BY MANAGEMENT OF THE GUT-LIVER AXIS

Our hypothesis about the mechanism of endotoxemia and its consequences related to complications in advanced liver cirrhosis are shown in Figure 2. Gram-negative bacteria and endotoxins are more likely than other types of bacteria to stimulate tumor necrosis factor and cytokines that would lead to the production of nitric oxide (NO)[138]. Endotoxemia in relation to bacterial translocation, causes induction of NO synthase leading to increased vascular NO production, which is the primary stimulus for the development of vasodilatation and its accompanying clinical manifestations in cirrhosis[99]. Nitric oxide is also a potent inducer of increased membrane permeability in the vascular endothelium and intestinal mucosa, possibly contributing to bacterial translocation[28,99]. In patients with advanced cirrhosis, there may be a vicious cycle among endotoxemia, induction of NO and increased intestinal permeability, which may further induce derangement of the hyperdynamic circulatory status and renal failure. New clues to improving

Table 1 Detection methods of endotoxemia

	Name of the test (manufacturer)	Pro	Con
LAL test	ToxinSensorTM Gel clot Endotoxin Assay Kit (GenScript) PYROGENTTM-5000 LAL Reagent (Lonza)	Good marker of BT	Not specific, react βd-Glucan, plasma preparation difficult
Chromogenic substrate assay	Toxicolr test (Seikagaku Corporation)	Good marker of BT available for both end-point assay and kinetic assay	Not specific, react βd-Glucan PCA method: poor endotoxin recovery plasama pretreatment reagent currently unavailable
	QCL1000 (BioWhittaker/Cambrex Lonza end-point assay Kinetic-QCL™ Kinetic Chromogenic LAL Assays (Lonza)	Good marker of BT available for both end-point assay and kinetic assay	Not specific, react βd-Glucan
	LAL Coatest, S-2423 (Kabi Vitrum Diagnostica, Chromogenix)	Good marker of BT available for both end-point assay and kinetic assay	Not specific, react βd-Glucan, not available now
	EndosafePTS (Charles River Lab)	Good marker of BT handy and quick	Not specific, react βd-Glucan (Concomitasnt use of Endosafe PTS glucan assay may be necessary for specificity)
	LAL chromogenic endpoint assay Hycult Biotech Mini-LAL assay (Hycult Biotechnology) ToxinSensor™ Chromogenic LAL Endotoxin Assay (GenScript) end-point method	Good marker of BT available for both end-point assay and kinetic assay	Not specific, react βd-Glucan
	Pyrochrome (Associates of Cape Cod Inc)	Easy plasma pretreatment (dilution and heatig) available for both end-point assay and kinetic assay	Not specific, react βd-Glucan
	Endospecy (Seikagaku Corporation)	Endotoxin-specific available for both end-point assay and kinetic assay	PCA method: poor endotoxin recovery plasama pretreatment reagent currently unavailable New PCA method: plasma pretreatment reagent currently unavailable
Turbidometric assay	Limulus ES II test (Wako) PYROSTAR™ ES-F LAL Reagent (Charles River Lab)	Endotoxin-specific	Unnable to detect spillover endotoxemia in cirrhosaas
Recombinant factor C (rFC) system assay	Recombinant factor C (rFC) system PyroGene (Lonza) fluorescent EndoLISA Hyglos ELISA EndoZyme® recombinant Factor C (rFC) Assaye HyGlos fluorescent	Endotoxin-specific	Very few reports, usefullness for liver disease unclear
Endotoxin activity assay	EAA™ (Spectral Dianostics Inc)	Received FDA clearance rapid diagnostic for endotoxin activity in human whole blood. Higher EAA™ levels are correlated with a higher risk of mortality, as well as an increasing risk for developing sepsis	Addition of methylprednisolone decreased the EAA levels. Indiredt endotoxin assay to reflect the primed state of polymorphonuclear leukocytes

LAL: Limulus amebocyte lysate; FDA: Food and Drug Administration.

prognosis of advanced liver cirrhosis may be found in better management of gut-liver axis.

Selective intestinal decontamination

Selective intestinal decontamination (SID) for management of complications of liver cirrhosis has a long and evolving history. At first, the word SID was not used but the trial to eliminate the endogenous source of gram-negative aerobic bacteria was reported early in 1982. Adachi *et al*[139] reported that oral administration of polymyxin B is useful in the treatment of hyperammonemia and endotoxemia in liver cirrhosis, as a poorly absorbed antibiotic and as an antiendotoxin agent. Similarly, Tarao *et al*[47] stated that paromomycin sulfate (2 g/d for 4 wk) is effective in the prevention of endotoxemia and the associated renal impairment in cirrhosis. Although the effect of the latter was not reproduced later in patients with alcoholic liver disease[140].

Long-term use of norfloxacin (400 mg/d, mean follow-up period 6.4 ± 0.6 mo) has been reported to eliminate aerobic *gram-negative bacilli* from the fecal flora without significant changes in other microorganisms throughout the study[141]. A double blind, placebo-controlled trial evaluating its efficacy in cirrhotics who recovered from an episode of SBP, revealed a significant reduction of SBP recurrence by the treatement (20% *vs* 68%) at one year of follow-up[141]. In cirrhotic patients with low ascitic fluid protein concentrations (≤ 1 g/dL) or hyperbilirubinemia (> 2.5mg/dL), long-term prophylactic treatment with norfloxacin was also effective in the prevention of the first episode of SBP (1.8% *vs* 16.9%)[142]. However, these promising results were followed by the problem of quinolone-resistant SBP[142,143]. Prior antibiotic therapy and norfloxacin prophylaxis is confirmed to increase the risk of carriage of methicillin-resistant Staphylococcus aureus[144]. After that, primary prophylaxis of SBP has been targeted to high-risk patients. When cirrhotics with low protein ascitic levels (< 1.5 g/dL), advanced

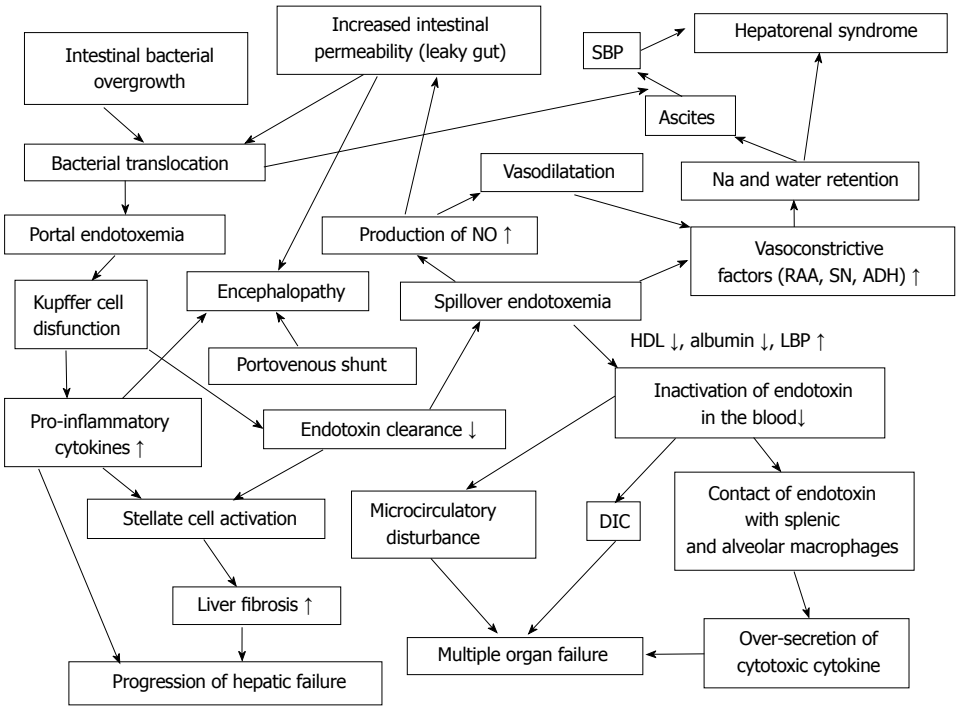

Figure 2 Mechanism of endotoxemia and its consequences in advanced liver cirrhosis (hypothesis). Depressed elimination of endotoxin by Kupffer cells (KCs) is considered to induce spillover endotoxemia and processing of endoxin by extrahepatic macrophages which secrete larger amount of TNF than KCs. The excessive cytokine response to endotoxin by splenic and alveolar macrophages may be important in the pathogenesis of ARDS and multiple organ failure. Endotoxemia enhances vascular NO production, which is the primary stimulus for the development of vasodilatation. Enhanced vasoconstrictive factors in response to vasodilatation and endotoxemia are responsible for ascites and hepatorenal syndrome. Hepatic encephalopathy is also closely related to inflammatory reaction attributable to leaky gut amd endotoxemia. RAA: Renin-angiotensin-aldosterone system; SN: Sympathetic nerves; ADH: Antiduretic hormone (vasopressin); SBP: Spontaneous bacterial peritonitis; NO: Nitric oxide; LBP: Lipopolysaccharide binding protein; HDL: High-density lipoprotein.

liver failure (Child-Pugh score $\geqslant$ 9 points with serum bilirubin level $\geqslant$ 3 mg/dL) or impaired renal function were selected, primary prophylaxis with norfloxacin reduced the incidence of SBP, delayed the development of hepatorenal syndrome, and improved survival[145].

Rifaximin is a minimally absorbed oral antimicrobial agent that is concentrated in the gastrointestinal tract[146]. It has broad-spectrum *in vitro* activity against gram-positive and gram-negative aerobic and anaerobic enteric bacteria, and has a low risk of inducing bacterial resistance[147-149]. Given its pharmacologic characteristics this drug has been used in the treatment of hepatic encephalopathy. Rifaximin has been compared with neomycin[150], paromomycin[151], and lactulose[152], showing similar results in both clinical improvement and reducing blood ammonia[153]. Recently its effect on advanced liver cirrhosis as SID agent has been intensively studied. Vlachogiannakos *et al*[154] reported that a 4-wk rifaximin regimen significantly ameliorated endotoxemia and lowered hepatic venous pressure gradient in patients with decompensated alcohol-related cirrhosis. Kalambokis *et al*[48] noted that rifaximin treatment reduced cardiac output and increased systemic vascular resistance, glomerular filtration rate and natriuresis, in association with decreases in plasma renin activity, endotoxin, IL-6, and TNF-α levels. These data supported that intestinal decontamination with rifaximin improved systemic hemodynamics and renal function in patients with advanced cirrhosis. Decrease of ascitic neutrophil count in cirrhotic patients with sterile ascites[155] and improvement of thrombocytopenia[156] ware also reported by the same group. Dănulescu *et al*[157] further reported that rifaximin causes a significant decrease in ascitic neutrophil count, producing a decrease in SBP frequency and improvement of life in cirrhotic patients with refractory ascites.

Several recent studies evaluated its effect on brain function based on the concept of gut-liver-brain axis. Rifaximin is associated with improved cognitive function and endotoxemia in minimal hepatic encephalopathy (MHE), which is accompanied by alteration of gut bacterial linkages with metabolites without significant change in microbial abundance[158]. A significant improvement in cognition including working memory and inhibitory control, and fractional anisotropy without effect on MD or MR spectroscopy, through modulation of fronto-parietal and subcortical activation and connectivity was seen after open-label rifaximin therapy in MHE[159].

Probiotics

Probiotics, lactose-fermenting *Lactobacilli* and *Bifidobacteria*, have been reported to stabilize mucosal barrier function and modulate the gut microflora, suppressing pathogenic microbial growth[70]. They acidify the gut lumen, compete with pathogenic bacteria for nutrients, and produce antimicrobial substance[70,160,161]. Administration of VSL#3, a probiotic combination

of eight strains of *Lactobacilli*, *Bifidobacteria* and *Streptococcus*, has been reported to reduce oxidative/nitrosative stress parameters in patients with alcoholic liver cirrhosis[162]. Cirrhotic subjects receiving *Escherichia coli Nissle* for 42 d showed an effectiveness in the restoration of normal colonic colonization and a trend of significant lowering of the endotoxemia and improvement of liver functions evaluated by Child-Pugh score[163]. Stadlbauer *et al*[164] proved that probiotics restore neutrophil phagocytic capacity in cirrhosis, possibly by changing IL-10 secretion and TLR4 expression, warranting larger randomized controlled and mechanistic studies. Probiotics are able to decrease the permeability of the intestinal wall, and decrease bacterial translocation and endotoxemia in animal models as well as in clinical studies, which is extremely important in the prevention of complications of liver cirrhosis and infection after liver transplantation[165]. Probiotics could limit oxidative and inflammatory liver damage and, in some situations, improve the histological state[165]. Recent meta-analysis could not confirm that probiotics are effective to hepatic encephalopathy[166]. Further clinical trials are needed to know an ideal probiotic therapy for this purpose[166].

Synbiotics

Synbiotic treatment was also associated with a significant reduction in endotoxemia[164]. The Child-Pugh functional class improved in nearly 50% of cases[164]. Synbiotic preparation consisting of 4 freeze-dried, non-urease-producing lactic acid bacteria and four fermentable fiber, Synbiotic 2000®, was effective to patients with liver cirrhosis and minimal hepatic encephalopathy[167]. Synbiotic treatment for 30 d significantly increased the fecal content of non-urease-producing *Lactobacillus* species, which was associated with a significant reduction in blood ammonia and endotoxin[167]. An improvement in Child-Pugh class occurred in 47% of patients receiving synbiotic preparation, compared with 29% or 8% of patients receiving fermentable fiber alone or placebo, respectively[167]. For the prevention of infections, this synbiotic regimen (Synbiotic 2000®) was more effective than fiber alone in reducing the incidence of bacterial infections in liver transplant recipients[168]. Additionally, Wan *et al*[169] reported that taurine and oat fiber achieved an additive inhibitory effect on intestinal endotoxin release in a rat liver ischemia/reperfusion model, which might be an effective approach for the treatment of intestinal endotoxemia.

Prebiotics

Lactitol and lactulose are synthetic non-absorbable disaccharides. They remain undigested until they reach the large bowel, where they are metabolized by colonic bacteria, generating acetic and lactic acids. The resulting lower pH may inhibit urease-producing intestinal bacteria and promote the growth of non-urease-producing *Lactobacilli*[70,170]. Chen *et al*[171] reported that lactitol increased beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli* with a significant decrease in plasma endotoxin levels in patients with chronic viral hepatic diseases.

New cocktails and others

Current approaches for hepatic encephalopathy include the use of non-absorbable antibiotics (*i.e.*, neomycin, paromomycin, metronidazole, or rifaximin) and non-absorbable disaccharides[70]. Probiotics may be a promising therapeutic option in the management of hepatic encephalopathy[167,172-174], although we need more clinical studies to get a final conclusion[166]. Some authors have shown that probiotics may positively modulate the gut microflora, reducing the amount of bacterial ammonia reaching the portal vein[70]. The long-term oral administration of *Enterococcus faecium SF 68* was equally effective as lactulose and its effect on mental status persisted longer than lactulose[172]. Oral intake of *Bifidobacterium longum* plus fructo-oligosaccharides for 90 d was also effective in biochemical and neuropsychological tests of cirrhotics with minimal hepatic encephalopathy[175].

CONCLUSION

It has been proposed that a "leaky gut" may be the cutting edge for the passage of toxins, antigens or bacteria into the body, and may play a pathogenic role in advanced liver cirrhosis and its complications. More attention should be paid to the role of intestinal bacteria and bacterial products in the field of hepatology. The usefulness and the limitations of selective intestinal decontamination should be more clearly defined. Rifaximin may be promising. However, more judicious combinations of probiotics and prebiotics should be explored. Infusion of albumin or HDL in addition to endotoxin adsorption may be theoretically effective for intractable endotoxemia. Moreover, adequate management of the gut-liver axis is even effective for prevention of fibrosis in alcoholic and nonalcoholic steatohepatitis. This seems to be a radical therapy to decrease liver cirrhosis itself. Readers interested in the topics are recommended to read recent excellent reviews[4,70,71,82,103,108]. The research in these fields may open a new possibility in the next decade.

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REVIEW

Endothelial dysfunction in cirrhosis: Role of inflammation and oxidative stress

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Abstract

This review describes the recent developments in the pathobiology of endothelial dysfunction (ED) in the context of cirrhosis with portal hypertension and defines novel strategies and potential targets for therapy. ED has prognostic implications by predicting unfavourable early hepatic events and mortality in patients with portal hypertension and advanced liver diseases. ED characterised by an impaired bioactivity of nitric oxide (NO) within the hepatic circulation and is mainly due to decreased bioavailability of NO and accelerated degradation of NO with reactive oxygen species. Furthermore, elevated inflammatory markers also inhibit NO synthesis and causes ED in cirrhotic liver. Therefore, improvement of NO availability in the hepatic circulation can be beneficial for the improvement of endothelial dysfunction and associated portal hypertension in patients with cirrhosis. Furthermore, therapeutic agents that are identified in increasing NO bioavailability through improvement of hepatic endothelial nitric oxide synthase (eNOS) activity and reduction in hepatic asymmetric dimethylarginine, an endogenous modulator of eNOS and a key mediator of elevated intrahepatic vascular tone in cirrhosis would be interesting therapeutic approaches in patients with endothelial dysfunction and portal hypertension in advanced liver diseases.

Key words: Asymmetric dimethylarginine; Endothelial function; Nitric oxide; Portal hypertension; Hepatic cirrhosis, Reactive oxygen species; Inflammation

Core tip: Endothelial dysfunction (ED) is a key and early relentless event in patients suffering from gastrointestinal bleeding in cirrhosis and involves in response to both vasoactive and vasoconstrictor substances. The one such vasoactive molecule, nitric oxide (NO) plays a prime role in maintaining normal hepatic vascular function and if there any defect in NO availability leads to ED and portal hypertension (PHT) could be of great utility in preventing and curing complications of PHT.

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INTRODUCTION

The endothelium is the largest organ and encompasses > 10^{13} endothelial cells in the body can able to generate both vasodilator [nitric oxide (NO), endothelium derived hyperpolarising factor (EDHF) and prostacyclin] and vasoconstrictor (endothelin-1, norepinephrine, leukotriene, thromboxane A_2 and angiotensin Ⅱ) substances and is essential for hepatic vascular homeostasis. Endothelium serves as a barrier to separate blood from the underlined tissue and thus maintains homeostasis at the vascular wall during physiological condition[1,2]. The salient features of normal healthy endothelium which including, regulation of vascular permeability, decrease in vascular tone, reduce in platelets adhesion and aggregation, prevention of thrombosis, inhibition of smooth muscle cell proliferation, inflammation and restricting leukocyte adhesion[3]. Indeed, many of these functions are mediated by endothelium driven NO[4]. The term endothelial dysfunction (ED) implicate a loss of function of numerous activities of the endothelium[5], mainly characterised by impairment of the production and release of endothelium driven vasodilatory factors including NO[4,6]. The hepatic vascular bed of cirrhotic liver exhibits ED and is now considered to play a key role in the initiation and advancement of liver cirrhosis[7]. The intrahepatic vasculature also displays increased sensitivity to vasoconstrictors in cirrhosis[8]. Furthermore, ED is also a common index for a wide variety of pathological conditions such as chronic renal failure, atherosclerosis, hypercholesterolemia, hypertension, diabetes and coronary artery disease[9,10].

HEPATIC CIRRHOSIS

Cirrhosis is a complication of many forms of chronic liver diseases and is a late stage of fibrosis, in which regenerative nodular formation surrounded by fibrous bands of the liver. The development of portal hypertension (PHT) (elevated pressure within the hepatic circulation) heralds the onset of most fatal complications of cirrhosis, which carry a poor prognosis and represent the first cause of death and need for liver transplantation in patients with cirrhosis[8,11]. The pathogenesis of PHT is predominantly related to a combination of structural and dynamic components that cause an increase in hepatic vascular resistance to portal blood flow[12]. The structural components such as fibrosis, regenerative nodule formation and vascular remodelling[13].

INTRAHEPATIC ENDOTHELIAL DYSFUNCTION IN CIRRHOSIS

Impaired endothelial dependent relaxation in the hepatic microcirculation due to reduced bioavailability of vasodilator, NO in cirrhotic liver contributes to increasing intra-hepatic vascular resistance, which culminating portal hypertension[14]. By contrast, in the splanchnic vascular bed, overproduction of NO contributes to increased endothelium dependent relaxation, leading to hyperdynamic circulatory disturbances, which observed in cirrhosis with portal hypertension[15,16]. Furthermore, increased vasoconstrictor agents such as thromboxane A_2 (TX A_2), a COX-1 derived prostanoids, and endothelin-1 are thought to associated with the pathogenesis of the dynamic component of the augmented intra-hepatic resistance and play a major role in the intrahepatic endothelial dysfunction of the cirrhotic liver[7,17]. Such imbalance between endogenous vasoconstrictor and vasodilator factors observed in the cirrhotic liver is thought to be similar to that found in other cardiovascular diseases[18]. The assessment of NO concentration in cirrhotic liver and systemic circulation is considered to be the prime indicative of endothelial dysfunction (ED).

NO AND ENDOTHELIAL DYSFUNCTION IN CIRRHOSIS

Endothelial dysfunction is thought to be a key event in the development of distinct human vascular diseases, including liver cirrhosis, hypertension, diabetes and atherosclerosis. Classically, ED has been considered to be the result of a decrease in bioavailability of NO in cirrhosis[14,19]. The amino acid, L-arginine, is the substrate of eNOS, the enzyme responsible for NO synthesis (Figure 1). Endothelial nitric oxide synthase (eNOS) driven nitric oxide (NO) is a potent vasodilator that plays a substantial role in maintaining vascular homeostasis in the normal intact liver[14,19], however when the liver fails, reduced intrahepatic eNOS activity triggers endothelial dysfunction contributes to the pathogenesis of PHT (Figure 1)[20]. It is an early and relentless event occurring after all forms of liver injury that leads to substantial morbidity and mortality in individuals with cirrhosis[11,21]. Reduced NO bioavailability makes a major contribution to endothelial dysfunction and is mainly due to reduced NO production or increased NO breakdown due to the chemical reaction with oxidant radicals[22]. Inflammation and oxidative stress are other important pathophysiological consequences that causes endothelial dysfunction and reduced NO bioavailability[22], which make an important contribution to the vascular structural changes in cirrhosis[23]. Furthermore, treatment with exogenous L-arginine

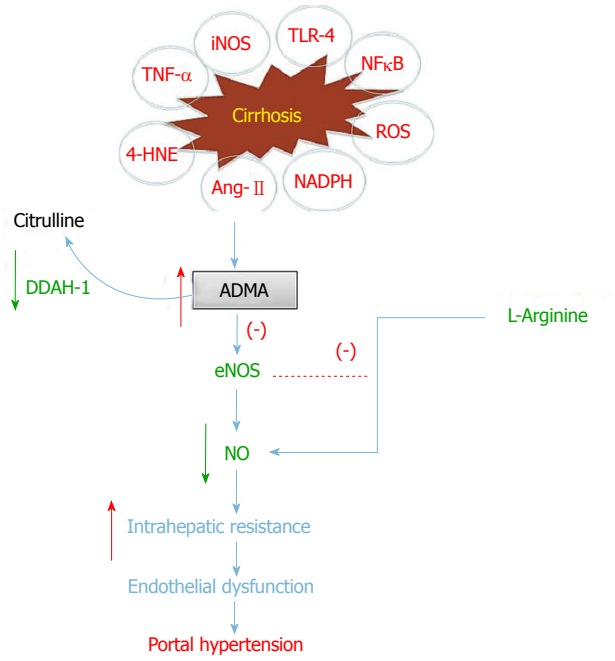

Figure 1 Schematic representation of the proposed role of inflammation and oxidative stress in mediating hepatic endothelial dysfunction in cirrhosis. Inflammation and oxidative stress are synergistically triggers accumulation of ADMA in the systemic circulation and the liver. Increased ADMA endogenously inhibits eNOS results in decreased hepatic NO production, which causes increased intrahepatic vascular resistance and endothelial dysfunction thus, portal hypertension in the context of cirrhosis. Inflammation and oxidative stress also inhibit ADMA hydrolysing enzyme, DDAH activity and promote methylarginine concentrations in the liver. L-arginine is the source for eNOS enzyme for NO production and is vasoprotective. TNF-α: Tumor necrosis factor α; NFkB; Nuclear factor kappa B; TLR: Toll like receptor; Ang II: Angiotensin II; NO: Nitric oxide; ADMA: Asymmetric dimethylarginine; eNOS: Endothelial nitric oxide synthase; DDAH: Dimethylarginine diaminohydrolase; iNOS: Inducible nitric oxide synthase; 4-HNE: 4-hydroxy-2-nonenal; NADPH; Nicotinamide adenine dinucleotide phosphate-oxidase; ROS: Reactive oxygen species.

has been shown to improve vascular function in liver cirrhosis suggesting that decreased substrate availability contributes to endothelial dysfunction[24]. Thus, impaired vascular uptake of L-arginine may play a key role in the pathogenesis of endothelial dysfunction in cirrhosis and should be explored as a potential therapeutic target[25].

Endogenous negative eNOS regulators

Several mechanisms are involved in regulating NO production following eNOS activation. Studies in relation to decreased hepatic eNOS activity have, to date, focused on inhibitors of eNOS activity such as asymmetric dimethylarginine (ADMA)[26,27] and caveolin-1 (major coat protein of endothelial caveolae)[28], or on the process affecting the post translational modification of eNOS[29]. Furthermore, trafficking and proper subcellular localization of eNOS are also critical for regulation of its activity[29,30]. One such protein, eNOS trafficking inducer (NOSTRIN), identified in a yeast two-hybrid approach, has been demonstrated to interact physically with eNOS *via* its C-terminal SH3 domain and regulated its function[29-31].

An emerging body of evidence indicated that ADMA, a deleterious endogenous inhibitor of NO synthases and thus presumed to be a marker of hepatic dysfunction in cirrhosis with PHT. One mechanism thought to be partially responsible for the reduction in NO and resultant ED in liver disease is an increase in the levels of the endogenous inhibitor of NOS, ADMA[19,32]. In this regard, our previous studies have shown evidence that increased ADMA contributes to reduced hepatic NO biosynthesis as a consequence of altered hepatic vascular function in cirrhosis[26,27,33]. In critically ill patients to whom admitted in ICU, hepatic dysfunction was associated with elevated ADMA levels and was identified as an independent predictor of mortality[34]. Furthermore, increased plasma ADMA was reported recently in biopsy proven non-alcoholic fatty liver disease (NAFLD) patients[35], hepatic vein of patients with compensated cirrhosis[36] and decreased following liver transplantation; thus the significant improvement of liver function[37] propose an important role for ADMA in clinical medicine. Elevated plasma ADMA has also recognised as an important risk factor for cardiovascular disease[38], coronary heart disease[39] and chronic renal failure[40]. Consequently, increased ADMA may be related to elevated activity of protein methyltransferase (PRMT), which is responsible for the methylation of arginine residues in cellular proteins[41]. Moreover, increased ADMA level was correlated with the severity of inflammation and levels of increased proinflammatory cytokine such as tumor necrosis factor (TNF)[42,43]. Laleman *et al*[44] showed in cirrhotic animals infusion of ADMA and NG-nitro-L-arginine methyl ester (L-NAME), other known inhibitors of NOS synergistically aggravated and resulted in paradoxical vasoconstriction, which associated with a further decrease in NOx levels. The pathophysiological increase in hepatic ADMA concentration observed in cirrhotic rats manifest decrease of hepatic eNOS activity.

Dimethylarginine diaminohydrolase

Furthermore, the intracellular levels of these methylated arginines are regulated through their metabolism to citrulline and dimethylamine by its hepatic specific enzyme called, dimethylarginine diaminohydrolase (DDAH)[41,45] (Figure 1). Two isoforms of DDAH have been identified and are widely expressed in human and rodent liver. DDAH 1 is an important isoform for the regulation of hepatic and systemic ADMA concentration and is present higher levels in tissue that expressing neuronal NOS (nNOS)[41,45]. The other DDAH isoform (DDAH 2) has an important effect in regulating NO activity, and is mainly found in tissue expressing eNOS and inducible NOS (iNOS)[41,45,46]. It is well known that increased intracellular DDAH plays a critical role in regulating tissue ADMA concentration[26,27,33,47], therefore alterations of DDAH activity and expression lead to change in intracellular ADMA concentrations and concomitant NO synthesis. *In vitro*, human umbilical vein endothelial cells (HUVECS) exposed to prolonged (48 h) TNF-α show eight fold increase of

ADMA, compared to control medium and associated DDAH activity was decreased to almost 60% of baseline values[48]. DDAH is a redox sensitive enzyme and is thus subject to inhibition by oxidants derived from endothelial superoxide[47,49], and antioxidant treatment corrects DDAH inhibition in *in vivo*[33,50]. Cirrhotic rat livers showed an increased O_2^- content compared to control rat livers and was ameliorated by adenoviral gene delivery of superoxide dismutase, increases NO bioavailability, improves intrahepatic endothelial function and reduces portal pressure[51]. In contrast to ADMA, SDMA, its vasoactive stereoisomer, has no effect on inhibition of NO synthases but competes with arginine for cellular transport across the y+ transporter[38,52]. Recently, Siroen and co-workers have shown that the human liver takes up substantial amounts of SDMA from the portal and systemic circulation and suggested that high plasma levels of SDMA may have hemodynamic consequences similar to those reported for ADMA[53]. However, in patients with alcoholic cirrhosis, noted plasma SDMA level was within the normal limit[54].

OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION IN CIRRHOSIS

In cirrhosis, oxidative stress induced mainly by an overproduction of reactive oxygen species (ROS)[55], which is a critical determinant of endothelial dysfunction and is due to disturbed balance between oxidant and antioxidant enzymes. Increased superoxide formation in the presence of equimolar concentrations of NO will lead to the formation of the potent ROS and reactive nitrogen species[56]. Furthermore, decreased NO bioavailability within the cirrhotic liver is mainly attributed by endothelial dysfunction and is mainly due to diminished eNOS activity as well as NO scavenging by increased release of superoxide (O_2^-)[55]. NO also binds to superoxide anion (O_2^-) to produce the most powerful oxidant peroxynitrite ($ONOO^-$). Consequently, peroxynitrite can cause oxidative damage, protein nitration and S-nitrosylation of biomolecules such as proteins, lipids and DNA[57,58]. In fact, peroxynitrite can also oxidise tetrahydrobiopterin (BH_4), an essential cofactor for eNOS to trihydrobiopterin radical (BH_3)[59], which can further disproportionate to dihydrobiopterin (BH_2). As a consequence, functional endothelial NOS is converted into a dysfunctional O_2^- generating enzyme[60] termed as eNOS uncoupling, contributes to ROS overproduction within the intrahepatic circulation. The generation of ROS and superoxide are the key mediators of damage to endothelial cells in cirrhosis[23,61]. BH_4 administration to cirrhotic rats increased hepatic NOS activity and cyclic guanosine monophosphate (cGMP) levels and significantly reduced ED, and subsequent portal pressure may represent a new therapy for ED in patients with cirrhosis[62,63]. Overwhelming evidences have also indicate that in cirrhosis with bacterial endotoxemia, excessive NO production by iNOS reacts with molecular oxygen (O_2^-), leading to the formation of peroxynitrite radical and causes massive hepatic tissue damage as well as fall in blood pressure induced in the vascular wall[27,64]. Furthermore, eNOS uncoupling is also caused by L-arginine depletion, increased endogenous eNOS inhibitors and S-glutathionylation of eNOS[65,66].

HEPATIC INFLAMMATION AND ENDOTHELIAL DYSFUNCTION IN CIRRHOSIS

Hepatic inflammation is a common trigger of liver diseases, associated with ED and causes increased hepatic vascular risk in cirrhosis[67]. There are several inflammatory mediators involved in downregulation of eNOS activity and NO bioavailability within the hepatic circulation resulting increased intrahepatic resistance and ED thus, PHT in patients with cirrhosis (Table 1).

TNF-α

TNF-α, a proinflammatory cytokine with the broad spectrum of deleterious effects is believed to exert vascular effects by increasing vascular permeability and causing vasodilatation, which mediated through NO dependent pathways[68]. In cirrhosis, increased systemic and hepatic TNF-α concentration has shown to associate with overwhelming NO production[47]. Interestingly, in cultured endothelial cells, TNF-α reduces NO bioavailability[48], and *in vivo* TNF-α may also directly alter endothelial vasomotor function[69]. Moreover, Plasma TNF-α concentration was shown to be significantly higher in alcoholic hepatitis (AH) patients who subsequently died than those who survived[70]. Patients with AH had significantly higher plasma TNF-α concentration than did patients with inactive cirrhosis or alcoholics having no liver disease[71,72]. It has also shown that lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, induces a marked TNF-α production *in vivo* in cirrhotic rats[73] and *ex vivo* in monocytes from cirrhotic patients[74]. Infliximab (anti-TNF antibody) treatment has been shown to reduce systemic TNF-α and a concomitant drop in portal pressure in alcoholic hepatitis patients with severe ED[75]. Our previous study also supported the above notion that treatment with anti-TNF improved hepatic DDAH enzyme function by decreasing ADMA level and a concomitant increase of hepatic eNOS activity and NO bioavailability in a bile duct-ligated cirrhotic rat[42]. Infliximab treatment also shown to beneficial in CCl_4, and high fat diet induced liver disease models[76,77]. Furthermore, anti-TNF treatment to the portal vein ligated rats significantly blunts the development of the hyperdynamic circulation and reduces portal pressure[75].

Nuclear factor-κB

Nuclear factor-κB (NFκB) is a ubiquitous transcription

Table 1 Factors affecting endothelial dysfunction in cirrhosis

Marker	Endothelial dysfunction	Ref.
Inflammatory marker		
TNF-α	Inhibition of NO synthesis	[47,75,78,84,99,104]
NFκB	Increase of ADMA	
TLR	Increase of Caveolin-1	
Ang II	Reduction of eNOS activity	
	Inhibition of DDAH enzyme	
	Upregulation of iNOS	
	Increase of superoxide production	
	Reduction of antioxidant capacity	
Oxidative marker		
4-HNE	Reduction of DDAH enzyme activity	[42,78,104]
NADPH	Decrease of NO bioavailability	
	Increase of ADMA levels	
	Increase of ROS generation	
Cyclooxygenase -derived prostanoids		
TXA_2	Reduction of intrahepatic nitrate/ nitrite	[110-112]
PGI_2	Upregulation of iNOS expression	
	Increase of intrahepatic resistance	
Other marker		
ET-1	Increase of inflammation	[122,130,138,139, 145]
LOX-1	Stimulation of ROS generation	
PARs		
Adiponectin		
Palmitic acid		

TNF-α: Tumor necrosis factor-α; NFκB; Nuclear factor kappa B; TLR: Toll like receptor; Ang II: Angiotensin II; NO: Nitric oxide; ADMA: Asymmetric dimethylarginine; eNOS: Endothelial nitric oxide synthase; DDAH: Dimethyl arginine diaminohydrolase; iNOS: Inducible nitric oxide synthase; 4-HNE: 4-hydroxy-2-nonenal; NADPH; Nicotinamide adenine dinucleotide phosphate-oxidase; ROS: Reactive oxygen species; TXA2: Thromboxane A2; PGI2: Prostaglandin I2; ET-1: Endothelin -1; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1; PARs: Protease activated receptors.

factor that activated by a variety of cytokines which including TNF α and is thought to be a key regulator of genes involved in inflammation[47,78]. In BDL cirrhotic rats, elevated plasma F2-isoprostanes correlated with an increase of TNF-α and constitutive activation of NFκB[79]. Osanai *et al*[80] showed in HUVECs that synthesised ADMA by PRMT-1 was further stimulated by shear stress *via* activation of the NFκB pathway. Recently ADMA induces TNF-α production *via* ROS/ NFκB dependent pathway also reported in human monocytes[81]. Activation of NFκB also increased in the liver of chronically HCV-infected patients compared with controls[82], which is in line with our previous observation that increased NFκB protein expression in BDL cirrhotic rats was downregulated following Infliximab treatment[47,78]. TNF-α facilitates the translocation of free NFκB from cytosol to the nucleus and the induction of iNOS gene expression. The overproduction of NO by iNOS is important in inflammation and causes ED and may associate with hyperdynamic circulation in cirrhosis[27,83,84]. In this context, recently Jalan *et al*[84] observed that an incubation of cirrhotic patient plasma or LPS with HUVECS showing increased iNOS activity. Thus, transjugular intrahepatic stent-shunt insertion (TIPSS) induced endotoxemia results in upregulation of the iNOS pathway in the endothelium of critically ill cirrhotic patients[84]. Hence, increased iNOS driven NO is marker for the treatment of inflammatory disorders, and its prevention is a target for the design of new drugs acting on iNOS[85,86]. In fact, inhibition of NFκB activation was initially considered important for designing NOS inhibitors, since NFκB mainly involved in iNOS expression during inflammatory conditions[47,85,86]. Therefore, the regulation of iNOS *via* the NFκB pathway is an important mechanism in inflammatory processes and potential site for intervention in inflammatory diseases.

Toll-like receptors

Toll-like receptors (TLRs) belong to the family of transmembrane pattern-recognition receptors that recognize pathogen-associated molecular patterns, which including LPS[87]. TLR4 expressed on both parenchymal and non-parenchymal cell types in the liver and its activation trigger hepatic innate immune signaling, and may contribute to endothelial dysfunction and intrahepatic vascular tone in patients with cirrhosis[8,87,88]. In addition, the potential role of TLR4 in mediating renal injury in patients with cirrhosis was described recently[89]. The seminal observations made in this study were that the renal expression of TLR4 and the excretion of TLR4 protein was significantly higher in patients with cirrhosis who presented with acute deterioration and had renal dysfunction compared with those that did not[89]. Furthermore, urinary TLR4 was associated with significantly greater risk of death in patients with renal dysfunction and in those with superimposed inflammation[89]. TLR4 expressed on the surface of several cell types, including endothelial cells and its activation shown to reduce NO concentration, resulting ED[90]. Accordingly, anti-TLR4 treatment improved endothelium-dependent relaxation, and improved NO[91]. TLR4 also contributes to the increased ROS production and ED in hypertension, diabetes and obesity[92]. Recently, Benhamou *et al*[93] revealed that both TLR2 and TLR4 in mediating endothelial dysfunction and vascular remodeling in primary arterial antiphospholipid syndrome. TRL4 signalling leads to activation of NF-κB[94], a pathway associated with endothelial injury[95], and increased TLR4 expression has also shown in advanced liver disease[89,96]. LPS induced TLR4 mediated proinflammatory signalling has also showed in human hepatic stellate cells (HSC)[97], and functional expression of TLR9 has detected in sinusoidal hepatic endothelial cells and hepatocytes[98]. Furthermore, several animal studies support the importance of TLR4 in hepatic fibrosis, and TLR4 knockout mice showing less fibrosis induced by BDL or carbon tetrachloride (CCl_4) compared to wild type[99].

These results suggested an important function of TLRs on the development of inflammatory pathology in hepatic cirrhosis. Stadlbauer *et al*[96] observed that in AH patients, the increased TLR 2, 4 and 9 expressions correlated with neutrophil dysfunction and endotoxemia, albumin an endotoxin scavenger attenuated these complaints by decreasing TLRs expression. Several lines of evidence exist implicating gut derived endotoxemia in the pathogenesis of portal hypertension[100]. Administration of norfloxacin, a selective gut decontaminant prophylactic reduced endotoxin levels, TLR4 expression and decreased NO-mediated forearm vasodilatation and improved survival in cirrhosis[94,101].

Angiotensin Ⅱ

The renin-angiotensin system (RAS) plays a key physiological role in regulating vascular function. In the pathophysiology, RAS has also shown to promote vascular injury by triggering ED, vascular remodelling and vascular inflammation[102,103]. Angiotensin (Ang) Ⅱ the core composition of the RAS involved in many chronic diseases, which including hepatic cirrhosis[104]. Increased Ang Ⅱ causes endothelial dysfunction, vasoconstriction, sodium water retention, elevated blood pressure, ROS generation, inflammatory mediators and pro-fibrotic cytokines[105]. The adverse effects of Ang II induced ED is mediated by interaction with the plasma membrane AT 1 receptors (Ang Ⅱ type 1 receptors) and causes NO reduction by inducing eNOS enzyme dysfunction and promoting NOS uncoupling. Thus, pharmacological inhibition of the production or actions of Ang Ⅱ receptor blockers now represents an effective strategy to delay the progression of endothelial dysfunction in experimental models and humans[105,106]. In this context, previous clinical studies have shown evidence that RAS play an important role in the elevation of the ADMA concentration in hypertensive patients, and blockade of Ang Ⅱ by ACEI or Ang Ⅱ receptor blocker (ARB) significantly attenuates the elevated level of ADMA, resulting in endothelial protection[107,108]. Pharmacological blockade of angiotensin Ⅱ receptors using the drug Candesartan cilextil (CC) may attenuate the progression of liver cirrhosis and endothelial dysfunction. In HUVECS, CC increased the eNOS protein level, inhibited the expression of nicotinamide adenine dinucleotide phosphate oxidase (Nox) subunits and Ang Ⅱ induced intracellular ROS and nitric oxide, and promoted the extracellular release of nitric oxide[109] suggesting that it augmented the bioavailability of nitric oxide. Thus, CC administration may attenuate ED and for the future therapeutic approach in portal hypertensive patients with cirrhosis. Ang Ⅱ can also activate NADPH oxidase (Nox), leading to increased ROS generation and commencing ED in cirrhosis[104].

VASOCONSTRICTORS AND ENDOTHELIAL DYSFUNCTION

Thromboxane

TXA_2, a vasoactive prostanoid and COX metabolite, increased intrahepatic vascular tone in cirrhosis, more specifically in the phenomenon of hyper responsiveness to vasoconstrictors and caused intrahepatic ED[110,111]. Administration of COX inhibitors such as flurbiprofen and nitroflurbiprofen (NO releasing COX inhibitor) to cirrhotic rats result in decreased hepatic TXA_2 production and an increased intrahepatic nitrate/nitrite (an index of NO synthesis) concentration thereby by attenuating intrahepatic vascular resistance, endothelial dysfunction, and hepatic hyper reactivity to vasoconstrictors[111]. TXA_2 and PGI_2, the other COX 2 derivatives may act simultaneously, producing a compensatory effect that reduces NO release and may limit the hyperdynamic circulation in cirrhosis[112]. The use of indomethacin, a COX inhibitor has shown to prevent liver fibrosis, and rise in portal hypertension in liver cirrhosis[113]. Indeed, study also shows that COX inhibitors could worsen the hyperdynamic circulation associated with liver cirrhosis[112]. TXA_2 induces vasoconstriction by activating the TXA_2/prostaglandin-endoperoxide (TP) receptor[114]. TP receptor ligands include TXA_2, PGH_2, and isoprostanes[115,116]. TXA_2 acts through its G-protein-coupled receptor leading to vasoconstriction by activating the RhoA/Rho-kinase pathway, and by increasing calcium levels in HSC[117]. Oral administration of terutroban, a specific antagonist of the TP-receptor[118] has shown to attenuates inflammation and oxidative stress and reduce RhoA/Rho-kinase-dependent signaling and restore NO bioavailability in endothelial cells[119] may represent a useful agent in the treatment of endothelial dysfunction in cirrhosis with portal hypertension.

Endothelin-1

A most potent vasoconstrictor endothelin (ET) -1 regarded as a key player in ED, primarily binding to G-protein coupled receptors such as ETA and ETB and acts in a paracrine fashion[120]. Previous study has shown that ETB receptors present in endothelial cell can elicit endothelium-dependent relaxation by improving NO release by contrast, ETA and ETB receptors present on the fibroblasts and smooth muscle cells trigger vasoconstriction and inflammation[121]. Cirrhotic rats with diabetes showed higher intrahepatic ET-1 vasoresponsiveness than normoglycemic cirrhotic rats[122]. ED also found in patients with insulin resistance (IR)[123,124]. In this context, IR can be triggered by ET-1 infusion in rats by activating phosphatidylinositol (PI) 3-kinase activity in smooth muscle cells in an ETA dependent manner and treatment with ETA receptor antagonists results in improvement of insulin sensitivity and associated

endothelial function *via* an inhibition of PI 3-kinase activity[125]. In this context, a very recent study pointed out that LPS stimulation to portal hypertensive rats showed enhanced renal vascular response to ET-1 through ETA overexpression[126].

OTHER MARKERS OF ENDOTHELIAL DYSFUNCTION

Lectin-like oxidised LDL receptor-1

Lectin-like oxidised LDL receptor-1 (LOX1) is a key receptor for oxidised low-density lipoprotein (Ox-LDL) and identified in endothelial cells, and considered as a marker of ED in various pathological setting[127,128]. LOX-1 promotes ROS generation augments endothelial adhesiveness to monocytes and inhibits NO synthesis[129]. The recent review by Lubrano *et al*[130] described in detail about the relationship between LOX1 and ROS. Furthermore, increased expression of LOX1 was found in the placenta of women with intrahepatic cholestasis during pregnancy[131]. LOX-1 polymorphism also associated with liver disease severity in non-alcoholic steatohepatitis[132] and could be a biomarker for patients with endothelial dysfunction in liver cirrhosis.

Protease activated receptors

Protease activated receptors (PARs) are G protein-coupled receptors which, mediating cellular effects of some proteases of the activated coagulation system such as thrombin, trypsin or metalloproteinase[133]. ECs express PAR1, PAR2 and PAR4[134]. Endothelial PARs play important roles in the crosstalk between coagulation and inflammation in sepsis[133]. Acute PAR1 activation causes an increase in vascular permeability, presumably due to direct endothelial contractile responses[133]. PAR1 deficiency and blockade has shown to reduce inflammation in a mouse model of colitis[135]. Moreover, activation of protease through its receptor following thrombus formation, hemorrhage and inflammation led to the conversion of ECs to a proinflammatory phenotype and may result in vascular lesion development[133]. Garcia *et al*[136] have shown in cultured ECs, PAR1 activation stimulates the production of prostacyclin and NO, consisting with other reports shown in *in vivo* that PAR1 activators cause hypotension when injected intravenously and cause NO mediated vasodilation[137]. In this context, Knight *et al*[138] and Sakata *et al*[139] reported that PAR2 promotes experimental liver fibrosis by increase of TGF β production in mice and to induce a profibrogenic phenotype in human HSCs. Thus, targeting PARs and using its antagonists in endothelial dysfunction with cirrhosis may represent a novel therapeutic approach in preventing portal hypertension in cirrhosis.

Adiponectin

Adiponectin is a protein hormone synthesized by adipose tissue. Plasma physiological concentration of adiponectin represents 0.05% of all plasma proteins and is a key component in the relationship between adiposity, inflammation and insulin resistance[140]. Previous studies have shown evidence that the association between hypoadiponectinemia and ED[141,142]. Wang *et al*[142] showed in ECs that adiponectin stimulating NO synthesis by activating AMPK mediated pathway. Furthermore, adiponectin knockout mice exhibited impaired endothelium dependent vasodilation and NO production[143]. Earlier published studies have pointed out that adiponectin administration significantly increases NO production by regulating eNOS enzyme activity and its phosphorylation and maintain endothelial function[141]. However, despite the hepatoprotective effect of adiponectin have shown in NAFLD and other chronic liver ailments, the plasma concentration of which increased in patients with cirrhosis of different aetiologies. Indeed, the factors related to elevated levels of adiponectin in cirrhosis are not yet completely understood. In this context, Tietge *et al*[144] showed an evidence that increased adiponectin levels in cirrhotic patients correlate exclusively with reduced liver function and altered hepatic haemodynamics. Furthermore, Salman *et al*[145] and Tacke *et al*[146] reported that an elevated adiponectin correlated with inflammation and liver damage and high levels were found in human cholestasis as well as in an animal model of cirrhosis[145,146]. Thus, adiponectin may serve as a novel biomarker for cholestasis in liver cirrhosis and agents that modifying adiponectin concentration in liver cirrhosis may use as a potential diagnostic tool but also as therapeutic target for ED in cirrhosis.

Free fatty acid

Liver plays a significant role in lipid homeostasis including several stages of lipid synthesis and transportation. Thus, it is reasonable to anticipate an abnormal lipid profile associated with the progression of hepatic dysfunction. Furthermore, hyperlipidaemia is main risk factor for ED, which is a common indicator in patients with hepatic cirrhosis[147]. Accumulated evidence indicates that increased hepatic and plasma free fatty acid (FFA) concentration led to hyperlipidemia and may cause ED in cirrhosis. Previously it has been shown that FFA trigger HUVECs apoptosis and inhibit cell cycle progression[148]. In this regard, palmitic acid a key FFA in the bloodstream, exposure to ECs causes cell necrosis and the release of proinflammatory cytokines[149,150] consistent with other report showing in cultured bovine retinal pericytes, in which palmitate can induce apoptosis by promoting oxidative stress[151]. Recently Ristic-Medic *et al*[152] observed in cirrhotic patients, increased levels of palmitic acid and total saturated fatty acids when compared to healthy controls. Thus, FFA play an important role in cirrhosis with ED and agents that reduce palmitic acid concentration would be used as a possible future target for therapy. One such agent,

Table 2 Classic and novel therapeutic strategies directing to improvement of endothelial dysfunction in cirrhosis

Therapeutic agent	Endothelial function	Ref.
Anti-inflammatory agents	Increase of NO bioavailability Reduction of ADMA Upregulation of eNOS activity Decrease of Inflammation	[25,42,75,111]
Vitamins	Improvement of eNOS activity Increase of NO bioavailability Scavenging of ROS generation Antioxidant function	[159,160,162]
Flavonoids	Increase of NO bioavailability Prevention of oxidative stress Improvement of antioxidant enzymes	[15,166,168]
Nuclear receptors	Increase of NO bioavailability Improvement of DDAH Reduction of ADMA Amelioration of hepatic vascular tone	[33,50,187]
Ammonia lowering agents	Detoxification of ammonia levels Increase of NO bioavailability Reduction of ADMA Upregulation DDAH expression	[27,189,190,192,201]
Statins	Decrease of total cholesterol Improvement of Akt-dependent eNOS phosphorylation Promoting NO biosynthesis Reduction of Ox-LDL Attenuation of inflammatory indices	[147,170,172,202,203]
Beta blockers	Amelioration of oxidative stress Attenuation of Inflammation Restoration of antioxidant enzymes	[194,196]
Angiotensin-receptor antagonists	Increase of NO Decrease of Ang-II mediated inflammation Decrease of TIMP-1, MMP-2 mediated fibrosis	[200,204]

NO: Nitric oxide; ADMA: Asymmetric dimethylarginine; eNOS: Endothelial nitric oxide synthase; DDAH: Dimethyl arginine diaminohydrolase; Ox-LDL: Oxidized low-density lipoprotein; Ang Ⅱ: Angiotensin Ⅱ; TIMP-1: Tissue inhibitor of metalloproteinase 1; MMP-2: Matrix metalloproteinase-2.

eicosapentaenoic acid (EPA), ω-3 polyunsaturated fatty acid (PUFA) is abundant in fish oil has shown to enhance the production of NO, *via* activating eNOS and improve normal vascular endothelium[153]. Very recently Lee *et al*[154] have demonstrated that treatment with EPA protects against palmitic acid induced ED through activation of the AMPK-eNOS mediated pathway. Highly purified EPA also shown to prevent the development of inflammation and hepatic fibrosis in rats[155,156]. In addition, peroxisome proliferator-activated receptor (PPAR)-α, a member of the nuclear receptor superfamily and a key regulator of fatty acid homeostasis[157], has been shown to improve endothelial dysfunction and portal pressure in cirrhotic rats[158]. The above indices, therefore, provide a rationale for novel insights into the pathophysiology of ED and the potential for the development of novel biomarkers and therapeutic approaches in patients with endothelial dysfunction and advanced liver disease.

EMERGING THERAPY FOR REVERSAL OF HEPATIC ED IN CIRRHOSIS

ED is an early event in the pathogenesis of cirrhosis with PHT and can be reversible with certain therapies (Table 2). Restoration of ED appears to be a crucial therapeutic target, since ED predicts most of the liver related problems in alcoholic liver disease (ALD), hepatorenal syndrome (HRS), hepatic encephalopathy (HE) and sepsis.

Antioxidant strategy (Vitamins and flavonoids)

Ascorbic acid (vitamin C) has been shown to improve the NO-dependent vasodilatation in vascular beds of patients with conditions characterized by marked ED in cirrhosis[159]. The other antioxidant α-tocopherol (vitamin E) has also shown to improvement of hepatic ED by suppressed hepatic ADMA and oxidative stress and improved hepatic NO in cirrhotic rats[160]. In addition, folic acid, a superoxide scavenging vitamin B9 and its active metabolite 5-methyltetrahydrofolate (5-MTHF) has been shown to restore ED in patients with many cardiovascular diseases[161]. Folic acid mainly involved in downregulating eNOS derived superoxide and eNOS uncoupling thereby improving regeneration of BH_4 from BH_2, by preventing BH4 oxidation, which results in increased NO[161]. The beneficial effects of 5-MTHF have shown in decompensated cirrhotic patients recently[162]. Superoxide dismutase (SOD) gene transfer also has shown to reduce portal pressure in CCl_4 cirrhotic rats with portal hypertension through reducing oxidative stress and increased NO bioavailability[61]. Tempol, a SOD mimetic reduces superoxide, increases nitric oxide, and reduces portal pressure in sinusoidal endothelial cells of cirrhotic livers[163]. Furthermore, flavonoids, an integral part of the human diet have been shown to confer protective effects on vascular endothelial function in humans[164], and its protective effects are chiefly ascribed to their antioxidant and vasodilatory actions[165]. Very recently, Hsu *et al*[15] found that green tea polyphenol decreases the severity of portosystemic collaterals and mesenteric angiogenesis in rats with liver cirrhosis. Furthermore, De Gottardi *et al*[166] observed in cirrhotic patients with portal hypertension that dark chocolate blunted the postprandial increase in hepatic venous pressure gradient (HVPG) by improving flow-mediated hepatic vasorelaxation and ameliorated systemic hypotension. In addition, Lin *et al*[167] showed quercetin supplementation has associated with multifactorial potential as well as down-regulation of NF-κB and TGF-β/Smad signalling, probably *via* interference with TLR signalling. Resveratrol, a natural polyphenolic flavonoid present higher amount in grapes has shown

to reduce portal pressure by attenuating ED. Moreover, resveratrol supplementation also results in reducing oxidative stress and upregulating eNOS expression without affecting systemic hemodynamics in cirrhotic rats[168].

Statins

Statins (HMG-CoA reductase inhibitors) lower serum cholesterol concentrations and exhibits beneficial therapeutic effects in cirrhotic patients, as evidenced by various clinical trials[169-171]. Abraldes *et al*[169] demonstrated that simvastatin improve hepatic NO generation and endothelial function and lowers portal pressure in patients with cirrhosis. Moreover, atorvastatin has been shown to prevent liver inflammation and HSC activation induced by Ang-II infusion[172]. Schwabl *et al*[172] found that pioglitazone, an insulin sensitiser decreases portosystemic shunting by modulating inflammation and angiogenesis in cirrhotic and non-cirrhotic portal hypertensive rats. Additionally, Sorafenib, a tyrosine kinase inhibitor approved in the treatment of hepatocellular carcinoma, has shown to have beneficial effects in reducing portal pressure in cirrhosis[173]. The other multitarget receptor tyrosine kinase inhibitors such as Sunitinib and Imatinib were also shown to use for the treatment of portal hypertension[174] and may have a potential role in regulating ED in cirrhosis through NO mediated mechanisms. However, further encouraging clinical studies of statins strategy to ED in cirrhosis needs to be explored.

Anti-inflammatory agents

Human serum albumin (HSA) is one of the most frequent treatments in patients with decompensated cirrhosis[175]. It also reduced the severity of other chronic liver diseases such as HE and HRS and improved survival of patients with spontaneous bacterial peritonitis (SBP)[175-177]. In addition, albumin has been demonstrated to have a clinically significant beneficial effect on ED and survival during experimental endotoxemia[178]. It also reduced sequential organ failure assessment (SOFA) score in critically ill patients with hypoalbuminemia[179]. Furthermore, pentoxifylline and N-acetylcysteine, the other known anti-inflammatory agents have shown to associated with reduced the risk of inflammation and ED in cirrhosis[180-182]. Antibiotics such as quinolones and rifaximin and high-density lipoprotein (HDL) treatment have shown to associate with the reduction of inflammation and portal pressure by neutralising portal bacterial endotoxin load[20,101,183]. Thus, anti-inflammatory agents would improve NO bioavailability and reduce ED, considered a potential therapeutic approach for the management of portal hypertension in cirrhosis.

Nuclear receptors

Obeticholic acid, a synthetic farnesoid X receptor (FXR) ligand belongs to a nuclear receptor superfamily of transcription factor, which plays an important role in bile acid and lipid metabolism[184,185] has also been the subject of considerable attention over recent years[50]. FXR agonists have numerous target genes including DDAH1[186]. Our previous study has shown evidence that obeticholic acid significantly increases hepatic DDAH-1 and eNOS activity and improved NO bioavailability in cirrhotic rats, leading to improvement in endothelial function and associated drop in portal pressure[33]. Similarly, a multi-centre phase 2a trial of obeticholic acid in decompensated cirrhotic patients show a trend towards a drop in portal pressure[187]. Another promising approach of transfection of cirrhotic liver with DDAH-1 decreased ED and portal hypertension in BDL rats[188]. Furthermore, other member of the nuclear receptor superfamily, PPAR- α also has shown to improve endothelial dysfunction and portal pressure in cirrhosis[158].

Ammonia lowering agents

AST-120, an oral adsorbent carbon microspheres and ammonia-lowering agent[189] has shown to reduce ED in adenine-induced uremic rats[190]. AST-120 treatment was also shown to prevent the progression of HE in cirrhotic rats[189] and chronic kidney disease (CKD) in a clinical setting[191], which may be used for the treatment of ED in cirrhosis. Indeed, further studies would be needed for describing the potential role of AST-120 on NO mediated ED in cirrhosis. Furthermore, we established a new promising therapy such as OCR-002 (ornithine-phenylacetate) for the treatment of HE[27,192]. OCR-002 is currently being advanced in the clinic and also effective in reducing PHT by lowering ammonia mediated inflammation and improving NO bioavailability[193] and may consider for the future therapeutic approach for the management of ED and associated PHT in patient with cirrhosis.

Non-selective β blockers

Carvedilol is a non-selective β blocker with alpha-1 adrenergic blocker activity has been shown to amelioration of oxidative stress and restoration of antioxidant enzyme activities, and attenuation of NF-κB mediated inflammation in chronic liver disease[194]. Interestingly, Reiberger *et al*[195] observed in BDL cirrhotic rat that nebivolol, a third generation beta-blocker increased splanchnic blood flow and portal pressure *via* NO mediated signalling. In this context, Ma *et al*[196] demonstrated in myocardial infraction that targeting NO with a nebivolol treatment improves diastolic dysfunction through reducing myocardial oxidative stress by enhancing 5'-AMP-activated protein kinase and Akt activation of NO biosynthesis. Moreover, long-term nebivolol administration reduces renal fibrosis and prevents endothelial dysfunction in a rat hypertensive model[197]. In this context, Mookerjee *et al*[11] have pointed out that specific controlled studies are addressing the use of β-blockers in patients with severe decompensation of cirrhosis with high risk of

sepsis and renal dysfunction are inadequate. Hence, further clinical studies on the effect of β-blockers through NO mediated pathway are challenging in liver cirrhosis.

Angiotensin-receptor antagonists

Additionally, Candesartan cilextil (CC), a selective angiotensin Ⅱ type Ⅰ (AT1) receptor antagonist widely used as an antihypertensive in clinical practice has shown to improve ED[198,199]. In addition, recent clinical study has shown evidence that CC administration was safe and well tolerated to compensated cirrhotic patients, without an underlying cause of renal failure or hepatic decompensation[200]. Given the substantial experimental evidence that CC has the potential beneficial effect in distinct human diseases by blocking Ang-Ⅱ mediated AT1 receptor and may improve ED and NO bioavailability and associated mechanism in cirrhosis.

CONCLUSION

In conclusion, this review has been discussed the involvement of various inflammatory and oxidative stress markers on the regulation of NO biosynthesis and associated ED. The therapeutic interventions, which including antioxidants, anti-inflammatory and ammonia lowering agents, bile acid receptors, statins, insulin sensitisers, beta blockers and ARBs have been shown to increasingly recognised to attenuate liver cirrhosis by decreasing inflammation, oxidative stress and promoting NO biosynthesis. Subsequently, the development of an ideal therapy based on the increase of hepatic NO synthesis through ADMA-DDAH pathway may improve endothelial function and reduce inflammation, subsequent portal pressure reduction and without compromising systemic arterial pressure in patients with advanced liver disease. Remarkably, DDAH-1 gene strategy to cirrhotic liver would advance future therapeutic attention in the context of improvement of NO synthesis and reduce inflammation and associated ED and portal pressure reduction in-patient with cirrhosis.

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MINIREVIEWS

Gender-based disparities in access to and outcomes of liver transplantation

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Abstract

Despite comprising 35% of transplants, the number of female transplant recipients has continued to decline. Accordingly, there is a growing attention to the issue of access to and outcomes of liver transplantation in women. The purpose of this review is to critically evaluate the published literature on etiologies contributing to gender-based disparities in liver transplantation focusing on the steps from chronic liver disease through transplantation including disparities in liver disease prevalence, access to liver transplant centers and transplant waiting list, receipt of liver transplantation once listed and disparities in post-liver transplantation outcomes. Our review finds factors contributing to this disparity may include gender differences in the etiology of underlying liver disease and patient and physician referral patterns, lifestyle and health care, but also utilization of an imperfect organ allocation system based on the model for end stage liver disease score and donor-recipient liver size matching. The review also highlights the need for further research in the area of gender disparity in order to develop appropriate approaches to address it and to improve allocation of this precious resource in the future.

Key words: Female gender; Liver transplantation; Creatinine; Model for end stage liver disease; Disparity

Core tip: Liver transplantation is a life-saving procedure for many patients with end stage liver disease therefore it is of utmost importance to ensure equity in its distribution. Recently, growing attention has been placed on the issue of gender disparity in access to and receipt of a liver transplant. Factors contributing to this disparity include important differences in the etiology of underlying liver disease and indications for liver transplant that differ by gender. Systematic bias against women also appears to exist in many of the crucial steps of organ allocation. Better understanding of those mechanisms and their solutions are needed to improve liver transplantation rates in women.

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INTRODUCTION

Chronic liver disease is the 12th leading cause of death in the United States with steadily increasing prevalence rates[1,2]. Notwithstanding medical therapies targeted to treat complications of end-stage liver disease, liver transplantation (LT) is the only life-saving procedure and is the treatment of choice for selected patients. In spite of the many improvements in liver organ allocation following the adoption of the model for end-stage liver disease (MELD) score in 2002, thousands of patients die awaiting liver transplantation every year as the supply of organs remains overwhelmed by the more than 17000 individuals on the waiting list. This burden is disproportionally exemplified in women, who comprised 35% of transplant recipients in 2013[3]. The proportion of female liver transplant recipients has continued to decline since the adoption of MELD in 2002. Data suggest that this proportion of women is also less likely to undergo liver transplantation once listed and have a greater probability of dying or becoming too sick to undergo liver transplantation compared to men[4]. Accordingly, there has been a fervid interest to identify the mechanisms behind gender discrepancies in disease burden and the process of liver transplantation (Table 1)[4-15]. The present review aims to evaluate the published literature on gender - based disparities in liver transplantation focusing on those areas of greatest significance to equity in LT, including the prevalence of chronic liver disease, access to LT, receipt of a liver transplant once listed and post-liver transplantation outcomes.

PREVALENCE OF CHRONIC LIVER DISEASE IN WOMEN

It is well established that etiologies of liver disease differ by gender. To determine an accurate epidemiology of chronic liver disease by gender in the United States has proven difficult however due to the lack of a national data collection system. Nonetheless, one can estimate prevalence rates by gender for individual diseases known to cause chronic liver disease. For example, women are 10 times more likely to have primary biliary cirrhosis than men and four times as likely to have autoimmune hepatitis[16,17]. Women are also more likely to present with alcohol and drug induced hepatotoxicity and acute liver failure as compared to men[18-20]. On the contrary, a National Health and Nutrition Examination Survey (NHANES) conducted between 2003 and 2010 found men to be significantly more likely to be chronically infected with hepatitis C virus than women[21]. Furthermore, gender also likely plays a role in disease progression for certain etiologies of chronic liver disease. Cross sectional studies have identified male sex as a risk factor for disease progression to cirrhosis by over 2.5 fold in patients with chronic hepatitis C[22]. Population prevalence of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) has been difficult to accurately establish[23]. Although the majority of studies based on the most recent NHANES data report NAFLD to be more prevalent in men than women, rates may vary depending on the diagnostic and staging tools utilized. Using data from the NASH Clinical Research Network (CRN), Younossi *et al*[2], reported that patients with biopsy proven NASH were more likely to be female than male while those using liver enzyme data or ultrasound to diagnose NAFLD report higher prevalence in men. Given the aging population, the increasing proportion of patients with NASH cirrhosis requiring LT, and the real possibility of curing most patients with chronic hepatitis C virus (HCV) liver disease, one would expect to see an increased demand for LT for women. The changing epidemiology of chronic liver disease along with the fact that disease severity may not be as accurately reflected in the MELD score for those diseases affecting primarily women makes the issue gender disparity in LT of growing concern.

GENDER DIFFERENCES IN CHRONIC LIVER DISEASE

The dominant mechanisms behind gender differences in the prevalence, natural history and outcomes of chronic liver disease (CLD) remains incompletely understood. Lifestyle choices regarding alcohol and intravenous drug use, as well as access to and use of medical care differ between men and women and may underlie some of the gender-based difference in CLD. Data from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) showed that the prevalence of alcohol abuse and alcohol dependence is 2-3 times higher in men *vs* women[24]. However, while men consume and misuse alcohol at significantly higher rates, women experience shorter time intervals between the onset of alcohol use and alcohol related complications[25]. Hence it is postulated that the gender discrepancies in the prevalence, disease progression and outcomes of chronic liver disease may not all lie in behavior, but be largely influenced by the biological differences. For example, total body water, levels of alcohol dehydrogenase and slower rates of alcohol metabolism all contribute to the increased susceptibility of women to the toxic effects of alcohol[26,27]. In addition, differences in estrogen receptor concentrations, which in animal models is increased in male livers exposed to alcohol and unchanged in female alcoholic livers, may protect males from the alcohol induced liver disease seen in females[28]. On the contrary, female gender may play a beneficial role in viral hepatitis as studies have shown that estrogen can prevent stellate cell activation which is responsible for liver fibrogenesis[29]. The fall of estrogen is accompanied by a rapid increase in pro-

Table 1 Characteristics of studies assessing the impact of gender on liver transplantation *n* (%)

Topic	Ref.	Number of female patients (%)	Data collection
Access to LT Center	Bryce *et al*[5]	66622 (46%)	Transplant Centers in Pennsylvania/ UNOS
Access to waiting list and LT	Moylan *et al*[4]	16262 (36%)	UNOS Database
	Volk *et al*[6]	19518 (39%)	SRTR
	Mathur *et al*[7]	28759 (36%)	SRTR
Renal function and MELD	Cholangitas *et al*[8]	140 (38%)	Royal Free Hospital
	Huo *et al*[9] Lim *et al*[10]	103 (22%)	Taipei Veterans General Hospital
	Mindikoglu *et al*[11]	379 (45%)	Mayo Clinic
		14530 (36%)	UNOS Database
	Myers *et al*[12]	14541 (36%)	UNOS Database
Donor recipient size mismatch	Lai *et al*[13]	12585 (36%)	UNOS Database
	Mindikoglu *et al*[14]	10741 (37%)	OPTN
Post LT outcome graft/patient survival	Duffy *et al*[15]	169 (58%)	Single Center UCLA

UNOS: United network for organ sharing; SRTR: Scientific registry transplant recipients; LT: Liver transplantation; MELD: Model for end-stage liver disease; OPTN: Organ Procurement and Transplantation Network; UCLA: University of California, Los Angeles.

inflammatory and anti-inflammatory cytokines[30]. Accordingly, the antifibrogenic role of estrogen may contribute to the discontinuous fibrosis progression in women compared to men with HCV as well as to why disease progression is significantly increased after menopause[31-33]. While the prevalence of NAFLD is higher in men than women of reproductive age, the protective effect of estrogen in NAFLD is eliminated after menopause[34]. Furthermore, after menopause, women have an increased risk of insulin resistance, hyperlipidemia and visceral fat, all of which are known risk factors for worsening NAFLD severity and could contribute to increased prevalence of NAFLD cirrhosis in women over time[35].

LIVER TRANSPLANT RATES: CHANGING PREVALENCE OF CLD

Chronic HCV and alcoholic liver disease together encompass the most common causes of cirrhosis, accounting for approximately one half of patients currently on the LT waitlist (Figure 1)[3]. NHANES data collected between 1988 and 2008 found that while the prevalence of alcoholic liver disease and HCV has remained stable over the past decade, the prevalence of NAFLD has steadily increased, reflecting the obesity epidemic[2]. Moreover, with continued strides in HCV treatment possibly leading to a subsequent decrease in liver transplantation rates for HCV cirrhosis and hepatocellular carcinoma (HCC), end stage liver disease secondary to NAFLD may change the spectrum of patients requiring liver transplantation. Moreover, as the prevalence of NAFLD is similar in post-menopausal women and men, women may have an increased representation on the LT waiting list in the United States in the near future.

LIVER TRANSPLANT REFERRAL

Gaining access to a life-saving liver transplant is a complicated and daunting prospect. Despite being chronically ill and even debilitated, patients must successfully navigate several challenging steps including first seeking medical care, referral to a hepatologist/ gastroenterologist, referral to a transplant center for evaluation, placement on the liver transplant waitlist and finally receipt of liver transplantation. While women comprise just over 35% of candidates on the LT waitlist (Figure 2), challenges in establishing an accurate epidemiology of chronic liver disease by gender makes it unclear if this percentage reflects variations of disease burden in women or gender differences amongst those seeking and receiving healthcare. It is thus unclear how or if female gender plays a role in the first step of liver transplantation: referral to a transplant center.

In the field of kidney transplantation, multiple studies have found that female sex is associated with a lower likelihood of inclusion on the transplant waiting list and suggest that gender disparity is not due to fewer women seeking health care or transplantation[36-38]. Thamer *et al*[39], in a national survey of nephrologists, found that men were more likely to be recommended for kidney transplant. Kucirka *et al*[40] found that women were more likely to be reported as unsuitable for kidney transplantation because of age or being medically unfit compared to their male counterparts. Unfortunately, information regarding LT referral patterns are limited. In one study from Pennsylvania, Bryce and colleagues found that with the exception of acute liver failure, the probability of being evaluated and listed for liver transplant was consistently lower for women[5]. The study was limited in that it did not assess individuals that may have been referred to a transplant center but never evaluated. Referral practice patterns are difficult to accurately assess in general and those of community physicians to liver transplant centers represent an even more troublesome and poorly studied area. Given the probable influence of physician variability in preferences and attitudes towards patient referral, screening and eligibility for LT, comprehensive evaluation of referral patterns are urgently needed to

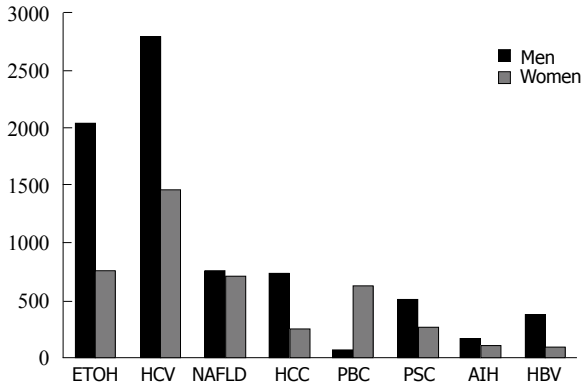

Figure 1 Primary cause of liver disease by gender, adult LT wait list candidates (2014). Based on Organ Procurement and Transplantation Network data as of July 1, 2014. Numbers of LT on the x-axis and etiologies of liver disease on the y-axis. PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; AIH: Autoimmune hepatitis; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease.

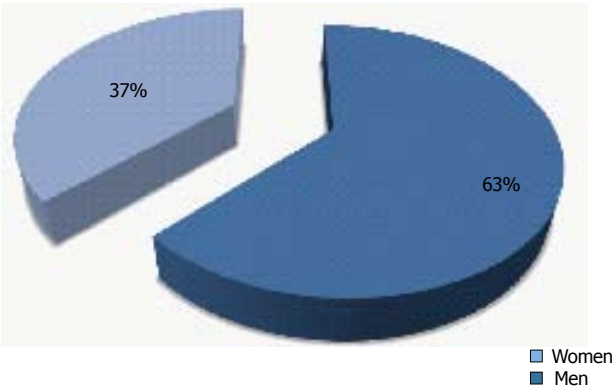

Figure 2 Adult Liver Transplantation Waiting List Candidates (2014). Based on Organ Procurement and Transplantation Network data as of November 28, 2014.

inform policies aimed at improving access to transplant centers for women.

LIVER TRANSPLANT RATES AND WAITLIST MORTALITY: INFLUENCE OF MELD

The current deceased donor liver transplantation (DDLT) organ allocation system in the United States is based on disease severity as measured by the MELD score as well as geographic proximity to available organs. Introduction and use of the MELD score in 2002 reflects the Institute of Medicine's recommendation for an objective system to assess disease severity with less subjective criteria and less emphasis on waiting list time[41]. Prior to 2002, patients with end-stage liver disease awaiting DDLT were stratified based on subjective assessments of disease severity as well as their hospital status and accumulated time on the waiting list. While the major goal of the MELD score was to ensure liver allografts went to the sickest patients first, another goal was to eliminate possible systematic biases such as referral patterns as well as gender and racial disparities in organ allocation. Using a large national database of liver transplantation from the Organ Procurement and Transplantation Network (OPTN), Moylan *et al*[4] noted an increased gender disparity in LT after the implementation of MELD. This study found that women were more likely than men to die while waiting for LT or to become too sick for LT in the post-MELD era. In addition, women were also less likely to receive a liver transplant within 3 years of listing in the pre-MELD and post-MELD group. Mechanisms behind this disparity were not able to be determined from that investigation but it was speculated that the use of creatinine as a marker of renal function, donor recipient size mismatch and geographic disparities could all contribute to women receiving less liver transplants than men.

RENAL FUNCTION AND MELD

Creatinine is a breakdown product of creatine phosphate during muscle metabolism and is influenced by age, total muscle mass and laboratory variation[42]. As a small and freely filtered solute, creatinine is the most widely used marker in estimating glomerular filtration rate (GFR) and underlying renal function. Men tend to have higher levels of measured serum creatinine as they have a greater mass of skeletal muscle compared to women[42]. In end stage liver disease, decreased hepatic creatinine synthesis, increased tubular creatinine secretion and decreased skeletal mass contribute to falsely low serum creatinine even in the presence of renal impairment[43]. Although not validated in patients with liver disease, the Modification of Diet in Renal Disease (MDRD) formula, which estimates GFR using four variables (creatinine, age, ethnicity and gender), is superior to other formulas[44]. Using the MDRD formula, the estimated GFR in women is lower compared to men with the same serum creatinine, age and race. Therefore although serum creatinine does not reflect underlying renal function in women, this difference is not taken into account when calculating the MELD score, which is based on serum creatinine and not GFR.

Several studies have investigated the use of creatinine in the MELD score and whether it may result in a systematic bias against women. When comparing creatinine, estimated GFR, and respective MELD scores in men and women with end stage liver disease, Cholangitas *et al*[8] found that a 2-3 point correction was needed for women with MELD scores > 19 in order to make them comparable with men and accurately assess renal function and hence overall disease severity. Similarly, Myers and colleagues found that women with end stage liver disease were less likely to undergo LT and had a greater three month mortality than men despite having lower creatinine but worse renal dysfunction based on estimated GFR[12].

In order to determine whether the use of creatinine was the source of a systematic bias against women, the investigators of this study revised the MELD score to include estimated GFR. Interestingly, this did not improve the difference in 3-mo mortality in women compared to men. This discrepancy may be explained by the suboptimal accuracy of the MDRD formula in estimating GFR in patients with end stage liver disease despite its superiority to alternative formulas. Compared to the gold standard for evaluation of renal function with direct measurement of GFR with inulin clearance or iothalamate, only 67% of GFR estimates were within 30% of measured GFR amongst pre-transplant patients[45]. A study by Lim *et al*[10] using ^{125}I-iothalamate for true GFR found that this was superior to creatinine in assessing mortality risk with a significant improvement of the discriminative ability when incorporated in the MELD score. Unfortunately, the prognostic impact was not evaluated between genders. Nonetheless, gender discrepancies in renal function and transplantation emphasized the inequities of the MELD score and stress the importance of examining alternative methods to evaluate renal function in patients with end stage liver disease.

DONOR-RECIPIENT SIZE MISMATCH

While data supports that renal function calculations and the use of creatinine in the MELD score contribute to decreased access to LT in women compared to men, other factors likely impact this disparity as well. An interesting study by Mindikoglu and colleagues investigated whether lower rates of LT in women *vs* men could be completely explained by MELD scores alone by comparing transplantation rates for each MELD score separately. They found that even within groups defined by MELD scores, women were generally transplanted at lower rates than men confirming that that suspicion that other factors play a role[11]. One of these factors is likely allograft size. Women are typically smaller than men in terms of total body mass as well as in liver size and overall height. It has been proposed that these differences lead to donor size mismatch and contribute to the gender disparity in LT[46].

Several published studies have looked at size differences and their contributions to this disparity. For example, Lai *et al*[13] used data from the OPTN to examine factors associated with differences in wait-list mortality between men and women in the MELD era and demonstrated that height, a surrogate marker for liver size, contributes to gender disparity. The investigators found that the majority of women were represented in the lowest quartile of the height distribution of wait-list candidates (< 165 cm). The mortality rates were 24% higher in this quartile and the rates of LT were significantly lower (P < 0.001). Again using OPTN data, Mindikoglu and colleagues found that size contributed to lower LT rates in women compared to men. Using median estimated liver volume and liver weights as surrogates for liver size, they found significantly lower volumes and weights in women men. After controlling for region, blood type and MELD score in a regression model including liver volume and weight, they found that women were significantly less likely to undergo LT than men suggesting that donor liver size mismatch significantly impacts gender disparity[14]. While the association between donor-recipient size and wait-list mortality remains poorly understood overall, a plausible explanation is that shorter and smaller women need smaller organs, which are preferentially offered to pediatric recipients. As a result, women may have to wait longer for a size appropriate allograft. This disparity in wait-list time may be reduced with the increased use of split-liver transplantation from deceased and living donors, but further research in this area is desperately needed.

GEOGRAPHIC DISPARITIES

Liver allograft distribution has historically been based on the geographic relationship between the hospital from which the organ is recovered and the transplant hospital in which the recipient is listed[47]. Unfortunately, this method of distribution has inadvertently created donor service areas (DSA) and regions with vastly discordant supply and demand ratios has contributed to significant geographic differences in access to liver transplantation[47]. Whether such geographic differences impact gender disparity in LT has therefore come under investigation. Mathur *et al*[7] evaluated this question using the OPTN regions with the largest sex-based disparities in transplant rates in the pre-MELD and MELD eras. They reported 16%-22% lower LT rates in women in regions 1, 2 and 10 during the pre-MELD era. In the MELD era, geographic disparity broadened with women exhibiting significantly lower LT rates in 6 of the 11 OPTN regions, with a maximum deficit of 35% in the Pacific Northwest (Region 6).

Despite a concerted effort by the United Network for Organ Sharing (UNOS) to change the liver allograft distribution policy, there has not been a significant reduction in the geographic variation in LT[47]. Broader sharing of organs has been proposed as a possible solution. The Share 15 policy, which ranks regional candidates with MELD > 15 higher than local candidates with MELD < 15, began in 2005 in the hopes of narrowing the range of median MELD at transplant among DSAs. As of yet, there are no studies investigating the effect of this policy in reducing gender-based geographic disparities. Nevertheless, as transplant needs and allograft supply differ widely amongst current regions, Gentry *et al*[48] proposed that broader sharing alone without redistricting may not be sufficient in reducing geographic disparities. But again, how redistricting affects women's access to LT remains unknown. The OPTN/UNOS Liver and Intestinal Organ

Transplantation Committee continue to investigate a number of approaches to reduce geographic disparities. Studies will also be needed to assess how these models affect gender-based disparities.

LIVER TRANSPLANTATION: OUTCOMES

Many factors contribute to outcomes after LT. The impact of gender on survival after LT varies with other donor and recipient factors such as race, age, ethnicity, as well as indication for LT. It also may vary by survival time and type of liver transplantation procedure performed. For example, female gender has been associated with advanced fibrosis and graft loss after LT for chronic HCV and this was increased with older donor age[49]. Long-term, women are reported to have improved survival than men however. In a small 20-year follow up study of LT recipients, women had improved survival over men[15]. One-year survival rates are similar between men and women with deceased donor liver transplantation, whereas they are increased compared to men in living donor liver transplantation[50]. The mechanisms behind these different survival rates by gender remain unclear.

Fortunately, the rates of re-transplantation continue to decrease. Women, however, continue to have slightly higher rates of re-transplantation compared to men as shown in one analysis of the 2009 Scientific Registry of Transplant Recipients[51]. The higher re-transplantation rate in women is speculated to be associated with the number of female candidates whose primary liver transplant was for diseases known to recur such as primary biliary cirrhosis, autoimmune hepatitis and NASH post-LT. Mathur *et al*[52] also found that female liver transplant recipients had 24% greater adjusted odds of receiving a low-quality liver allograft compared to their male counterparts which may affect both survival and re-transplantation rates. The etiology of the difference in quality of allografts for women recipients is unclear but felt possibly to relate to selection of shorter and older donors more often for women than men. Despite the utilization of poorer quality allografts in women, outcomes remain comparable across gender but warrant further longitudinal analysis as the epidemiology of LT changes.

CONCLUSION

LT is a life-saving procedure for many patients with end stage liver disease. Given its substantial and sustained improved survival rates over the last three decades, ensuring equity in its use remains crucial. Recent research has shed light on gender disparity that now exists in the LT process. The factors contributing to this disparity are many and include not only important gender differences in the etiology of underlying liver disease and patient and physician referral patterns, lifestyle and health care, but also utilization of an imperfect organ allocation system based on the MELD score and donor-recipient liver size matching which create bias against women at several steps. These biases include the use of creatinine as an imperfect marker of renal function in women, the need for smaller, less available liver allografts for the majority of women awaiting LT and geographic barriers to their distribution. This review highlights areas that require continued research to better understand such gender differences so that the disparity can be addressed and resolved. These steps will be of ultimate importance as we strive to improve the lives of all patients with end stage liver disease both pre- and post-liver transplantation.

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MINIREVIEWS

Magnetic resonance imaging of the cirrhotic liver: An update

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Abstract

Noninvasive imaging has become the standard for hepatocellular carcinoma (HCC) diagnosis in cirrhotic livers. In this review paper, we go over the basics of MR imaging in cirrhotic livers and describe the imaging appearance of a spectrum of hepatic nodules marking the progression from regenerative nodules to low- and high-grade dysplastic nodules, and ultimately to HCCs. We detail and illustrate the typical imaging appearances of different types of HCC including focal, multifocal, massive, diffuse/infiltrative, and intra-hepatic metastases; with emphasis on the diagnostic value of MR in imaging these lesions. We also shed some light on liver imaging reporting and data system, and the role of different magnetic resonance imaging (MRI) contrast agents and future MRI techniques including the use of advanced MR pulse sequences and utilization of hepatocyte-specific MRI contrast agents, and how they might contribute to improving the diagnostic performance of MRI in early stage HCC diagnosis.

Key words: Magnetic resonance imaging; Hepatocellular carcinoma; Hepatic nodules; Liver imaging reporting and data system; Dysplastic nodules; Regenerative nodules

Core tip: Noninvasive imaging has become the standard for hepatocellular carcinoma (HCC) diagnosis in cirrhotic patients. Typical imaging features of HCC, including increased arterial enhancement and delayed washout, provide very high specificity and acceptable sensitivity in characterizing even very small nodules. Diagnostic limitations apply to detecting hypovascular HCCs and differentiating high-grade dysplastic nodules from early HCCs. New techniques such as diffusion-weighted images, T2*, and hepatocyte-specific magnetic resonance imaging contrast agents, are being currently evaluated, which might improve future detection and characterization of hepatic lesions when combined with the current standard imaging protocols with dynamic imaging.

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INTRODUCTION

Every year, hepatocellular carcinoma (HCC) is diagnosed in more than 500000 people worldwide; with approximately 20000 new cases in the United States[1,2]. HCC is already the fifth most common neoplasm worldwide and is the third most common cause of cancer-related death, after lung and stomach cancers[1].

HCC rarely occurs before the age of 40 years, reaching a peak at approximately 70 years of age, and is two to four times more prevalent in men[3].

Most of the burden of disease (85%) occurs in developing countries, with the highest incidence rates reported in regions such as Southeast Asia and sub-Saharan Africa; where infection with hepatitis B virus (HBV) is endemic[1]. On the other hand, HCC related to hepatitis C virus (HCV) infection and secondary cirrhosis has become the fastest-rising cause of cancer-related death in the developed countries[2].

Patients diagnosed at an early stage are eligible for potentially curative therapies; including surgery (resection and liver transplantation) and locoregional ablative options (radiofrequency, microwave ablation, or ethanol injection). With this stage-driven strategies, 5-year survival rates range between 50%-70%[4]. However, very poor prognosis is observed with advanced HCC.

Therefore, an effort to diagnose HCC at early stages is being taken with the implementation of screening programs that may lead to earlier implementation of treatment.

Correlation with alpha-fetoprotein levels may sometimes be useful; however, not all tumors express alpha-fetoprotein. Additionally, mildly elevated alpha-fetoprotein levels may be seen in patients with chronic liver disease or in patients with cirrhosis but no HCC[5].

Ultrasound (US) is widely used, and represents the first imaging modality of screening by various international society consensuses; essentially because of the ease of access, lack of ionizing radiation, and lower cost compared with computed tomography (CT) and magnetic resonance imaging (MRI). The role of gray-scale US in cirrhotic patients in clinical practice is screening and surveillance, rather than accurate diagnosis of HCC (Figure 1). According to the updated American Association for the Study of Liver Diseases (AASLD) guidelines[6], the diagnostic algorithm of HCC starts from suspected nodules found on US surveillance. However, reported sensitivity and specificity is variable[7] and studies have shown a significant lower detection rate of HCC compared with CT and MRI[8]. Additionally, the technique is poor to detect small HCCs. At present, the real cost-effectiveness of US is not known, as it is common to find HCC in patients with prior negative US while receiving appropriate surveillance[9,10].

The use of CT for the detection of HCC requires intravenous iodinated-contrast administration and a minimum of a triphasic technique to evaluate the characteristic findings of increased arterial enhancement and late washout of typical HCCs. Several studies have shown higher sensitivities of gadolinium-enhanced MR imaging compared to CT for the detection of HCC of all sizes[11], while other studies have suggested a lower sensitivity of CT for detecting dysplastic nodules, small HCCs, and diffuse HCC compared with MRI[7,10].

The relatively short interval follow-up that is advocated for this patient population raises concern regarding the cumulative radiation dose and increased risk of worsening renal function due to the necessary repeated administration of intravenous contrast material[12].

Recent technological development of MRI scanners allowed high-quality multiphasic dynamic imaging of the entire liver[13]. Additionally, the superb contrast resolution and development of liver specific contrast agents rendered MR an important imaging modality for assessing cirrhosis and its complications, especially HCC. However, despite being an optimal imaging technique for the comprehensive evaluation of the liver[14,15], MRI has been used mainly as a problem solving technique[14].

Several studies have demonstrated a trend to increased sensitivity and specificity of dynamic MRI over dynamic CT for the detection and characterization of HCC of all sizes with reported sensitivities of 76%, 61%, 90% and 77% for MRI *vs* 61%, 52%, 78% and 54% for CT, respectively[11,16-18]. An optimized, dynamic T1-weighted gradient recalled echo (GRE) with individually tailored arterial phase timing, has shown very high sensitivity and specificity (> 90%-95%)[19].

The MRI sensitivity vary with tumor size; however, it was estimated to be about 100% in HCCs larger than 2 cm[20]. The detection of small tumors remains challenging, and MRI also outperforms CT in this area, with reported sensitivities for the detection of HCCs measuring 1-2 cm of 84% and 47% for MRI *vs* 85% and 68% for CT, respectively[11,21].

To date, validated CT and MRI criteria for the diagnosis of HCC are based on the hemodynamic features of the nodules and include arterial hyperenhancement and delayed washout[22]. The most recent recommendations by the AASLD state that a diagnosis of HCC can be made if a nodule larger than 1 cm shows typical hemodynamic features of HCC on either dynamic CT or MRI[6]. In our opinion, this reduces MRI and CT to a minimum common denominator; because despite the greater sensitivity of dynamic MRI for the detection of small HCCs; which might be explained by inherit superior contrast resolution of MRI and superior paramagnetic effect of intravenous gadolinium-based contrast agents, the diagnosis of HCC using only hemodynamic criteria is not without its limitation, as small HCCs frequently show atypical enhancement patterns[23]. One study showed that the majority of

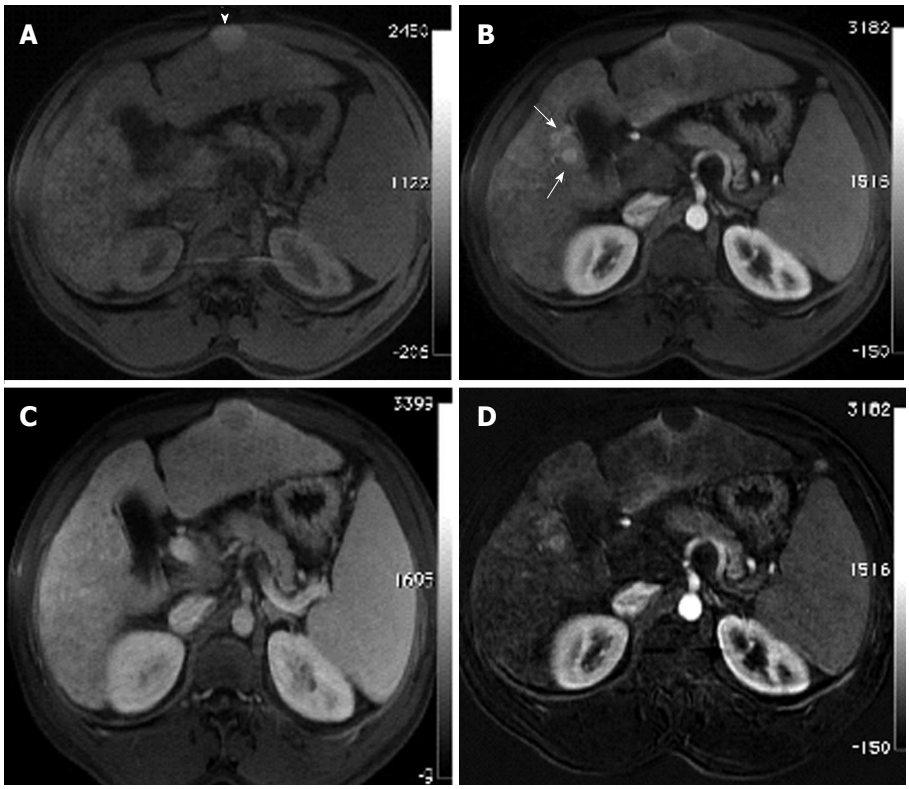

Figure 1 Incidentally discovered solitary left hepatic lobe nodule on screening ultrasound, referred as suspicious nodule for HCC, for MRI evaluation. A: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) late hepatic arterial and (C) delayed phases; D: Post-processed subtracted arterial image. The known left hepatic lobe nodule demonstrate increased intrinsic T1 signal (arrowhead, A), without appreciable increased arterial enhancement (B), confirmed on subtraction images (D), in keeping with a macro-regenerative nodule. However, the MRI reveals multiple small foci at hepatic segment #5 that show increased arterial enhancement (arrows, B) and delayed washout (D) in keeping with multiple small HCCs. HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; GRE: Gradient recalled echo.

HCCs less than 2 cm showed arterial hypervascularity regardless of washout[24].

MRI provides multi-parametric data on anatomical abnormality with both T1- and T2-weighted sequences, and provides functional sequences such as diffusion-weighted images (DWI) and contrast uptake with the use of liver-specific hepatobiliary contrast agents, providing cellular information of the hepatocellular nodules that can improve lesion detection and characterization. Table 1 shows a summary of a wide-spectrum of lesions in cirrhotic liver and their imaging appearances on MRI.

In this article, we provide an overview of the basic MRI techniques used for assessment of cirrhotic nodules. We also shed some light on liver imaging reporting and data system (Li-RADS), and the role of different MRI contrast agents and future MR imaging techniques including the use of advanced MR pulse sequences and utilization of hepatocyte-specific MRI contrast agents, and how they might contribute to improving the diagnostic performance of MRI in early stage HCC diagnosis.

PROTOCOL

An adequate imaging protocol has to be standardized to allow repeatability and consistency. The standard imaging techniques are based on dynamic fat-suppressed post-contrast T1-weighted 3D GRE sequences, combined with fat-suppressed and non fat-suppressed T2-weighted sequences. T2-weighted images are usually acquired with single-shot fast spin-echo (SSFSE) technique due to its robustness to motion. Chemical shift imaging is acquired with breath-hold dual-echo spoiled GRE. Additional sequences may be added to the protocol (see below).

Since detection of HCC relies on dynamic fat-suppressed post-contrast T1-weighted 3D GRE sequences and proper timing of the arterial phase is critical for optimizing sensitivity for HCC detection (Figure 2). We routinely use real time bolus-triggering method in order to consistently achieve adequate arterial phase images. An optimal arterial phase is recognized when contrast is present in the portal veins and absent in the hepatic veins; referred to as late-hepatic arterial phase or hepatic-arterial dominant phase. Post-processed subtraction arterial imaging may be utilized and may carry an additional value for detecting subtle early enhancement; which can be observed in cases of nodules with increased intrinsic signal on T1-weighted images, nodules with microscopic fat (Figure 3), or small lesions in a

Table 1 Summary of a wide-spectrum of lesions in cirrhotic liver and their imaging appearances on magnetic resonance imaging

Imaging sequences	RNs	Siderotic nodules (RNs or LGDNs)	LGDNs[1]	AP Shunts[2]	HGDNs	HCCs
T1-weighted images	Iso- or hyperintense[3]	Hypointense[4]	Iso- or hyperintense	Isointense	Iso- or slightly hyperintense	Ranging from hypo- to hyperintense
T2-weighted images	Iso- or hypointense	Hypointense	Iso- or hypointense	Isointense	Isointense	Iso- to mildly hyperintense
DWI	Isointense	Hypointense	Iso- or hypointense	Isointense	Isointense	Iso- to hyperintense[5]
Post-Gadolinium Dynamic images (arterial and delayed images)	Iso-enhancement on the hepatic arterial phase, and no delayed washout	Iso-enhancement on the hepatic arterial phase, and no delayed washout	Iso-enhancement on the hepatic arterial phase, and no delayed washout	Hyper-enhancement on the arterial phase, and no show delayed washout[6]	Usually hyper-enhancement on the arterial phase and can be mistaken for HCC. These nodules do not show delayed washout	Usually hyper-enhancement on the arterial phase and delayed washout (hypointense), with or without pseudocapsule enhancement[7]
Hepatobiliary phase images	Iso- to slightly hyperintense	Hypointense	Iso- to slightly hyperintense	Isointense	Isointense	Hypointense[8]

[1]RNs and LGDNs tend to be indistinguishable on MRI; [2]AP shunts might be indistinguishable from HGNDs and small, early HCCs. Relying on additional features and short-term follow-up can help in making this distinction; [3]The exact cause for this hyperintensity is believed to be due to the presence of binding proteins; [4]These nodules show lower signal intensity on longer TE T1-weighted GRE sequences, due to susceptibility artifact; [5]Usually hyperintense, especially if > 2 cm; [6]AP shuns are usually easy to differentiate from other hypervascular lesions when they show a triangular or linear configuration. When AP shunts show round configuration, they can be indistinguishable from HGDNs and HCCs; [7]HCCs ≤ 1.5 cm are frequently isointense on T1- and T2-weighted images and are detected only on the arterial phase. Early stage HCC, especially tumors ≤ 2 cm, may also appear isointense, or less likely, hypointense on the arterial phase; [8]Some HCCs may appear isointense or hyperintense on the hepatobiliary phase; especially well-differentiated and moderately-differentiated HCCs. LGDNs: Low-grade dysplastic nodules; AP: Arterio-portal; HGDNs: High-grade dysplastic nodules; HCCs: Hepatocellular carcinomas; RNs: Regenerative nodules; DWI: Diffusion weighted images.

background of heterogeneous background hepatic parenchymal enhancement.

HEPATOCARCINOGENESIS

In cirrhotic liver the stepwise development of cancer from areas of regeneration to overt development of HCC is called "multistep hepatocarcinogenesis" and is the widely accepted main mechanism of hepatocarcinogenesis. *De novo* hepatocarcinogenesis also is presumed to occur as an alternative pathway. Even in such cases, later progression to overt HCC takes place in a multistep fashion[25]. Accepted imaging diagnosis of HCC is primarily based on sequential changes in the intra-nodular blood supply during hepatocarcinogenesis; regenerative nodules (RN) show similar blood supply to normal liver, borderline lesions such as dysplastic nodules (DN) or early HCCs show wide variations of blood supply, and advanced HCCs are supplied by abnormal arteries alone[25,26].

High-grade DN is a lesion with strong malignant potential, being recognized as a precursor of HCC. DN and early HCCs are recognized as lesions in the "gray zone"[27] as although usually being hypervascular they tend to show no washout on late phases, hindering the diagnosis[24].

Another source of HCC misinterpretation is on iso-enhancement of HCC on arterial phase images due to the iso or hypovascularity of the lesion[28] (Figure 4). Additionally, misdiagnosed HCCs on MRI may be due to poor patient compliance, especially from the inability to suspend respiration, which is becoming less of a problem with the new advancement in developing faster and motion robust sequences.

MRI FEATURES OF CIRRHOTIC NODULES

Dominant nodules are frequently identified during an imaging surveillance program in patients with liver cirrhosis. The change in vascularity observed in hepatic nodules during the multistep hepatocarcinogenesis correlates with the development of malignancy and determines their distinguishing imaging characteristics.

RN

RNs consist of proliferating normal liver cells surrounded by a fibrous stroma[29]. A RN is described as containing one or more portal tracts located in a liver that is abnormal whether because of cirrhosis or other disease[30]. The blood supply of a RN continues to be largely from the portal vein, with minimal contribution from the hepatic artery[31]. This explains why there is no hyper-enhancement on the hepatic arterial phase on MR images. Because of their histopathological nature, as described above, RNs are often indistinct on T1- and T2-weighted images. However, they can have higher T1 signal intensity compared to background liver tissue. The explanation for this increase in signal is not exactly known; it has been proposed to be due to the presence of metal-binding proteins, proteins *per se*, or lipid[32,33] (Figure 5). RNs may occasionally contain iron (siderotic nodules), which will show decreased T1- and T2 signal intensities due to susceptibility effects[34].

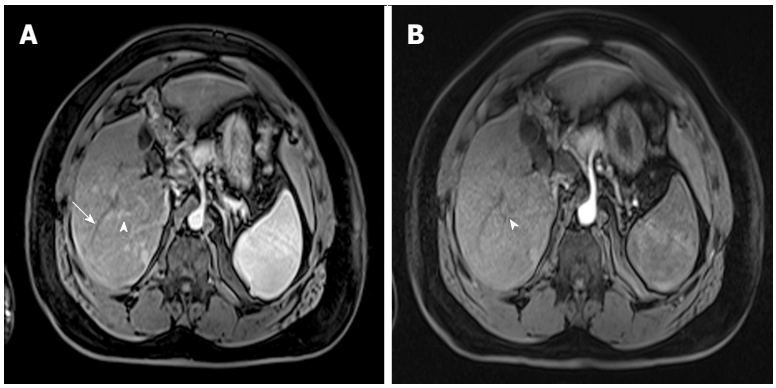

Figure 2 Value of proper timing for detecting hypervascular hepatic lesions. A-B: Post-contrast fat-suppressed 3D-GRE T1-weighted images acquired 4 mo apart. A: Initial scanning shows contrast in the portal vain branches (arrowhead, A), without opacification of the hepatic veins (arrow, A), suggesting late hepatic arterial phase timing; the optimal time for detecting hypervascular pathologies, with demonstration of multiple lesions; B: A subsequent scan acquired 4 mo later shows contrast in the hepatic artery without opacification of the portal vein branches (arrowhead, B), suggesting an early arterial timing, without evidence of hypervascular lesions. A subsequent scan was acquired (not shown); which confirmed the persistence of these hypervascular lesions. GRE: Gradient recalled echo.

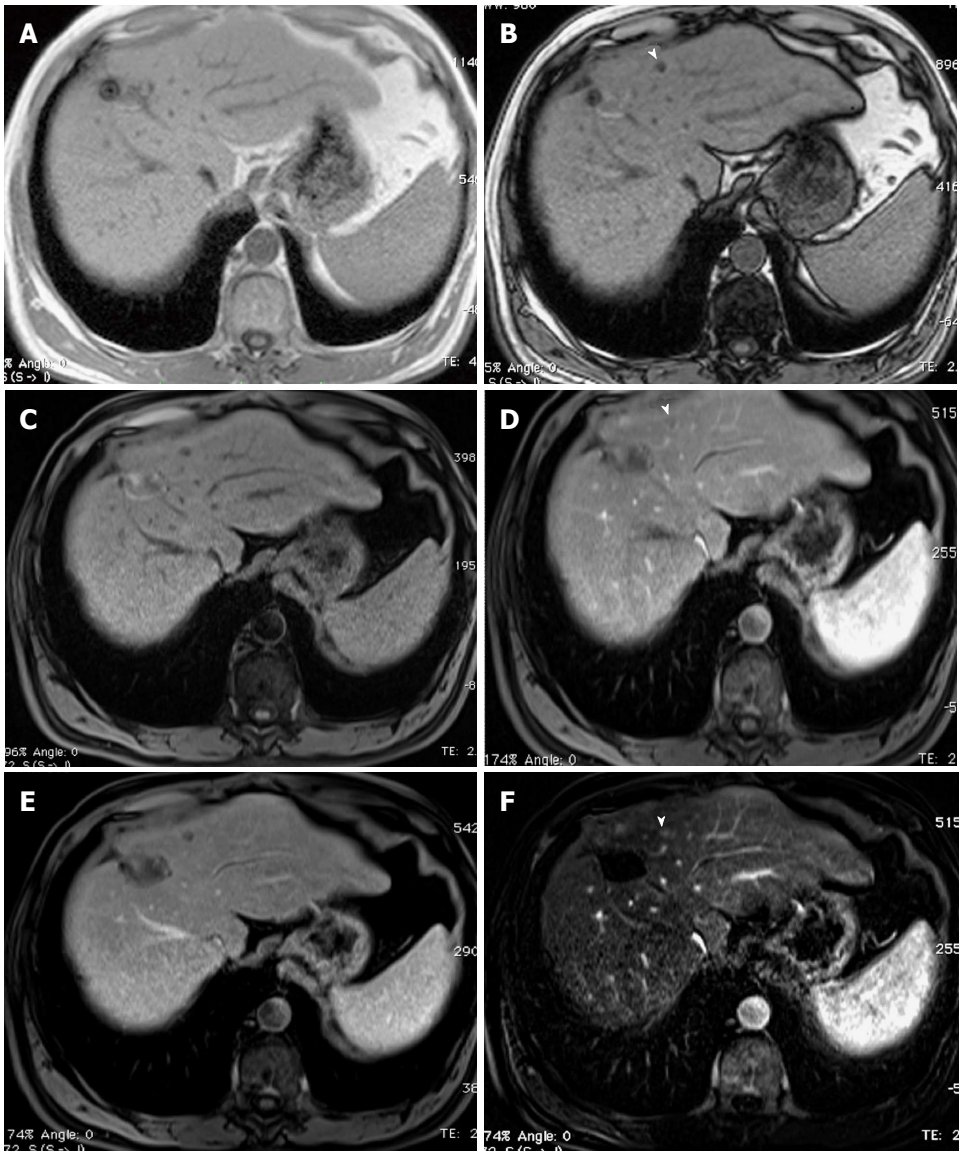

Figure 3 Small fat-containing hepatocellular carcinoma; the value of subtraction images. A: In-phase; B: Opposed-phase GRE T1 weighted images; C-E: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (D) late hepatic arterial and (E) delayed phases; F: Post-processed subtractions arterial phase image. There is a small left hepatic nodule, which demonstrates drop of signal intensity on opposed-phase (arrowhead, B) and pre-contrast images (C) compared to the in-phase images (A), suggesting the presence of fat, with possible minimal increased arterial enhancement (arrowhead, D), confirmed on subtraction images (arrowhead, F), and washout on delayed images (E) in keeping with a small fat-containing hepatocellular carcinoma. GRE: Gradient recalled echo.

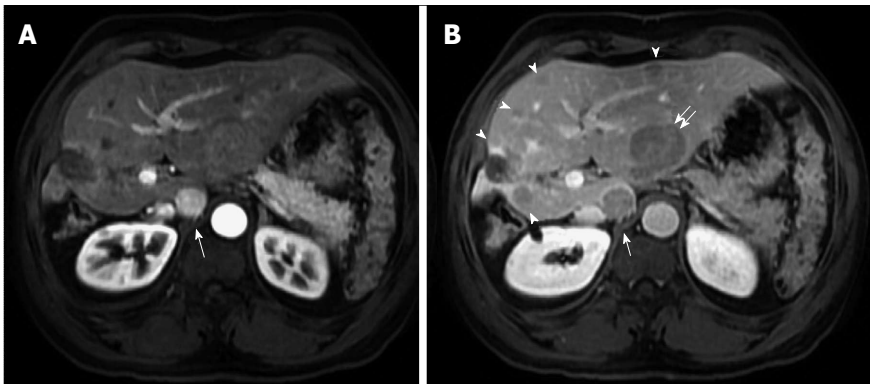

Figure 4 Hypervascular and non-hypervascular hepatocellular carcinomas. Post contrast fat-suppressed 3D-GRE T1-weighted images during the (A) late hepatic arterial and (B) delayed phases. There is a focal hepatic lesion medial to the inferior vena cava, which demonstrates intensely increased arterial enhancement (arrow, A) and washout on delayed images (arrow, B) in keeping with a hypervascular HCC. Additionally, there are multiple foci of delayed washout throughout the liver (arrowheads, B), the largest of which is seen at the left hepatic lobe (double-arrow, B), with variable degrees of arterial enhancement, in keeping with multiple hypo- and iso- vascular HCCs. Of note are the hypertrophic changes of the left hepatic lobe as well as atrophic and post-interventional changes of the right hepatic lobe. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo.

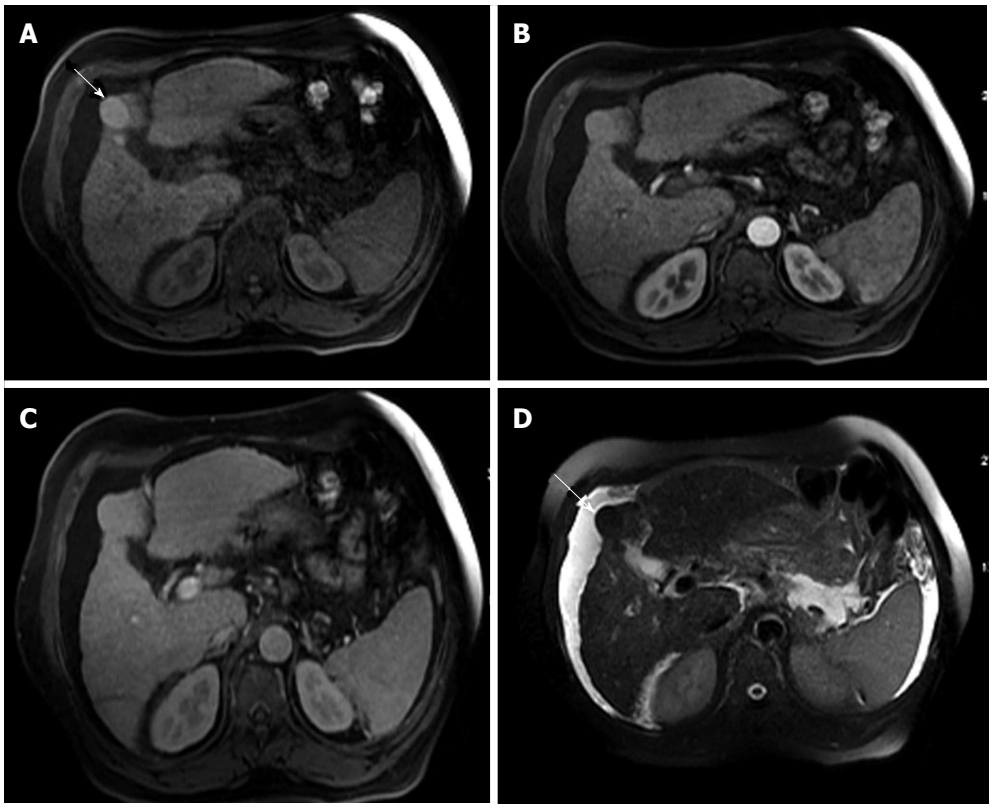

Figure 5 Dominant regenerative hepatic nodule. A-C: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) late hepatic arterial and (C) poral venous phases; D: Fat-suppressed SSFSE T2-weighted image. There is a subcapsular, partially exophytic nodule at hepatic segment #5, which demonstrates increased intrinsic T1 signal on pre-contrast images (arrow, A) and isosignal intensity to background liver parenchyma on post-contrast images (C), without appreciable increased arterial enhancement (B) or increased T2 signal intensity (arrow, D) in keeping with a dominant regenerative nodule. GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

Chemical shift imaging aids in the characterization of hyperintense T1-weighted nodules. Fatty nodules show drop of signal intensity on the opposed-phase T1-weighted sequence, due to destruction of the magnitude vector within the same voxel, exerted by fat and water molecules having opposite directions and resulting in decreased signal intensity; indicative of intracellular (microscopic fat).

Chemical shift imaging aids also in the diagnosis of siderotic nodules, showing drop of signal on the sequence with the longer echo-time (TE), which could be during the in-phase or opposed phase, depending on the MR machine used for imaging and its field strength, due to susceptibility effects resulting from proton de-phasing exerted by the presence of iron (Figure 6).

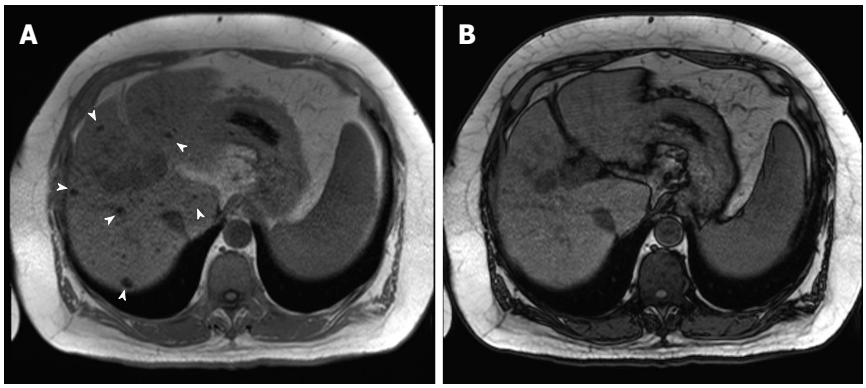

Figure 6 Multiple siderotic hepatic nodules. In-phase (TE = 4.9 ms) (A) and opposed-phase (TE = 2.4 ms) (B) GRE T1 weighted images. There are multiple small nodules seen through out the liver, which demonstrate isosignal intensity on the in-phase images (arrowheads, A), without corresponding abnormalities on the opposed-phase images in keeping with Multiple siderotic hepatic nodules. GRE: Gradient recalled echo.

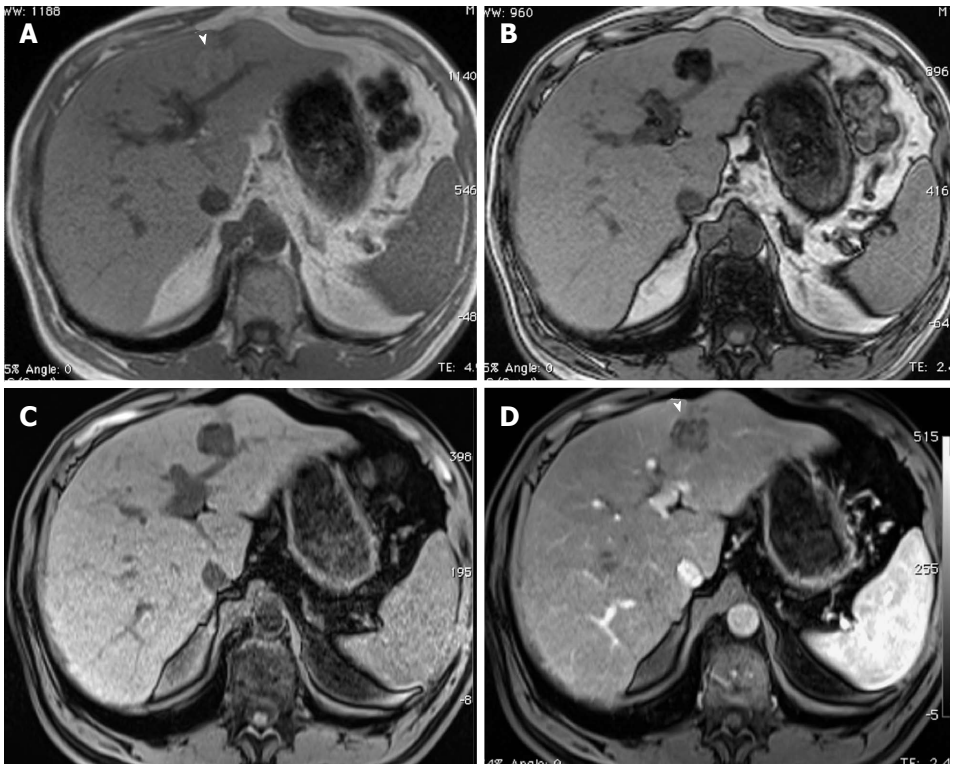

Figure 7 Large, fat-containing hepatocellular carcinoma. In-phase (A) and opposed-phase (B) GRE T1 weighted images. Pre- (C) and post-contrast fat-suppressed 3D-GRE T1-weighted images during late hepatic arterial phase (D). There is a prominent left hepatic nodule, which demonstrates minimally increased intrinsic T1 signal on the in-phase images (arrowhead, A) and low signal intensity on the opposed-phase (B) and pre-contrast images (C), indicating the presence of fat. The lesion demonstrates heterogeneous mildly increased arterial enhancement (arrowhead, D) in keeping with a fat-containing HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo.

Several studies have shown that nodules with high signal intensity on T1-weighted images are in most cases benign. In younger patients with numerous macro-nodules, almost all of these lesions follow a benign course[35]. In patients with cirrhosis, small hyperintense hepatic lesions on T1-weighted images without hyper-enhancement on the arterial-phase images usually show no interval growth or disappear during serial imaging[36]. Regardless of their intrinsic signal features, a reliable finding of RNs is the absence of enhancement on the arterial phase, compared with the background hepatic parenchyma.

A notable exception are fat-containing, large size (> 1.5 cm) nodules (hyperintense on T1-weighted in-phase images with drop of signal on the opposed-phase T1-weighted images), which strongly suggest malignancy (Figure 7). Otherwise, the presence of numerous nodules < 1 cm suggests benignity[37].

DNs

DNs are defined as regenerative nodules containing atypical cells with nuclear crowding and architectural derangement and a variable number of unpaired arterioles or capillaries without definite histologic

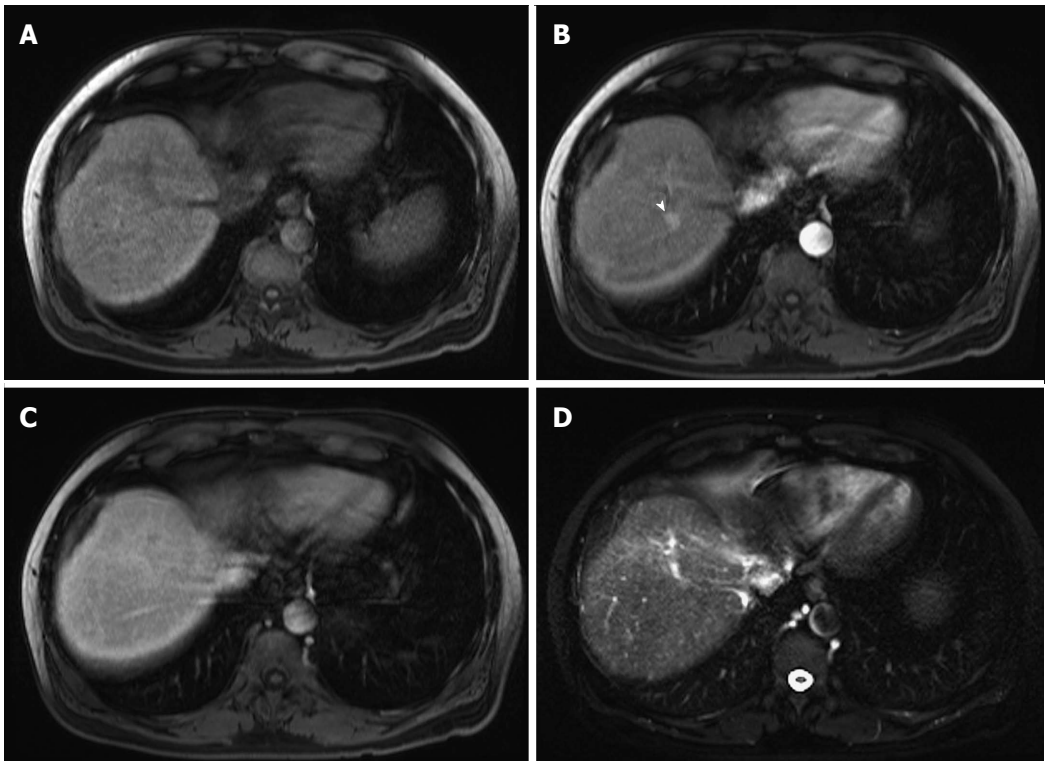

Figure 8 High-grade dysplastic nodules. Pre- (A) and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) Late hepatic arterial; and (C) Delayed phases; (D) Fat-suppressed SSFSE T2-weighted image. There is a nodule at hepatic segment #7, which demonstrates iso T1 signal intensity (A), increased arterial enhancement (arrowhead, B), without definite washout or corresponding T2 signal abnormality in keeping with a HGDN. However, early HCC cannot be totally excluded and short-term follow-up should be recommended, especially in patients with hepatitis C infection. HGDN: High-grade dysplastic nodules; HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

signs of malignancy[38]. High-grade dysplastic nodules (HGDNs) display at least moderate atypia and occasional mitosis[30]. DNs primarily display T1 and T2 isointensity to the background liver parenchyma, but T1 hyperintensity is also possible as described above with RNs[39]. Low-grade dysplastic nodules (LGDNs) primarily display enhancement characteristics similar to that of the background liver parenchyma on all dynamic phases; because they remain mainly supplied by the portal circulation. LGDNs are not considered premalignant lesions. As lesions progress, their blood supply becomes more arterialized, giving the typical hypervascular features of HCC[40]. Unfortunately, the portal and arterial supply to LGDNs and HGDNs is variable and inconsistent[39]. They may even be associated with increased alpha-fetoprotein despite not being malignant[41].

HGDNs are considered premalignant lesions[30] and tend to show intense early enhancement after gadolinium injection and fade to isointensity[42], without washout (Figure 8), because supply from the portal venous system remains comparable with the background liver[43,44].

The development of HCC within a DN has been reported within as short as 4 mo[45]. Usually it is seen as an increase in size and development of washout on delayed imaging, allowing definite diagnosis of HCC (Figure 9). Early studies have also reported DNs with "a nodule within a nodule" appearance. This classic MR description is a focus of increased T2 signal intensity within a T2 low-signal-intensity nodule, which may or may not demonstrate arterial hyper-enhancement on dynamic MR images[46].

According to the latest guidelines from the EASL and AASLD practice guidelines, DNs should not be treated or managed as cancers[47]. In our clinical practice, we advise more frequent surveillance imaging (usually 3 mo) as there is an increased risk of progression to HCC.

ARTERIOPORTAL SHUNTS

Arterioportal (AP) shunts usually demonstrate enhancement on the arterial phase and mostly fade back to isointensity on the portal venous or delayed images (Figure 10). They are sometimes easily distinguished from HGDNs/early HCCs by their subcapsular location and wedge- or comma shaped configuration. However, they may sometimes become main mimickers of HGDN/early HCCs; posing as a potential differential diagnosis when they are round or oval in configuration[48].

HCC

The EASL and AASLD have proposed and validated

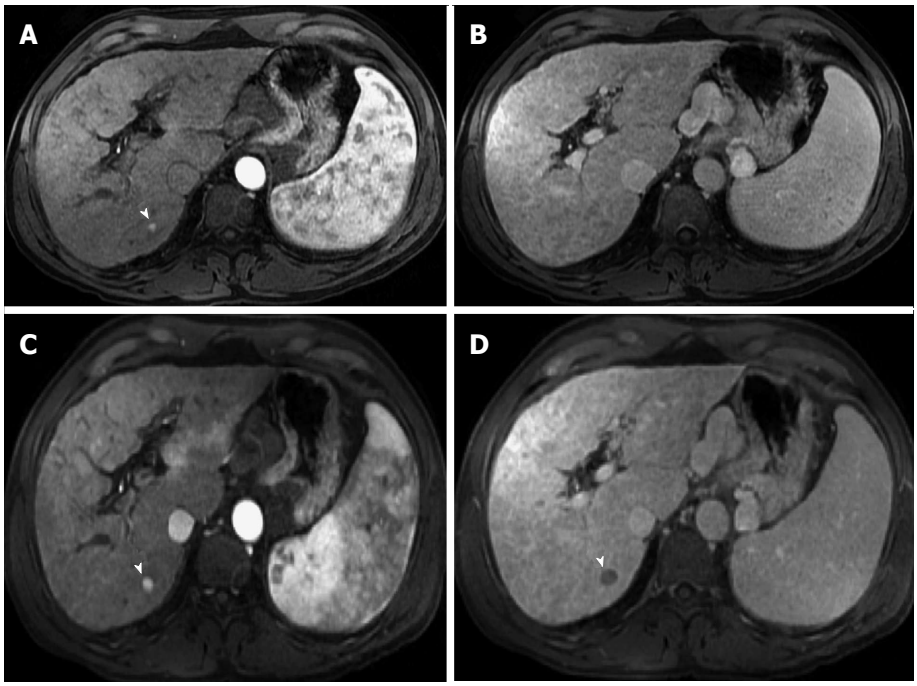

Figure 9 Dysplastic nodule progressing into an hepatocellular carcinoma. Post-contrast fat-suppressed 3D-GRE T1-weighted images during the late hepatic arterial (A and C) and delayed phases (B and D). There is a small right hepatic lobe nodule, which demonstrates increased arterial enhancement (arrowhead, A) and fades out on the delayed images (B) on the initial examination. On the 4-month follow-up study, there is evidence of interval growth (arrowhead, C, D) and development of clear delayed washout (arrowhead, D) both of which are signs of progression into HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo.

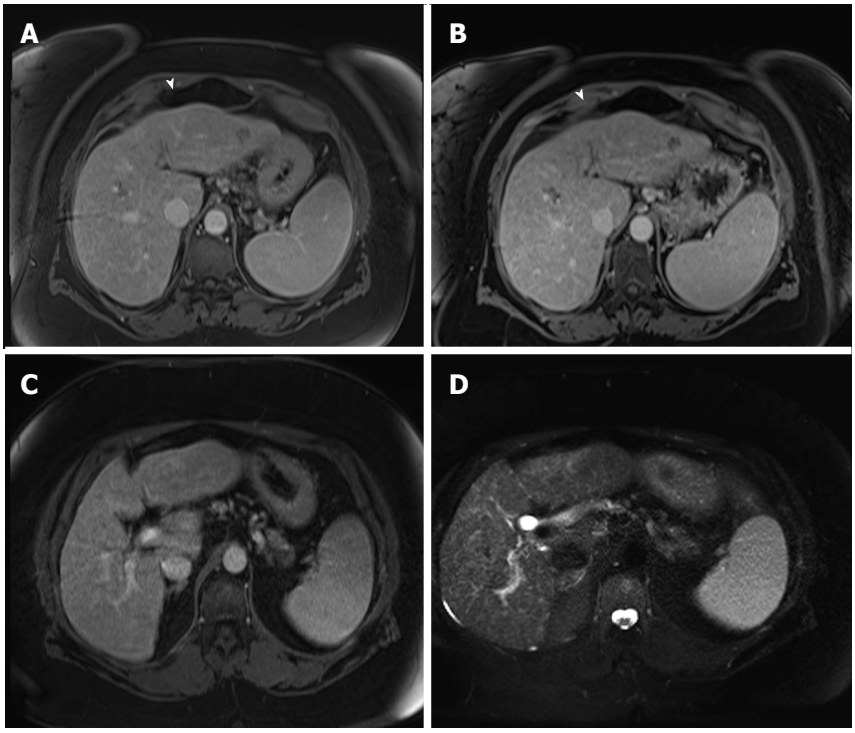

Figure 10 Arterioportal shunt. Post-contrast fat-suppressed 3D-GRE T1-weighted images during the late hepatic arterial (A and B) and delayed phases (C); D: Fat-suppressed SSFSE T2-weighted image. There is a convoluted linear area of increased arterial enhancement with a vessel leading to it (arrowhead, A, B), which does not demonstrate delayed washout (C), or corresponding T2 signal abnormality in keeping with an AP shunt. SSFSE: Single-shot fast spin-echo; GRE: Gradient recalled echo.

imaging criteria for the diagnosis of HCC in cirrhotic patients, which correspond to the typical HCC features including arterial hyper-enhancement and delayed washout[49] (Figure 11). HCCs may show a variety of MR imaging features; reflective of the variable characteristics of the tumor's architecture, grading,

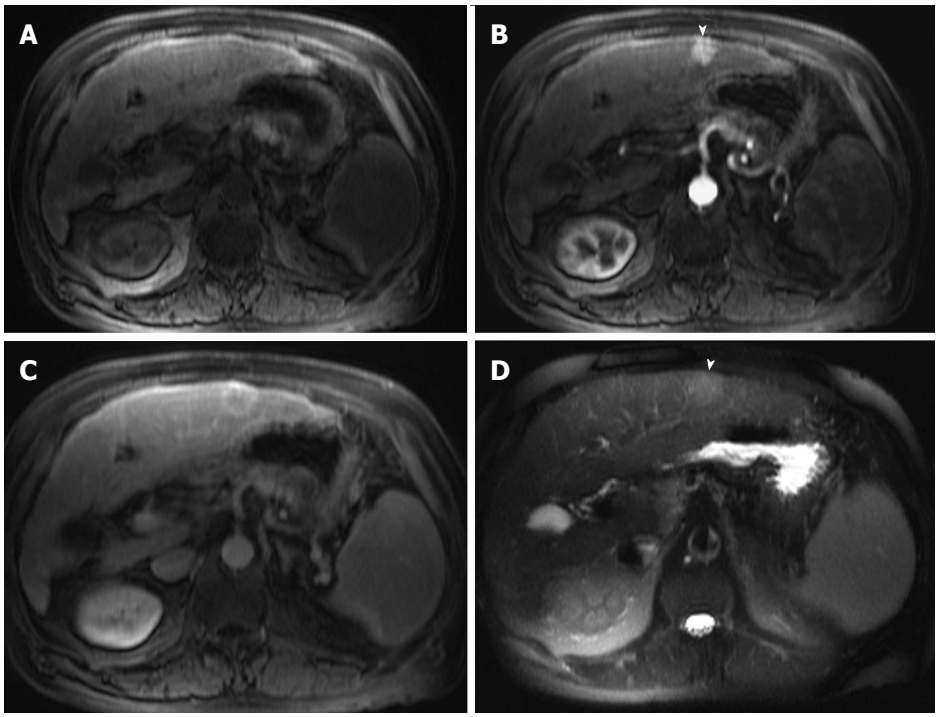

Figure 11 Classical hepatocellular carcinoma. A: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the late hepatic arterial (B) and delayed phases (C); D: Fat-suppressed SSFSE T2-weighted image. There is a peripheral well defined left hepatic lobe nodule, which demonstrates iso T1 signal intensity (A), increased arterial enhancement (arrowhead, B), delayed washout and pseudocapsule enhancement (C), and mildly increased T2 signal (arrowhead, D) in keeping with classical features of HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

stromal components, and intracellular content[22].

Arterial hyper-enhancement is the most common and important imaging finding in the diagnosis of HCC[50]. While considered a reliable feature, it can be seen in HGDNs and AP shunts. Arterial hyper-enhancement can also be seen in a variety of benign and malignant hepatic lesions, including hemangiomas and focal nodular hyperplasia and hypervascular metastases. However, these liver lesions are infrequent in the setting of hepatic cirrhosis[51].

Because arterial hyper-enhancement can be observed with other lesions and nodules, additional imaging criteria are needed to decrease the false-positive rate and increase sensitivity, while maintaining high specificity for the diagnosis of HCC[49]. Therefore, delayed washout, among other secondary features, is used for this purpose.

The key distinguishing feature of HCC is the development of delayed "washout"; defined as arterially enhancing nodules becoming hypointense compared to the background liver on the delayed phase imaging (not to be confused with "fade out", which is defined as arterially enhancing nodules becoming isointense to background liver on delayed phase imaging).

HCCs greater than 2 cm in size tend to show washout[52,53], which explains the high diagnostic sensitivity for tumors this size. However, for HCCs smaller than 2 cm the sensitivity is lower. This is not due to hypovascular HCCs, which are uncommon, but rather to hypervascular HCCs that do not show washout on delayed images[24,49,54] (Figure 12). In one series of 60 HCCs, smaller than 2 cm, 85% of these lesions were hypervascular, and only 61.7% of which showed washout[24]. Similarly, in another series, 51 out of 131 HCCs showed arterial hyper-enhancement without clear wash-out on delayed images[54].

Delayed pseudo-capsule enhancement of hepatic nodules aids in the diagnosis of HCC, and can be helpful in lesions that do not show classical features of HCC on dynamic imaging (Figure 13).

Since it is extremely difficult to perform biopsy of small nodules that are only visible on arterial phase images, we usually prefer close follow-up. Generally we advocate that lesions measuring 1-2 cm are re-imaged at a 3-mo interval to assess for lesion interval growth or development of washout. The lack of interval growth on short-term follow-ups does not exclude the possibility of malignancy, as HCC may demonstrate slow growth. Therefore, only nodules that are stable for 2 years are considered benign[14]. However, it is worth emphasizing the value of direct comparison and lesion measurement between both the current and older prior examination to demonstrate undetected subtle changes in size on short-term followups; which is indicative of slow growth, a feature of early well-differentiated HCC (Figure 14).

MORPHOLOGIC HCC SUB-TYPES

HCCs can manifest as different morphologic types including focal (nodular), massive, and diffuse/

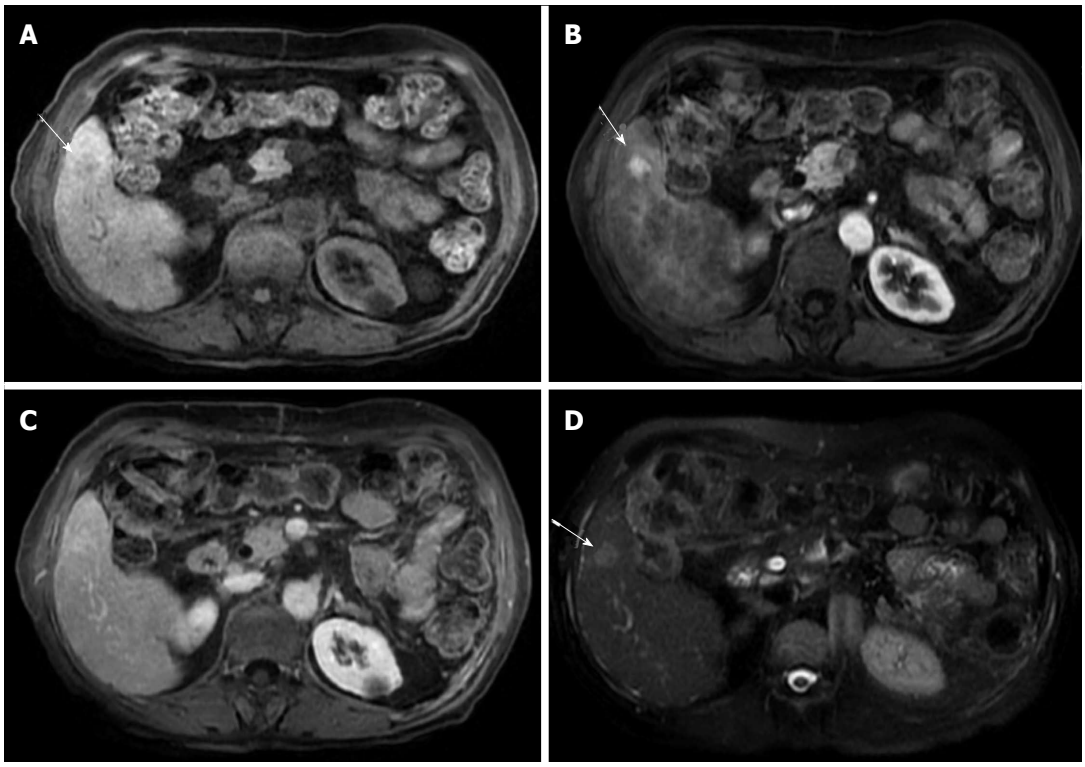

Figure 12 Small, non-washing-out hepatocellular carcinoma. A: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the late hepatic arterial (B) and delayed phases (C). D: Fat-suppressed SSFSE T2-weighted image. There is a small nodule at hepatic segment #5, which demonstrates minimally decreased T1 signal on pre-contrast images (arrow, A) and increased arterial enhancement (arrow, B). The nodule demonstrates iso to slightly increased signal on the delayed phase images, without clear washout (C), but mildly increased T2 signal intensity (arrow, D) in keeping with an HCC. Note that T2 signal alteration increased the accuracy of diagnosing HCC in this patient, despite the lack of delayed washout (also see Figure 17). HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

infiltrative[55,56].

Nodular type is the most common encountered type and usually presents as encapsulated focal nodule with well-defined margins. Nodular type can be further classified as solitary or multi-focal.

Massive tumors are well-defined tumors large enough to often render these patients non-eligible for loco-regional ablative therapies or hepatic transplantation.

Multi-focal nodular subtype is an advanced type and shows similar features to solitary nodular subtype on conventional and dynamic MRI. Additional features that are not commonly seen with solitary focal lesions, but are noted with multi-focal HCC and other aggressive subtypes include portal venous thrombosis and in intrahepatic metastases[26].

Diffuse HCCs are usually large and have ill-defined boundaries without clear demarcation. They usually present with very high alpha-fetoprotein levels and are almost always associated with portal venous thrombus; which can be bland or most of the time tumoral in nature; based on the presence of neovascularity on the arterial imaging. Diffuse HCCs can be extremely subtle, and therefore difficult to demonstrate by imaging alone as they can blend with the background cirrhotic parenchyma; preventing early diagnosis and leading to advanced disease at presentation with often distant metastatic disease.

One study by Kneuertz *et al*[56] evaluated 147 patients with advanced HCCs (75 with infiltrative disease and 72 patients with multi-focal disease). In that study, failure to display a discrete mass was observed in 42.7% of patients, low signal on T1-weighted images was observed in 55.7%, high signal on T2- weighted images was observed in 80.3% of patients. They also demonstrated mild miliary pattern of enhancement on arterial phase imaging in 16.4% of patients, with delayed washout in 50.8%.

Diffuse HCCs can be difficult to differentiate from areas of confluent fibrosis on CT. However, the combined additive advantage of T2-weighted imaging, DWI, and delayed imaging can be used to enhance the diagnostic accuracy of diagnosis on MRI, which display more distinct lobulated margins, with poorly defined amorphous infiltration surrounding thrombosed portal veins, and clearly depict internal reticulation throughout the tumor[57,58].

Additionally, post-contrast delayed imaging demonstrates heterogeneous washout[59] , allowing differentiation between confluent fibrosis as this shows increase enhancement over time Another distinctive feature from confluent fibrosis is the presence of regional tumor thrombus that is almost invariably present in patients with diffuse HCC[57,60,61] (Figure 15).

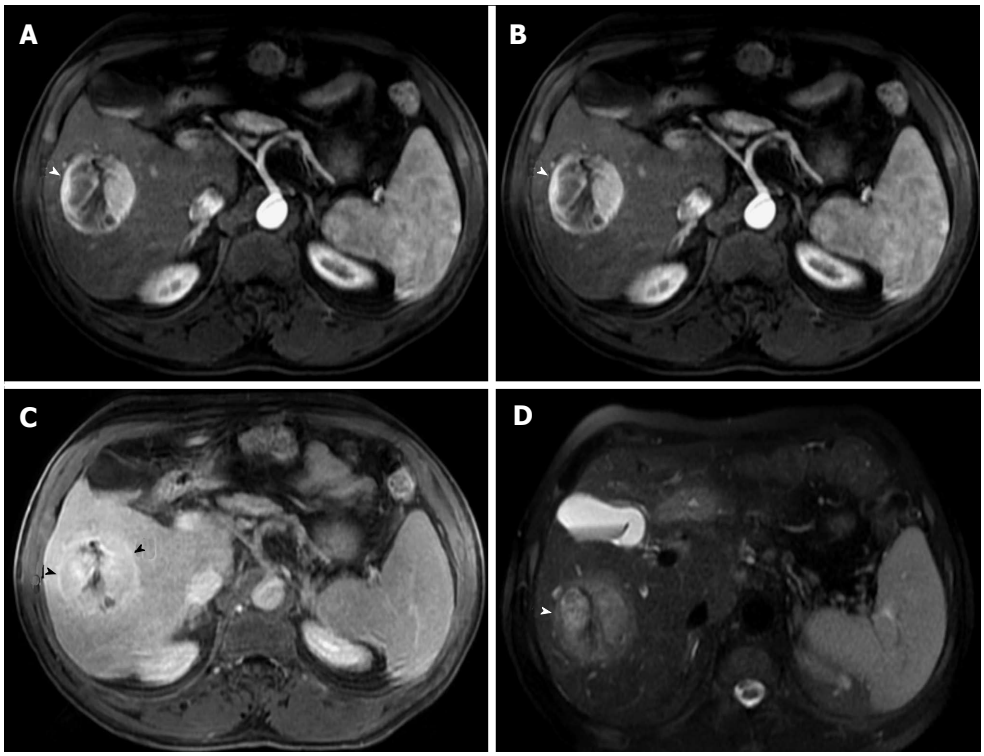

Figure 13 Large hepatocellular carcinoma with delayed pseudocapsule enhancement but no intralesional washout. Post-contrast fat-suppressed 3D-GRE T1-weighted images during the (A) late hepatic arterial, (B) poral venous, and (C) delayed phases; D: Fat-suppressed SSFSE T2-weighted image. There is a large right hepatic lobe mass, which demonstrates heterogeneous, intensely increased arterial enhancement (arrowhead, A) with progressive fading throughout the subsequent images (B), but no clear washout (C). The lesion demonstrates delayed pseudocapsular enhancement (black arrowheads, C) and mildly increased T2 signal intensity (arrowhead, D) in keeping with a large HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

Multiple small satellite nodules associated with the main tumor or multiple small recurrent tumors of moderate or poor differentiation are regarded as intrahepatic metastases[62]. The clinical significance about intrahepatic metastases is that they require immediate curative or palliative interventions even when smaller than 1 cm; as such lesions are likely to display aggressive behavior, unlike single or multicentric primary tumors of the same size[63].

A rare variant of nodular morphologic subtype is lesions with rim-enhancement on arterial imaging on initial MRI (Figure 16) has been described in the literature[64], suggesting a more progressive behavior with rapid interval growth and disease worsening; therefore, requiring prompt therapy and short-term follow-up.

FUTURE DIRECTIONS

T2-weighted imaging

The appearance of HCC on T2-weighted images is variable. Early reports suggested that HCC displayed high or equivalent signal intensity compared to the liver parenchyma on T2-weighted images[65,66].

Other researchers reported that both non-enhanced T1- and T2-weighted sequences may contribute in the characterization of cirrhotic nodules; however, minimally increasing the detection rate[67].

More recent studies have shown that the addition of T2-weighted imaging to gadolinium-enhanced T1-weighted 3D-GRE dynamic imaging improves the diagnostic performance of MRI in the detection of HCC compared to dynamic MR imaging alone. This is especially true for lesions smaller than 1 or 2 cm (Figure 12), which may show hypervascularity but might not display any washout, distinguishing them from HGDNs[54,68-70] (Figure 17).

HCCs tend to show minimal to mildly increased signal intensity on T2-weighted images, as opposed to intra-hepatic cholangiocarcinoma or mixed HCC-cholangiocarcinoma; both of which are increasingly being reported in patients with cirrhosis, and tend to show moderately increased signal intensity on T2-weighted images with evidence of increased vascularity on arterial phase imaging and progressive contrast enhancement throughout subsequence phases. Such distinction is clinically important as those lesions are associated with a poor prognosis and a high rate of tumor recurrence after liver transplantation, and have higher risk of nodal and distant metastatic disease[71].

T2-weighted imaging is also helpful in the detection of lymphadenopathy in patients with focal hepatic lesions[70].

Diffusion-weighted imaging

The possibility of performing functional imaging sequences is an additional advantage of MRI over CT[72]. With technological advances in hardware and

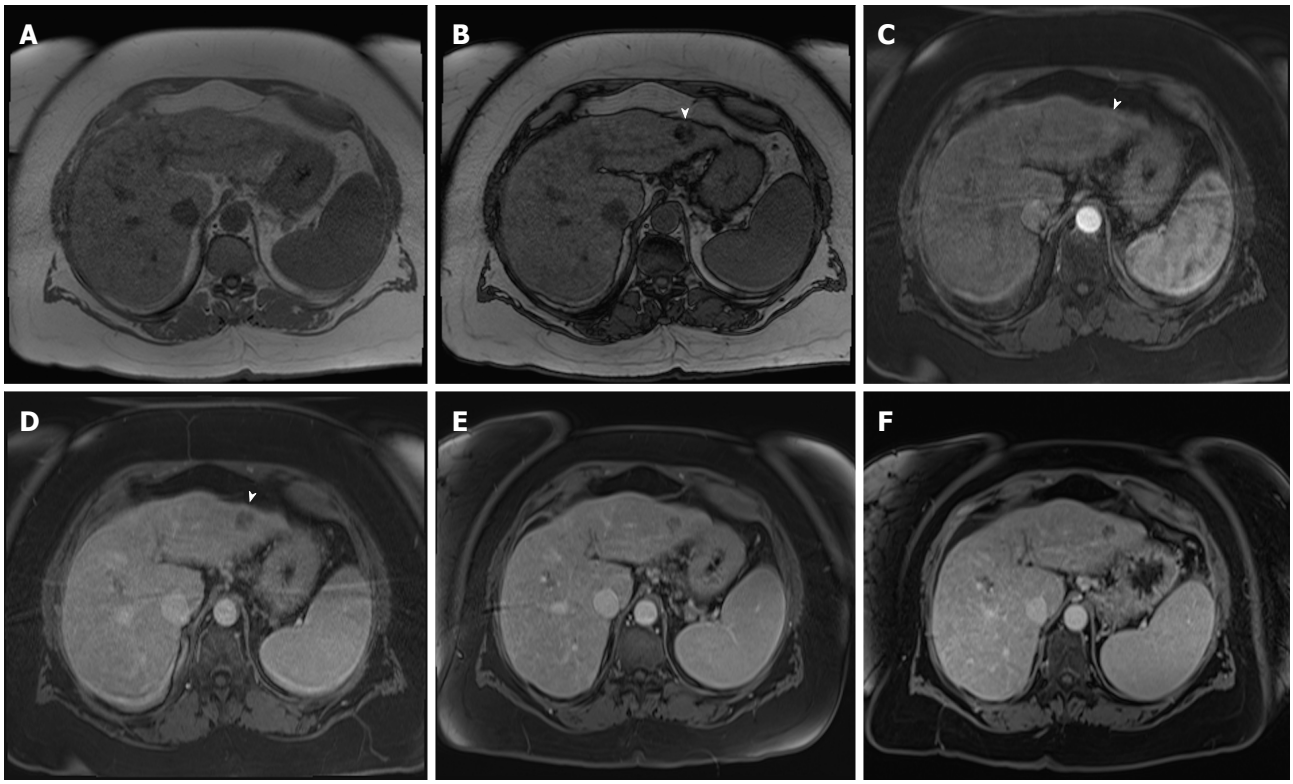

Figure 14 Slow growing, well-differentiated, fat-containing, small hepatocellular carcinoma. In-phase (A) and opposed-phase GRE T1 weighted images (B); Post-contrast fat-suppressed 3D-GRE T1-weighted images during the late hepatic (C) arterial and delayed phases (D). E, F: Post-contrast fat-suppressed 3D-GRE T1-weighted delayed images from prior examinations performed 1 and 2 years prior, respectively. The is a small left hepatic lobe nodule, which demonstrates drop of signal on the opposed-phase image (arrowhead, B) compared to the in-phase image (A), increased arterial enhancement (arrowhead, C), and delayed washout (arrowhead,D). The lesion does not demonstrate significant change in size from the immediate prior examination (E). However, when compared with a more remote examination (F), substantial interval growth can be appreciated consistent with a slow growing, well-differentiated, fat-containing, small HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo.

software, DWI can be readily applied to liver imaging with improved image quality. DWI is an imaging technique based on differences in the Brownian motion (diffusibility) of water molecules within tissues. In highly cellular tissues such as tumors, the diffusion of water protons is restricted. Therefore, both qualitative and quantitative variables reflect tissue cellularity and cellular membrane integrity[49,73-75]. DWI is useful for detecting small focal liver lesions in general[49,73-75].

A limited number of small studies have shown encouraging results suggesting that DWI has a good diagnostic performance in the detection of HCC in patients with chronic liver disease and equivalent to conventional contrast-enhanced for lesions greater than 2 cm in size[49,76]. Currently, the limitation of DWI is primary lesion characterization rather than lesion detection[49,76].

The greatest benefit relies on the combined use of DWI with conventional dynamic MRI; providing higher sensitivities than dynamic MRI alone in the detection of small HCC lesions in patients with chronic liver disease[77,78] (Figure 18). Therefore, an additional acquisition of DWI is being implemented in abdominal protocols[77].

In a recent study a new MRI criteria was proposed, combining the features of lesions after gadolinium-based contrast agents administration and hyperintensity on DWI[49]. This significantly increased the sensitivity for the diagnosis of HCC compared to conventional hemodynamic criteria, irrespective of tumor size. However, further larger prospective studies are still needed to establish its definitive role for detecting HCC in patients with chronic liver diseases.

T2-weighted imaging*

The performance of liver MRI is highly dependent on gadolinium administration[79]. The revised recommendations refrain from the utilization of intravenous gadolinium-based contrast agents in patients with poor renal function[80]. One recent report has suggested that T2*-weighted MRI may offer the potential for diagnosing HCC in patients with liver cirrhosis[81].

The proposed mechanism for the visualization of HCC on the T2*-weighted sequence is attributed to the combination of the high sensitivity of this sequence to the presence of iron and iron differential deposition in the hepatic parenchyma. On T2*-weighted MRI, hepatic iron causes progressive signal loss with longer TEs, whereas HCCs demonstrate only slight signal loss[81].

One limitation of this sequence is the appearance of lesions after chemoembolization, which potentially

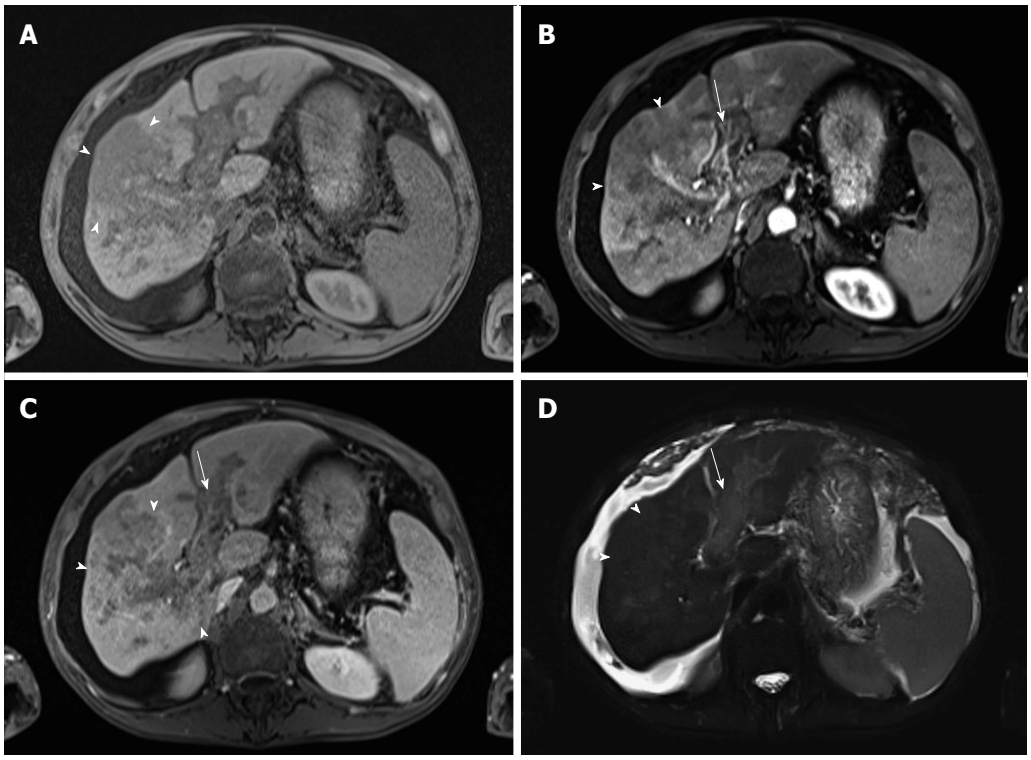

Figure 15 Diffuse hepatocellular carcinoma associated with tumor thrombus. (A) Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) late hepatic arterial and (C) delayed phases; (D) Fat-suppressed SSFSE T2-weighted image. There is a large ill-defined area involving the right hepatic lobe, which shows decreased T1 signal in pre-contrast images (arrowheads, A), heterogeneous mildly increased arterial enhancement (arrowheads, B), and heterogeneous delayed washout with permeative appearance (arrowheads, C), and heterogeneous mildly increased T2 signal (arrowhead, D) in keeping with HCC. There is also evidence of expansion of the portal vein branches, abnormal increased arterial enhancement (arrow, B), lack of opacification and washout on the delayed images (arrow, C), and mildly increased T2 signal (arrow, D) simulating the behavior of the primary tumor in keeping with diffuse tumor thrombus, a finding almost invariably associated with diffuse HCC subtype. Also of note is the mild to moderate ascites and omental hypertrophy secondary to portal hypertension. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo.

reduces the diagnostic performance of the sequence[82].

The addition of a T2*-weighted sequence to a routine liver MRI protocol might lead to additional improved specificity[83], although future studies are likely indicated to determine the full diagnostic performance of T2*-weighted MRI in a larger patient population.

MRI CONTRAST AGENTS

Contrast agents used in cirrhosis-associated hepatic nodules MR evaluation are divided into three types: extracellular Gadolinium-based contrast agents (GBCAs), super-paramagnetic iron-oxide (SPIO) particles, and Gadolinium-based hepatobiliary contrast agents.

Extracellular GBCAs are paramagnetic contrast agents that generate T1-shortening and provide information about tissue vascularity[38]. SPIO particles and hepatobiliary agents are liver-specific contrast agents. SPIO particles are taken up by Kupffer cells within the reticuloendothelial system (RES), and the hepatobiliary agents are taken up by hepatocytes and are excreted *via* the bile ducts[84].

Despite early promising results of SPIO particles for diagnosing HCC, later evidence reveal that is less efficient than dynamic MRI using conventional extracellular GBCAs in the detection and characterization of HCC[85]. Additionally, there are currently no commercially available intravenous SPIO particles contrast agents in the market.

More recently, two hepatobiliary agents; gadobenate dimeglumine and gadoxetic acid were introduced to the market, combining extracellular properties with liver-specific properties, allowing both dynamic and hepatobiliary imaging. Gadoxetic acid is more highly liver-specific; approximately 50% of the injected dose is taken up by functioning hepatocytes and is excreted in bile, allowing delayed uptake imaging within 20 min from the time of injection, compared with an uptake of 3%-5% for gadobenate dimeglumine, which allows for delayed uptake imaging within two hours[13].

The hepatocyte uptake manifests as an increased signal in the hepatic parenchyma on T1-weighted images resulting in improved lesion-to-liver contrast as less well-differentiated HCCs contain hampered functioning hepatocytes. HCCs exhibit hypointensity on hepatobiliary phase images (Figure 18), except for some well-differentiated HCCs that may retain the contrast agent. Nevertheless, characterization of liver lesions depicted with hepatobiliary phase imaging must be performed in conjunction with routine dynamic

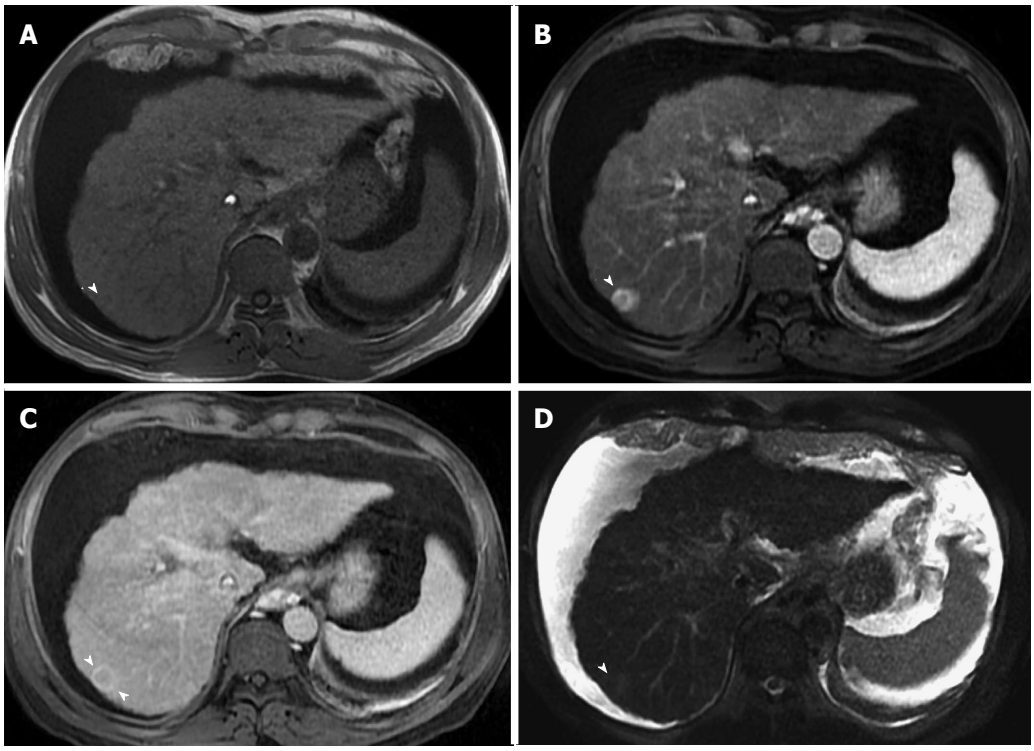

Figure 16 Ring-enhancing hepatocellular carcinoma, indicative of a more aggressive course. A: In-phase GRE T1 weighted image; B: Post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) late hepatic arterial and (C) delayed phases; (D) Fat-suppressed SSFSE T2-weighted image. There is a small nodule at hepatic segment #7, which demonstrates iso to slightly low T1 signal (arrowhead, A), heterogeneous increased arterial enhancement, predominantly peripheral (arrowhead, B), washout and pseudocapsule enhancement on delayed images (arrowhead, C), and mildly increased T2 signal intensity (arrowhead, D) in keeping with ring-enhancing HCC. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

sequences to improve accuracy[86].

Gadoxetic acid-enhanced MRI has several advantages in imaging the cirrhotic liver including: (1) higher sensitivity for the diagnosis of HCC, especially for lesions $\leqslant$ 2 cm[86-91]; (2) improved characterization of arterially enhancing lesions without definite washout on subsequent imaging[89,92]; (3) distinguishing arterially enhancing pseudo-lesions from HCC[92,93]; and (4) detection of lesions that are isointense to the background hepatic parenchyma on all sequences, apart from the hepatobiliary phase, that are at high risk of transforming to hypervascular HCC[94,95].

However, some limitations to the use of gadoxetic acid-enhanced MRI in the liver cirrhosis have been proposed, especially pertaining to the fact that some patients with cirrhosis can show less optimal lesion-to-liver contrast on early dynamic imaging and poor venous enhancement, which may hamper the diagnosis of HCC and assessment of the porto-spleno-mesenteric venous system patency[86].

Despite the recognized potential advantages of combined morphological and functional analysis of the liver, the inclusion of hepatobiliary contrast agents in international guidelines, besides the Japan Society of Hepatology, is still pending. Recently updated guidelines from the EASL[47] and the AASLD[6] make no contrast agent recommendations.

Overall, continued investigations with more direct comparative analysis between gadoxetic acid and other extracellular agents are warrant.

LI-RADS

LI-RADS was developed by the American College of Radiology[96]; with the aim of standardizing terminology and criteria for interpreting and reporting findings of CT and MRI examinations of the liver in patients with cirrhosis or increased risk of HCC; by using use a carefully chosen and agreed-on vocabulary, or lexicon, that differentiates hepatic histologic entities. It has been developed to provide a framework for assigning degrees of concern on imaging findings[97]. The LI-RADS classifies lesions to five categories ranging from definitely benign to definitely HCC. It uses arterial hyper-enhancement, washout, capsule, and interval growth as ancillary findings[96]. It currently, however, does not apply to hepatobiliary gadolinium-based agents[97].

CONCLUSION

Noninvasive imaging has become the standard for HCC diagnosis in cirrhotic patients. Typical imaging features of HCC such as increased arterial enhancement and delayed washout provide very high specificity and acceptable sensitivity even in nodules ranging from 1-2 cm in diameter. However, limitations apply specifically to hypovascular HCCs and in the differentiating HGDNs

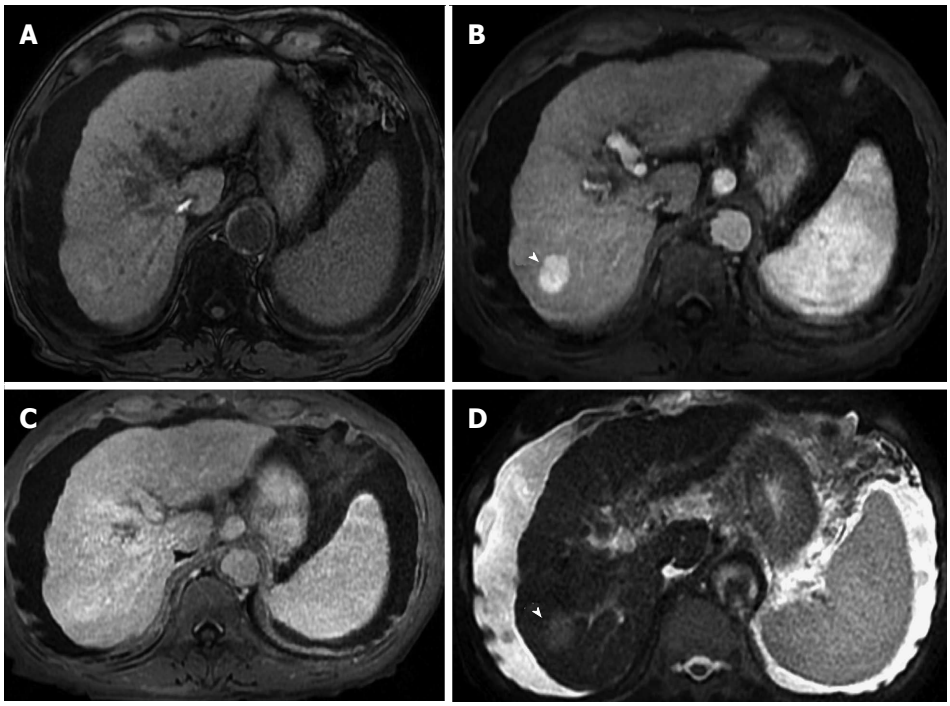

Figure 17 Large, non-washing-out hepatocellular carcinoma. A: Pre- and post-contrast fat-suppressed 3D-GRE T1-weighted images during the (B) late hepatic arterial and (C) delayed phases; D: Fat-suppressed SSFSE T2-weighted image. There is a sizable right hepatic lobe nodule, which demonstrates iso T1 signal intensity on pre-contrast images (A); increased arterial enhancement (arrowhead, B); becomes iso to slightly hyperintense compared to background liver, without definite washout, on the delayed images (C); and demonstrates mildly increased T2 signal intensity in keeping with HCC. Note that T2 sinal alteration increased the accuracy of diagnosing HCC in this patient, despite the lack of delayed washout (also see Figure 12). HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

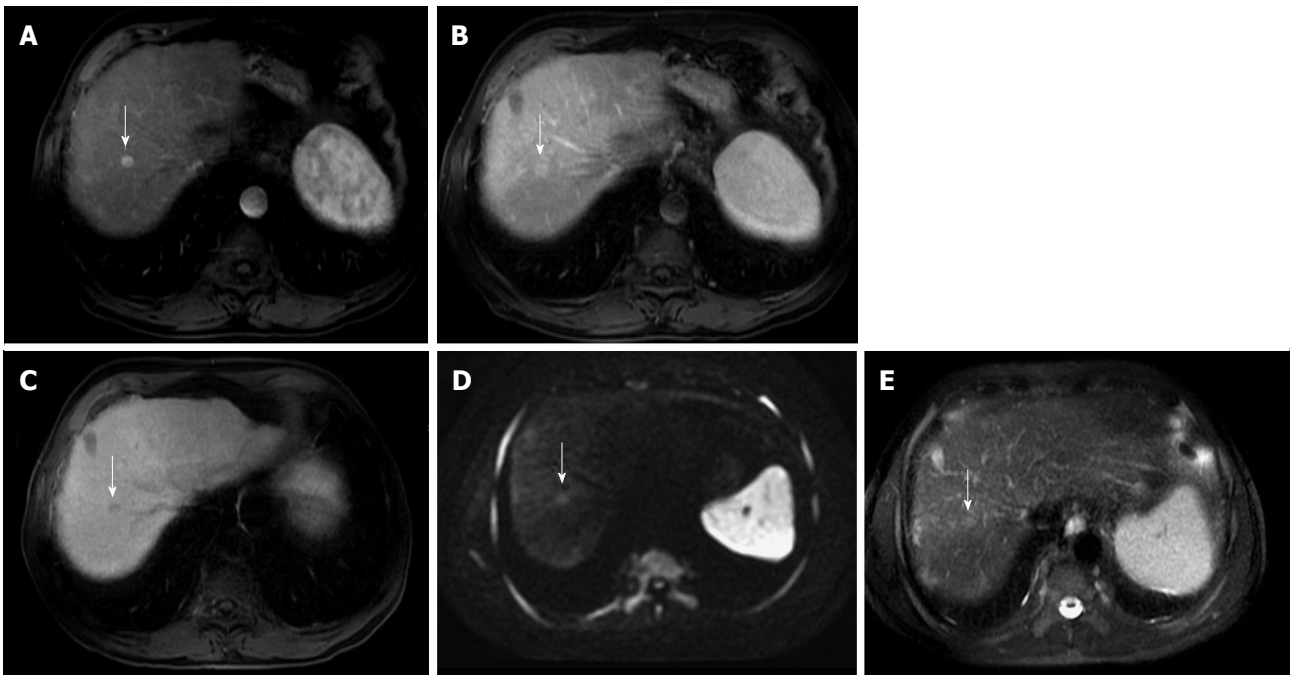

Figure 18 Small hepatocellular carcinoma showing the value of diffusion weighted image and hepatocyte-specific agents. A: Post-contrast fat-suppressed 3D-GRE T1-weighted images during the (A) late hepatic arterial, (B) delayed, and (C) hepatobiliary phases; D: Diffusion weighted image (DWI) (b = 50); E: Fat-suppressed SSFSE T2-weighted image. There is a small right hepatic lobe nodule, which demonstrates increased arterial enhancement (arrow, A), fading out on the delayed images (arrow, B), and demonstrates significantly decreased uptake on the hepatobiliary phase (arrow, C). The lesion also demonstrates clear increased signal on DWI (arrow, D) and subtle increased T2 signal (arrow, E). The constellation of findings is consistent with HCC. The decreased uptake on the hepatobiliary phase and increased signal on DWI increased the accuracy of HCC diagnosis in this patient with a small hypervascular nodule. HCC: Hepatocellular carcinoma; GRE: Gradient recalled echo; SSFSE: Single-shot fast spin-echo.

from early HCCs. In this review paper, we went over the basics of MR imaging of cirrhotic livers and described future directions, including the addition of new techniques such as DWI, T2*, and hepatocyte-

specific MRI contrast agents, in order to improve HCC detection rate in conjunction with the reference standard of optimized dynamic GRE T1-weighted imaging, with individually tailored arterial phase timing.

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MINIREVIEWS

Hepatitis B in healthcare workers: Transmission events and guidance for management

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Abstract

Hepatitis B virus (HBV) is the most efficiently transmissible of the bloodborne viruses that are important in healthcare settings. Healthcare workers (HCWs) are at risk for exposure to HBV from infected patients and, if infected, are similarly at risk of transmitting HBV to patients. Published cases of HBV transmission from HCW to patient are relatively rare, having decreased in frequency following the introduction of standard (universal) precautions, adoption of enhanced percutaneous injury precautions such as double-gloving in surgery, and routine HBV vaccination of HCWs. Here we review published cases of HCW-to-patient transmission of HBV, details of which have helped to guide the creation of formal guidelines for the management of HBV-infected HCWs. We also compare the published guidelines for the management of HBV-infected HCWs from various governing bodies, focusing on their differences with regard to vaccination requirements, viral load limits, frequency of monitoring, and restrictions on practice. Importantly, while there are differences among the recommendations from governing bodies, no guidelines uniformly restrict HBV-infected HCWs from performing invasive or exposure-prone procedures.

Key words: Hepatitis B; Healthcare worker; Blood-borne pathogens; Transmission; Invasive procedures

Core tip: Reports of transmission of hepatitis B virus (HBV) from infected healthcare workers (HCWs) to patients have been rare but are highly instructive when they do occur. These events have helped instruct formal recommendations for the management of HBV-infected HCWs. However, guidelines from various governing bodies differ in their recommendations for the monitoring of infected HCWs, as well as in their restriction of the practice of invasive, exposure-prone procedures.

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INTRODUCTION

Hepatitis B virus (HBV) is one of numerous blood-borne pathogens known to be transmissible in healthcare settings. HBV, among the blood-borne viruses including hepatitis C virus (HCV) and human immunodeficiency virus (HIV), is of particular importance because it is the most efficiently transmissible following percutaneous exposure. The incidence of transmission *via* needlestick injury in one study was approximately 2% with HBV e antigen (HBeAg)-negative blood and 19% with HBeAg-positive blood[1]. Thus, healthcare workers (HCWs) are at risk for exposure to HBV from infected patients, and correspondingly, HBV-infected HCWs may potentially transmit HBV to patients. Fortunately, reported instances of HCW-to-patient transmission of HBV have been rare and have substantially decreased in frequency over the past four decades. Here, we examine the limited data available on the prevalence of HBV infection in HCWs, review published cases of HCW-to-patient transmission of HBV, and evaluate the guidelines and recommendations for the management of HBV-infected HCWs with a particular focus on the variability of guidelines across geographic regions and governing bodies.

PREVALENCE OF HBV INFECTION IN HEALTHCARE WORKERS

Since the development of a vaccine to prevent acute HBV infection in the early 1980s, the incidence of acute HBV infection in the general population of the United States has sharply fallen. The Centers for Disease Control and Prevention (CDC) estimates that the incidence of new HBV infections had fallen 5-fold between 1980 and 2010, from 208000 to 38000 new infections per year[2].

Historically, HCWs shouldered the burden of HBV infection in the United States. A study conducted in the United States Army between 1972 and 1974 found a HBV seropositivity rate of 5.8% among officers involved in direct patient care, compared to a seropositivity rate of 2.8% in those in non-patient care-oriented positions[3]. A serologic study conducted in physicians between 1975 and 1976 found serologic evidence of prior HBV infection in 18% of subjects, with a higher rate among pathologists (27%) and surgeons (28%)[4]. Following the 1982 Advisory Committee on Immunization Practices (ACIP) recommendation for HBV vaccination for HCWs[5], studies continued to demonstrate that HCWs were at increased risk for HBV infection, although the rates of seropositivity steadily declined. A voluntary study of 943 HCWs at a large urban academic medical center conducted over 8 mo in 1991 demonstrated HBV core antigen (HBcAg) positivity in 6.2% of HCWs compared to 1.8% in the comparator group of local blood donors[6]. Though the current prevalence of HBV in HCWs is not known, it likely mirrors that of the general population, significantly decreasing following the introduction of routine infant vaccination, catch-up adolescent vaccination, and pre-employment vaccination for HCWs who may potentially be exposed to blood or bodily fluids.

REVIEW OF PUBLISHED CASES OF HEALTHCARE WORKER-TO-PATIENT TRANSMISSION OF HBV

Table 1 summarizes the published cases of HCW-to-patient transmission of HBV[7-39]. Confirmed transmissions are defined as cases where the HCW and patient(s) were epidemiologically linked and genetic relatedness of the viruses was confirmed through partial or complete DNA sequencing. Probable transmissions are defined as cases in which the subtype of HBV infecting the HCW and patient were identical in investigations of epidemiologically-linked HCW and patient HBV infections. Possible transmissions are defined as cases in which epidemiologic links were established, infected patients had no other risk factors for HBV acquisition but virologic subtyping data was not available to confirm transmission. It should be noted that, based on the availability of molecular technology at the time, chronologically earlier reports were limited in their ability to confirm transmission. Additionally, earlier reports often do not include the HBV viral burden of the transmitting HCW.

Summarizing published cases of HCW-to-patient transmission of HBV, recognized breaches in infection control practices were implicated in the transmission event in a notable minority of cases. Early reports of transmission occurred in association with dental procedures during which the dentist or oral surgeon did not wear gloves[8,14,16,22], which was not a standard recommended practice until the 1980s[40]. In addition, several early reports of HBV transmission occurred during surgical procedures where the surgeon did not routinely double-glove, which was not a standard recommended practice until the early 2000s[41,42]. It is also notable that transmission of HBV from infected HCWs in primary care or other specialties that do not perform exposure-prone procedures (EPPs) is exceedingly rare. When transmissions did occur with these providers, they were more likely to be associated with breaches in infection control practices, such as reuse of syringes for access of indwelling arterial catheters[10], reuse of subdermal electroencephalogram electrodes[31], or failure to wear gloves in the setting of a skin condition involving the hands of the provider[10,18,20]. Overall, in the 35 cases in which HBeAg testing results were available,

Table 1 Published cases of healthcare worker-to-patient transmission of hepatitis B virus

Ref.	Year	Location	Type of provider	HBeAg status	Viral load	HBV status known to provider	HBV status known to institution	No. of patients infected	Breach in infection control identified
[7]	1969	United States	Nurse	Not done	Not done	No	No	11[a] (11 possible)	None
[8]	1969-1974	United States	Oral surgeon	Positive	Not done	Not specified	Not specified	55 (10 probable, 45 possible)	HCW did not wear gloves
[9]	1973-1977	Switzerland	General practitioner	Positive	Not done	Yes	Not specified	41 (41 possible)	None
[10]	1974	United States	Respiratory therapist	Positive	Not done	No	No	4 (4 probable)	HCW did not wear gloves, had an exudative dermatitis on hands, and reused syringes when accessing indwelling arterial catheters
[11]	1975	United States	Oral surgeon	Not done	Not done	Not specified	Not specified	43 (43 probable)	None
[12]	1976-1979	United Kingdom	Surgical registrar	Positive	Not done	No	No	9 (7 probable, 2 possible)	None
[13]	1977-1978	United Kingdom	Surgical registrar, gynecologic surgery	Positive	Not done	No	No	8 (6 probable, 2 possible)	None
[14]	1978	United States	Dentist	Positive	Not done	Yes	Not specified	6 (2 probable, 4 possible)	HCW did not wear gloves
[15]	1978	Norway	Cardiac surgeon	Positive	Not done	No	No	5 (5 probable)	None
[16]	1978-1979	United States	Oral surgeon	Positive	Not done	No	No	12 (4 probable, 8 possible)	HCW did not wear gloves and had a generalized eczematous dermatitis, including hand involvement
[17]	1979-1980	United States	Obstetrician-gynecologist	Positive	Not done	Yes	Yes	4 (1 probable, 3 possible)	HCW held needle in hand rather than a needle holder when suturing, noted several episodes of blood on hands after removing gloves
[18]	1979-1981	The Netherlands	Cardiac surgeon	Not reported	Not done	Not specified	Not specified	3 (3 probable)	None
[18]	1979-1981	The Netherlands	Perfusion technician	Positive	Not done	Not specified	Not specified	11 (8 probable, 3 possible)	Bleeding warts on HCW's hands
[19]	1980	United States	Oral surgeon	Not done	Not done	Not specified	Not specified	3 (3 probable)	None
[20]	1980-1983	United Kingdom	Perfusion technician	Positive	Not done	Yes	Not specified	6 (6 probable)	HCW did not wear gloves, and had cuts and abrasions on hands
[20]	1980-1983	United Kingdom	Surgical registrar	Not reported	Not done	Not specified	Not specified	5 (5 possible)	None
[20]	1980-1983	United Kingdom	House officer	Not reported	Not done	Not specified	Not specified	1 (1 possible)	None
[21]	1984	United States	Obstetrician-gynecologist	Positive	Not done	Not specified	Not specified	6 (6 probable)	None
[22]	1984-1985	United States	Dentist	Positive	Not done	No	No	24 (6 probable, 18 possible)	HCW did not wear gloves
[23]	1987	United States	General surgeon	Positive	Not done	Yes	Not specified	5 (3 probable, 2 possible)	None
[24]	1987	United Kingdom	Obstetrician-gynecologist	Positive	Not done	No	No	22 (6 probable, 16 possible)	None

[25]	1988	United Kingdom	General surgeon	Negative	1×10^7 copies/mL	No	No	1 (1 confirmed)	None
[25]	1988	United Kingdom	Obstetrician-gynecologist, trainee	Negative	4.4×10^6 copies/mL	No	No	3 (3 confirmed)	None
[25]	1988	United Kingdom	Obstetrician-gynecologist, trainee	Negative	5.5×10^6 copies/mL	Yes	Not specified	1 (1 confirmed)	None
[25]	1988	United Kingdom	General surgeon, urologist, clinical assistant	Negative	2.5×10^5 copies/mL	No	No	1 (1 confirmed)	None
[26]	1988	United Kingdom	Cardiothoracic surgeon, trainee	Positive	Not done	No	No	17 (9 probable, 8 possible)	None
[27]	1991	United Kingdom	Surgeon	Positive	Not done	No	No	3 (3 possible)	None
[28]	1991	Canada	Orthopedic surgeon	Positive	Not done	Yes	Yes	2 (1 probable, 1 possible)	None
[29]	1991-1992	United States	Thoracic surgeon	Positive	1×10^9 copies/mL	Yes	Not specified	19 (9 confirmed, 4 probable, 6 possible)	None
[30]	1991-1993	United Kingdom	Cardiothoracic surgeon	Positive	Not done	Yes	No	20 (14 confirmed, 6 probable)	None
[31]	1991-1996	Canada	Electroencep-halogram technician	Positive	Not done	No	No	75 (4 confirmed, 71 possible)	HCW did not wear gloves and used reusable subdermal EEG electrodes
[32]	1993	United Kingdom	General surgeon	Positive	Not done	No	No	2 (2 confirmed)	None
[33]	1993-1994	United Kingdom	General surgeon, trainee	Positive	Not done	Not specified	Not specified	11 (1 confirmed, 10 possible)	None
[33]	1994	United Kingdom	General surgeon, trainee	Positive	Not done	Not specified	Not specified	2 (2 possible)	None
[33]	1994	United Kingdom	Urologist, trainee	Positive	Not done	Not specified	Not specified	1 (1 possible)	None
[34]	1995-1999	The Netherlands	General surgeon	Positive	5×10^9 GE/mL	No	No	28 (8 confirmed, 20 possible)	HCW noted glove perforations
[35]	1996	United Kingdom	Orthopedic surgeon	Negative but anti-HB e positive (pre-core mutant)	Not done	Yes	Yes	1 (1 confirmed)	None
[36]	1999	United Kingdom	Cardiothoracic surgeon	Negative but anti-HB e positive (pre-core mutant)	1.03×10^6 GE/mL	Yes	Yes	2 (2 confirmed)	None
[37]	2001	United Kingdom	General surgeon	Negative	$> 10^6$ copies/mL	No	No	3 (3 confirmed)	None
[38]	2009	United States	Orthopedic surgeon	Positive	> 17.9 million IU/mL	No	No	8 (2 confirmed, 6 possible)	None
[39]	2010	Japan	Obstetrician-gynecologist	Positive	1.6×10^9 copies/mL	No	No	1 (1 confirmed)	None

[a]Cases included only admitted pts with a dx of icteric "serum hepatitis". HBeAg: Hepatitis B virus e antigen; HBV: Hepatitis B virus; GE: Genome equivalents; HCW: Healthcare worker; EEG: Electroencephalogram.

the vast majority (77%) of transmissions occurred as a result of an HBeAg-positive HCW. The lowest measured viral load at which transmission occurred was 2.5×10^5 copies/mL, which notably occurred in a HCW with HBeAg-seronegative chronic HBV[25]. In another study that included six HBeAg-seronegative surgeons who had previously been implicated in HCW-to-patient transmission events, all were viremic and the lowest HBV DNA viral load measured was 4×10^4 copies/mL[43]. Accordingly, the authors suggest that this viral load may represent a lower limit above which HBV transmission during invasive procedures cannot be definitively ruled out[43]. However, confidence in this viral load has some limitations, as it was measured at

least 3 mo after the transmission event occurred.

REVIEW OF GUIDELINES FOR MANAGEMENT OF HEPATITIS B VIRUS-INFECTED HEALTHCARE WORKERS

Guidelines for the management of HBV-infected HCWs are largely based on the anecdotal data gleaned from cases of HCW-to-patient transmission and attempt to ethically balance the risk of viral transmission to the patient with the right of the infected HCW to perform his/her work in a safe manner without loss of the right to confidentiality about his/her own health issues. Here we summarize the guidelines on the management of HBV-infected HCWs from various governing bodies worldwide. Table 2 summarizes the recommendations, allowing direct comparison of guidelines on key factors, including screening and vaccination for HBV in HCWs, monitoring recommendations for HBV-infected HCWs, and restrictions on practice for those HCWs if any. Although there are slight differences among guidelines, it is important to note that none universally prohibits the practice of invasive procedures by an HBV-infected HCW. Table 3 summarizes each governing body's definition and categorization of EPPs where delineated.

The Society for Healthcare Epidemiology of America, in guidelines updated in 2010[44], recommends that HCWs with a positive HBeAg or circulating HBV burden of greater than or equal to 10^4 genome equivalents (GE) per mL of blood be prohibited from performing certain pre-defined high-risk (Category Ⅲ) EPPs and use double-gloving for all invasive procedures, for all contact with mucous membranes or non-intact skin, and for all instances in patient care for which gloving is recommended otherwise[44]. For HCWs with a circulating HBV burden of less than 10^4 GE/mL of blood, it is suggested that providers may perform all Category Ⅰ and Ⅱ (minimal- and low-risk) and Category Ⅲ (high-risk) procedures as long as they (1) have not transmitted HBV infection to patients; (2) obtain advice from an expert review panel; (3) undergo routine follow-up by occupational medicine including twice yearly viral testing to ensure that viral burden remains less than 10^4 GE/mL; (4) receive follow-up care by a personal physician who has expertise in the management of HBV infection and who may communicate with the expert review panel about the HCW's clinical status; (5) consult with an expert about optimal infection control procedures and strictly adhere to them; and (6) agree to and sign a contract or letter from the expert review panel that characterizes their responsibilities[44].

CDC guidelines from 2012 recommend that all HCWs receive HBV vaccination followed by assessment of hepatitis B surface antibody (anti-HBs) status and, in the case of non-response to vaccination, revaccination[45]. If HCWs do not achieve protective levels of anti-HBs after a second three-dose series of HBV vaccine, they should be tested for HBV surface antigen (HBsAg) and HBV core antibody (anti-HBc) to determine if previously or chronically infected. Pre-vaccination serology is recommended for HCWs who were born to mothers from endemic countries, who are sexually active men who have sex with men, and/or who perform EPPs[45]. CDC guidelines for management of chronic HBV infection apply only to those HCWs who perform EPPs. They recommend that HBV-infected HCWs that perform EPPs may continue to do so if a low (less than 1000 IU/mL or 5000 GE/mL) or undetectable HBV viral load is documented every 6 mo. If the viral load is above the recommended threshold, performance of EPPs should be restricted until subsequent retesting occurs[45]. CDC also recommends that institutions have written policies and procedures in place for the management of HBV-infected HCWs, including the ability to form an expert review panel to assist with management of these providers[45].

In its most recent guidelines, updated in 2003, the American College of Surgeons (ACS) recommends that surgeons know their HBV immunization and antibody status[46]. Surgeons who do not have protective anti-HBs levels should be vaccinated, with follow-up documentation of seroconversion. Failure to seroconvert should prompt a second vaccination series. Surgeons with antibody to HBV should know their HBsAg status and, if positive, their HBeAg status. ACS recommends that surgeons who are HBeAg-positive or have high viral loads be guided by expert panels regarding their continuation of clinical practice. These guidelines do not specify what constitutes a high viral load, the viral load limit at which a surgeon's practice should be restricted, or what procedures constitute EPPs[46].

Canadian guidelines, set forth by the Laboratory Centre for Disease Control of Health Canada in 1998, recommend mandatory HBV immunization of HCWs who perform EPPs with follow-up testing 4-8 wk later for anti-HBs response[47]. Those who are found to be non-responders on post-immunization testing should be screened for infection annually, with HBsAg and anti-HBc. HCWs who perform EPPs and test positive for HBsAg should undergo testing for HBeAg and if positive, should be referred to an expert panel and cease practice pending the panel's recommendations. If HBeAg-negative, they may continue to practice but should still be referred to an expert panel. Provided that the HCW follows recommendations set forth by the expert panel, disclosure of HBV status to patients before an EPP is not recommended.

The United Kingdom Department of Health (UKDH) released guidelines for management of HBV-infected HCWs in 2000[48], with an update focusing on HBV-infected HCWs on antiviral therapy in 2007[49]. UKDH recommends that all HCWs who perform EPPs be immunized against HBV and be tested for anti-HBs

Table 2 Guidelines for management of hepatitis B virus-infected healthcare workers

	CDC	SHEA	ACS	Canada	UK	Europe	Australia
Screening	All HCWs at risk for HBV infection should be tested	Not addressed in guideline	All surgeons should know their HBV status	Mandatory for all HCWs who perform EPPs	Mandatory for all HCWs who perform EPPs, can be done post-vaccination	Mandatory for all HCWs who perform EPPs, can be done post-vaccination	Annual testing recommended for all HCWs who perform EPPs
Vaccination	All HCWs susceptible to HBV infection should be vaccinated	Not addressed in guideline	All surgeons who are antibody negative should be vaccinated	Mandatory for all HCWs who perform EPPs	Mandatory for all HCWs who perform EPPs	Recommended for all HCWs	Recommended for all HCWs
Post-vaccination serology	Recommended	Not addressed in guideline	Recommended	Recommended	Recommended	Recommended	Not addressed in guideline
Frequency of testing/ monitoring	Every 6 mo	Every 6 mo	Not specified	Every 12 mo	Every 12 mo, or every 3 mo while on antiviral therapy	Every 12 mo if HBeAg negative, every 3 mo if HBeAg positive or on antiviral therapy	Every 3 mo if on antiviral therapy, every 12 mo if cleared HBsAg
Viral load limit	1000 IU/mL or 5000 GE/mL	10^4 GE/mL	Not specified	Not specified	10^3 GE/mL	10^4 GE/mL	Undetectable by PCR assay
HBeAg	Not required to be negative	Not required to be negative	Not required to be negative	Not required to be negative	Must be negative	Not required to be negative	Not addressed in guideline
Restriction of practice	EPPs restricted if viral load greater than set threshold	Category Ⅲ procedures restricted if viral burden greater than or equal to 10^4 GE/mL or HBeAg positive	Determined by expert panel	Determined by expert panel	If HBeAg positive or if viral load greater than 10^3 GE/mL	If viral load greater than 10^4 GE/mL	If HBV DNA level detectable
Definition of EPPs	Yes	Yes	No	Yes	Yes	Yes	Yes
Expert panel recommended	Yes	Yes	Yes, if HBeAg positive or high viral load	Yes, if HBsAg positive	No, recommend monitoring by an occupational health physician	No	Yes
Pre-emptive patient notification	No	No	Not specified	No	No	Optional for HCWs with HBV DNA levels above the cut-off level in order to continue practicing EPPs	No

CDC: Centers for Disease Control and Prevention, United States; SHEA: Society for Healthcare Epidemiology of America, United States; ACS: American College of Surgeons, United States; Canada: Laboratory Centre for Disease Control of Health Canada; UK: Department of Health, United Kingdom; Europe: European Consensus Group; Australia: Australian Government, Department of Health and Aging; HCW: Healthcare worker; HBV: Hepatitis B virus; IU: International units; GE: Genome equivalents; HBeAg: Hepatitis B e antigen; EPP: Exposure-prone procedure; PCR: Polymerase chain reaction; HBsAg: Hepatitis B surface antigen.

response post-vaccination[50]. If there is failure to respond to one vaccination series, the HCW should be tested for HBsAg, and if negative, should be tested for anti-HBc to determine if they have had prior infection or are true vaccine non-responders[50]. The guidelines recommend that all HCWs who are HBsAg-positive be tested for HBeAg[48]. Those that are HBeAg-positive or those who are HBeAg-negative but with a HBV viral load greater than 10^3 GE/mL should be restricted from performing EPPs[48]. If HBeAg is negative and viral load is less than 10^3 GE/mL without antiviral treatment, annual monitoring should be conducted, and practice should not be restricted[48]. The 2007 guidelines, developed in response to advances in antiviral treatment for HBV infection, recommends that HCWs who are HBeAg-negative should be permitted to perform EPPs while on antiviral therapy as long as their viral load remains < 10^3 GE/mL on monitoring done every 3 mo, and their pretreatment viral load was < 10^5 GE/mL[49]. Though the UKDH guidelines do not recommend formation of an expert panel to guide management of HBV-infected HCWs, they do recommend the involvement of an occupational health physician[48].

The European Consensus group guidelines published in 2003 recommend HBV vaccination of all HCWs in contact with patients, blood, or other bodily fluids, with vaccine response tested one month post-vaccination[51]. Non-responders should undergo additional vaccine series and if they continue to fail to respond, an investigation into their HBV status is recommended based on their job functions. Specifically

Table 3 Categories of exposure-prone procedures

CDC
Category Ⅰ. Procedures known or likely to pose an increased risk of percutaneous injury to a healthcare provider that have resulted in provider-to-patient transmission of HBV. These procedures are limited to major abdominal, cardiothoracic, and orthopedic surgery, repair of major traumatic injuries, abdominal and vaginal hysterectomy, caesarean section, vaginal deliveries, and major oral or maxillofacial surgery. Techniques that have been demonstrated to increase the risk for healthcare provider percutaneous injury and provider-to-patient blood exposure include: digital palpation of a needle tip in a body cavity and/or the simultaneous presence of a health care provider's fingers and a needle or other sharp instrument or object in a poorly visualized or highly confined anatomic site
Category Ⅱ. These procedures pose low or no risk for percutaneous injury to a HCW or, if a percutaneous injury occurs, it usually happens outside of a patient's body and generally does not pose a risk for provider-to-patient blood exposure. These include: surgical and obstetrical/gynecologic procedures that do not involve the techniques listed for Category I, the use of needles or other sharp devices when the HCW's hands are outside a body cavity, dental procedures other than major oral or maxillofacial surgery, insertion of tubes, endoscopic or bronchoscopic procedures, internal examination with a gloved hand that does not involve the use of sharp devices, and procedures that involve external physical touch
SHEA
Category Ⅰ. Procedures with *de minimis* risk of bloodborne virus transmission: regular history-taking and/or physical or dental examinations; routine dental preventive procedures, diagnostic procedures, orthodontic procedures, prosthetic procedures, cosmetic procedures not requiring local anesthesia; routine rectal or vaginal examination; minor surface suturing; elective peripheral phlebotomy; lower gastrointestinal tract endoscopic examinations and procedures; hands-off supervision during surgical procedures and computer-aided remote or robotic surgical procedures; and psychiatric evaluations
Category Ⅱ. Procedures for which bloodborne virus transmission is theoretically possible but unlikely: locally anesthetized ophthalmologic surgery; locally anesthetized operative, prosthetic, and endodontic dental procedures; periodontal scaling and root planing; minor oral surgical procedures; minor local procedures under local anesthesia; percutaneous cardiac procedures; percutaneous and other minor orthopedic procedures; subcutaneous pacemaker implantation; bronchoscopy; insertion and maintenance of epidural and spinal anesthesia lines; minor gynecological procedures; male urological procedures; upper gastrointestinal tract endoscopic procedures; minor vascular procedures; amputations; breast augmentation or reduction; minimum-exposure plastic surgical procedures; total and subtotal thyroidectomy and/or biopsy; endoscopic ear, nose, and throat surgery and simple ear and nasal procedures; ophthalmic surgery; assistance with an uncomplicated vaginal delivery; laparoscopic procedures; thorascopic procedures; nasal endoscopic procedures; routine arthroscopic procedures; plastic surgery; insertion of, maintenance of, and drug administration into arterial and central venous lines; endotracheal intubation and use of laryngeal mask; and obtainment and use of venous and arterial access devices that occur under complete antiseptic technique, using universal precautions, "no-sharp" technique, and newly gloved hands
Category Ⅲ. Procedures for which there is definite risk of bloodborne virus transmission or that have been classified previously as "exposure-prone:" general surgery; general oral surgery; cardiothoracic surgery; open extensive head and neck surgery involving bones; neurosurgery, other intracranial procedures, and open-spine surgery; nonelective procedures performed in the emergency department; obstetrical/gynecological surgery; orthopedic procedures; extensive plastic surgery; transplantation surgery except skin and corneal transplantation; trauma surgery; interactions with patients in situations during which the risk of the patient biting the physician is significant; and any open surgical procedure with a duration of more than 3 h, probably necessitating glove change
ACS
Not provided
Canada
Procedures during which transmission of HBV, HCV, or HIV from a HCW to patients is most likely to occur and includes the following: (1) digital palpation of a needle tip in a body cavity or the simultaneous presence of the HCW's fingers and a needle or other sharp instrument or object in a blind or highly confined anatomic site; (2) repair of major traumatic injuries; or (3) major cutting or removal of any oral or perioral tissue, including tooth structures, during which there is potential for the patient's open tissues to be exposed to the blood of an injured HCW
UK
Exposure-prone procedures are those invasive procedures where there is a risk that injury to the worker may result in the exposure of the patient's open tissues to the blood of the worker. These include procedures where the worker's gloved hands may be in contact with sharp instruments, needle tips or sharp tissues inside a patient's open body cavity, wound, or confined anatomical space where the hands or fingertips may not be completely visible at all times
Europe
Exposure-prone procedures are invasive procedures where there is potential for contact between the skin of the HCW and sharp surgical instruments, needles, or sharp tissues in body cavities or poorly visualized/confined body sites
Australia
Category 1: A procedure where the hands and fingertips of the HCW are visible and outside of the body most of the time and the possibility of injury to the worker's gloved hands from sharp instruments and/or tissues is slight
Category 2: A procedure where the fingertips of the HCW may not be visible at all times but injury to the worker's gloved hands from sharp instruments and/or tissues is unlikely. If injury occurs it is likely to be noticed and acted upon quickly to avoid the HCW's blood contaminating a patient's open tissues
Category 3: A procedure where the fingertips are out of sight for a significant part of the procedure, or during certain critical stages, and in which there is a distinct risk of injury to the worker's gloved hands from sharp instruments and/or tissues. In such circumstances it is possible that exposure of the patient's open tissues to the HCW's blood may go unnoticed or would not be noticed immediately

CDC: Centers for Disease Control and Prevention, United States; SHEA: Society for Healthcare Epidemiology of America, United States; ACS: American College of Surgeons, United States; Canada: Laboratory Centre for Disease Control of Health Canada; UK: Department of Health, United Kingdom; Europe: European Consensus Group; Australia: Australian Government, Department of Health and Aging; HCW: Healthcare worker; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

for HCWs who perform EPPs, the group recommends proof of anti-HBs response and if negative or unavailable, they should receive a booster dose of HBV vaccination, with vaccine response tested one month later. Non-responders should be investigated for HBV infection, with testing of HBsAg or anti-HBc, and only

those who are HBsAg-negative should be permitted to proceed with EPPs. HCWs who are HBsAg-positive should have HBeAg tested and if HBeAg-negative, can proceed with performing EPPs if the HBV DNA level is less than 10^4 GE/mL. The HBV DNA level should be monitored annually. If HBeAg-positive, the HCW should not perform EPPs unless their viral load is below the designated cut-off and they are evaluated by an expert panel that has approved the performance of EPPs. In this case, it is recommended that the HBV viral load be monitored every 3 mo.

Finally, Australian guidelines from 2012[52] recommend that all HCWs be vaccinated against HBV and that all HCWs who perform EPPs be tested for HBV and other blood-borne pathogens annually. A HCW is not permitted to perform EPPs if HBV DNA is detectable by an approved polymerase chain reaction assay. If the HCW is HBsAg-positive and on antiviral therapy, they are permitted to perform EPPs as long as HBV DNA is undetectable *via* testing every 3 mo. If HBsAg becomes negative on two consecutive occasions, the HCW may perform EPPs but will require annual testing thereafter. The guidelines also recommend formation of an expert review panel to advise on the management of HBV-infected HCWs.

The majority of governing bodies recommend the formation of an expert review panel or committee to assist with management and monitoring of HCWs infected with HBV and other bloodborne viruses. Guidelines generally recommend the inclusion of individuals who have expertise in the infected HCW's specialty and the procedures they perform, healthcare epidemiologists, infectious disease specialists, hepatologists, occupational medicine physicians, hospital administrators, human resources personnel, and the HCW's primary physician. Other suggested panel members include a public health official if such issues are managed at the state level, legal counsel, and experts in ethics.

CONCLUSION

HBV infection among HCWs is of particular concern given its high transmissibility relative to other blood-borne viruses, including documented transmissions from infected HCW to patient. Fortunately, instances of HCW-to-patient transmission of HBV have been relatively rare and have substantially decreased in frequency over the past four decades, presumably due to more vigilant screening and vaccination of HCWs, the use of universal precautions and double-gloving during EPPs, and formal recommendations from governing bodies on the appropriate restrictions of practice of infected HCWs. Our review of the published cases of HCW-to-patient transmission of HBV provides historical data for these formal recommendations. Our review also highlights the differences between recommendations for management of HBV-infected HCWs by various governing bodies, though a common feature is that no governing body uniformly prohibits the practice of EPPs by an HBV-infected HCW. While HBV is highly transmissible through parenteral and mucous membrane exposures, the formal recommendations set forth by the various governing bodies discussed above have helped to codify the manner in which we manage HBV-infected HCWs, thus reducing the risk of transmission to patients while balancing the need to protect the private health information of HCWs and their ability to continue to perform the work for which they are trained to do.

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MiR-122 in hepatitis B virus and hepatitis C virus dual infection

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Abstract

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the most common causes of chronic liver diseases and hepatocelluar carcinomas. Over the past few years, the liver-enriched microRNA-122 (miR-122) has been shown to differentially regulate viral replication of HBV and HCV. It is notable that the level of miR-122 is positively and negatively regulated by HCV and HBV, respectively. Consistent with the well-documented phenomenon that miR-122 promotes HCV accumulation, inhibition of miR-122 has been shown as an effective therapy for the treatment of HCV infection in both chimpanzees and humans. On the other hand, miR-122 is also known to block HBV replication, and HBV has recently been shown to inhibit miR-122 expression; such a reciprocal inhibition between miR-122 and HBV suggests an intriguing possibility that miR-122 replacement may represent a potential therapy for treatment of HBV infection. As HBV and HCV have shared transmission routes, dual infection is not an uncommon scenario, which is associated with more advanced liver disease than either HBV or HCV mono-infection. Thus, there is a clear need to further understand the interaction between HBV and HCV and to delineate the role of miR-122 in HBV/HCV dual infection in order to devise effective therapy. This review summarizes the current understanding of HBV/HCV dual infection, focusing on the pathobiological role and therapeutic potential of miR-122.

Key words: MiR-122; Hepatitis B virus; Hepatitis C virus; Hepatitis B virus/hepatitis C virus dual infection

Core tip: This paper summarizes direct and indirect interactions between hepatitis B virus (HBV) and hepatitis C virus (HCV), and the pathobiological role and therapeutic potential of liver specific miR-122 in HBV/HCV dual infection.

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are a major health problem globally. Approximately 350 million and 170 million people are infected with HBV and HCV worldwide, respectively[1]. Although the exact number of patients with HBV/HCV dual infection is unknown, about 2%-10% of patients with chronic HCV are found to be hepatitis B surface antigen (HBsAg) positive and 5%-20% of patients with chronic HBV are anti-HCV positive[2]. Several studies have reported that HBV/HCV dual infection accelerates the progression of chronic liver disease including fibrosis, cirrhosis and hepatocelluar carcinoma (HCC)[3,4].

Although HBV/HCV dual infection is not uncommon, it is rare to have both HBV and HCV actively replicating in the same patient[5]. In general, HBV DNA and HCV RNA levels are lower in dual infected patients compared to their mono-infected patients[4,6]. This is in accordance with the described phenomenon of "viral interference", *i.e.*, infection by a first virus results in resistance of cells to infection by a second virus[7]. Indeed, there is growing evidence of reciprocal inhibitions between HBV and HCV. These reciprocal inhibitions may be mediated by several mechanisms including direct interference between two viruses, indirect inhibition through host gene regulation, and innate/adaptive host immune responses. Recently, the liver-enriched microRNA (miRNA)-122 (miR-122) has been implicated in the regulation of both HBV and HCV infections.

MiRNAs are small noncoding RNAs which are implicated in various biological processes through destabilization or translational inhibition of protein encoding mRNAs. Several miRNAs have been shown to regulate the replication and life cycle of HBV and HCV, respectively[8,9]. Among these, the liver-specific miRNA, miR-122, is perhaps the best known miRNA implicated in HCV or HBV infection. Notably, although miR-122 has been shown to enhance HCV replication, it is also known to inhibit HBV replication. However, to date, the role of miR-122 in HBV/HCV dual infection has not yet been defined. This review summarizes the current understanding of the direct and indirect reciprocal interactions in HBV/HCV dual infection, and discusses the emerging role of miR-122 in this intricate process.

NATURAL HISTORY OF DUAL INFECTION WITH HBV AND HCV

HBV/HCV dual infection can be classified into four types based on clinical features[2,10]: acute dual infection, occult HBV infection, and super-infection by either virus in patients with preexisting chronic hepatitis. Given the different modalities of HBV/HCV dual infection, its actual prevalence may be underestimated on the basis of the available clinical data. For instance, the predicted prevalence may vary depending on the relative sensitivity of both HBsAg and HBV DNA assay in case of occult HBV infection. Presently, the exact global prevalence of HBV/HCV dual infection is largely unknown.

The viral dominance and prevalence of HBV/HCV dual infection vary depending on the ethnicity and geographic region. Nguyen *et al*[11] analyzed 115 patients with HBV/HCV dual infection in multiethnic study and found that HBV viral dominance pattern is significantly higher in Asian patients than non-Asian patients (38% *vs* 10%; $P = 0.02$). In contrast, HCV viral dominance pattern is more common in North American and European patients than in Asian patients. Simultaneous infection of HBV and HCV is rare, and it is mostly found in the situations such as accidental needle-stick injury, blood transfusion and injection drug users[12,13]. Mimms *et al*[14] showed that delayed appearance of HBsAg and decreased alanine aminotransferase (ALT) levels in simultaneous dual infected patients compared to mono-infected patients, suggesting that HCV inhibits HBV activity. Occult HBV infection, defined as the presence of HBV DNA in the liver and/or in the serum from patients with undetectable HBsAg[15], has been frequently identified in patients with chronic HCV infection[16]. Several lines of evidences suggest that occult HBV infection may accelerate the progression of liver disease including HCC in chronic HCV infected patients[17-20]. For example, the prevalence of occult HBV infection is significantly higher (61.6% *vs* 36.3%) in HCV positive patients with HCC than non-HCC chronic HCV infected patients[17]. The incidence of HCC is also significantly higher (14% *vs* 1.4%) in chronic HCV patients with occult HBV compared to HCV patients without occult HBV[19]. HCV superinfection is common in areas where HBV is prevalent such as Asian countries[21,22]. Several studies have shown that HCV superinfection results in suppression of HBV replication and elimination of HBsAg[23,24]. However, acute HCV superinfection in patients with chronic hepatitis B is associated with worse prognosis in terms of higher incidence of cirrhosis and HCC compared to chronic hepatitis B alone[21]. Although HBV superinfection in patients with chronic hepatitis C is less common, several studies have shown that acute HBV superinfection leads to suppression of HCV replication. Sagnelli *et al*[25] showed that the patients who had been HCV RNA positive for at least 1 year before HBV superinfection became HCV RNA negative during HBV infection, but more severe clinical presentation (development of portosystemic encephalopathy, ascites or prothrombin activity lower than 25%) were observed in HBV superinfected patients[25,26].

Therefore, the evidence is compelling that HBV/HCV dual infection exhibits reciprocal inhibition of viral replication, yet more aggressive clinical course of liver disease and higher risk of HCC compared to

HBV or HCV mono-infection.

RECIPROCAL INHIBITION BETWEEN HBV AND HCV

Clinical and *in vivo* animal studies have shown that the HBV and HCV affect their replication each other. Patients with HBV/HCV dual infection show significantly lower titer of HCV RNA compared to patients with HCV mono-infection[27]. Decreased HBsAg and HBV DNA levels are seen in chronic hepatitis B patients co-infected with HCV[28]. Likewise, HCV super-infection results in significant reduction in the titer of serum HBsAg in chronic HBV-infected chimpanzee[29]. Conversely, in chronic HCV-infected chimpanzee, HBV infection is delayed and attenuated compared to HCV-negative chimpanzee[30]. These reciprocal inhibitions between HBV and HCV may account for various viral profiles including occult infection and viral dominance in HBV/HCV dual infection.

Direct viral interaction

Studying the direct interaction between HBV and HCV requires appropriate *in vitro* and *in vivo* model systems. At present, only few cell systems are susceptible to hepatotropic viral infections, even in the case of HBV or HCV mono-infection. While human primary hepatocytes are susceptible to both HBV and HCV infection, they are restricted to infection with acute phase, due to their short life span and the loss of hepatocyte features during cell culture process[31]. As an alternative, heterologous overexpression of viral proteins has been utilized to study HBV and HCV viral protein interactions. Ectopic expression of HCV core protein has been shown to interrupt HBV X-protein (HBx)-mediated trans-activation of HBV genome through direct binding to HBx[32]. HCV core protein has also been shown to form complex with HBV polymerase, thereby inhibiting HBV transcription and viral encapsidation[32]. Recently, Bellecave *et al*[33] established an inducible HBV replicating Huh7 cell system that has consistent replicating subgenomic HCV RNA. In that system, tetracycline-induced HBV replication did not alter the replication of HCV, nor the subcellular localization of HCV proteins. Similar results were also observed in a newly established the HCC cell line, HLCZ01 (derived from HCC tissue of HCV-infected male patient; the HLCZ01 cells are susceptible to the entire life cycle of both HBV and HCV including virus entry, replication and viral particle production)[34]. In that system, HLCZ01 cells were infected with HBV for 10 d and then infected with HCV for another 6 d; following HCV infection, the intracellular HCV RNA gradually increased while the HBV DNA copies were not changed by HCV infection. Additionally, simultaneous infection of HLCZ01 cells by HBV and HCV did not affect either HBV or HCV replication[34]. Thus, the data on the direct interaction between HBV and HCV are rather conflicting; such an inconsistency may relate to different experimental conditions (such as cell system, viral genotypes, or duration of infection, *etc*.), although it points toward the possibility that the documented "viral interference" during HBV/HCV dual infection may be largely mediated by other mechanisms (such as indirect molecular interaction).

Indirect molecular interaction

Infection with HBV or HCV alters host gene expression and cellular phenotypes through diverse mechanism such as integration into host genomes and viral-host protein interaction[35]. Several studies have reported that the gene expression profiles in patients with chronic HBV and HCV are different[36,37]. In patients with chronic hepatitis B, the genes related to pro-apoptotic signaling and DNA repair response are up-regulated, whereas the genes related to immune reaction, lipid metabolism and epidermal growth factor receptor signaling are up-regulated in patients with chronic hepatitis C[37]. While these differentially regulated genes may reflect the difference in the pathogenesis of chronic hepatitis B and C, it is possible that these host genes may mediate indirect molecular interaction between HBV and HCV in the setting of HBV/HCV dual infection.

HCV NS5A has been shown to activate PI3K-Akt pathway through direct binding with p85 regulatory subunit of PI3K[38]. HCV E2 has also been reported to activate PI3K-Akt pathway *via* interaction with CD81 and claudin-1; the resulting PI3K-Akt activation further enhances HCV infectivity[39]. Constitutively active Akt1 results in decreased HBV replication, while inhibition of PI3K-Akt promotes HBV RNA transcription and DNA replication in HepG2.2.15 cells[40]. These findings suggest that HCV may inhibit HBV replication through activating PI3K-Akt pathway in patients with HBV/HCV dual infection. In addition, several studies suggest that HCV core and NS2 proteins inhibit the transcription of HBV through interacting with nuclear receptor family and affecting other cellular transcription factors[41,42].

EMERGING ROLE OF MIR-122 IN HBV/HCV DUAL INFECTION

MiR-122 is implicated in diverse aspects of hepatic functions, including hepatic lipid and cholesterol metabolism and regulation of hepatitis C and B viruses[43]. On the subject of HCV regulation, miR-122 is known to positively regulate HCV replication through direct interaction with the 5′ UTR of the HCV genome. In contrast to the general phenomenon that miRNA-mRNA complex leads to either mRNA degradation or inhibition of translation, miR-122 is unique in that it stabilizes HCV viral RNA by protecting the 5′ terminus of the HCV genome from degradation by the host exonuclease, Xrn-1 and also stimulates HCV translation[44]. Given that the miR-122 promotes the

accumulation of HCV, miR-122 represents an attractive therapeutic target for the treatment of HCV infection. Indeed, silencing of miR-122 has been shown to significantly inhibit HCV replication in cultured liver cells and chronically HCV-infected chimpanzee model[45,46]. A recent clinical study has shown that miravirsen, an antisense inhibitor of miR-122, significantly prolonged the reduction of HCV RNA in patients with chronic HCV genotype 1 infection[47].

On the subject of HBV regulation, miR-122 is known to inhibit the gene expression and replication of HBV. Chen *et al*[48] showed that miR-122 binds to highly conserved region of HBV pregenomic RNA, which is also a bicistronic mRNA encoding the HBV polymerase and core protein, thereby leading to inhibition of HBV gene expression and replication. MiR-122 also inhibits HBV replication by regulating the activity of p53 and its association with HBV enhancer *via* directly targeting of cyclin G1[49]. These findings suggest that liver-specific miR-122 is a critical regulator of viral replication in both HBV and HCV by either directly affecting viral RNA or modulating host gene expression.

The level of miRNAs is tightly controlled by transcriptional or post transcriptional regulation of biogenesis[50]. Although the expression of miR-122 is transcriptionally regulated by liver-enriched transcription factors including HNF4 and C/EBPα[51,52], it can be regulated by HBV and HCV infection. Recent studies have shown that the abundance of miR-122 is different in HCC patients with HBV versus HCV. The levels of miR-122 in patients from HBV-associated HCC is significantly lower compared to HCV-associated HCC[53]. Down-regulation of miR-122 is observed in a stable HBV-expressing cell line, HepG2.2.15, compared to its parental cell line, HepG2[54,55]. In addition, HBV infection also decreases the levels of miR-122 in human hepatocyte and HepaRG cells[56]. Recent evidence suggests that HBx is an important negative regulator of miR-122 expression, highlighted by the fact that HBx binds to peroxisome proliferator activated receptor gamma and inhibits the transcription of miR-122[56]. A separate study shows that HBx decreases miR-122 level post-transcriptionally through downregulation of Germline development 2[55].

Acute HCV infection has been reported to increase the level of miR-122 in cultured cells and chimpanzee models. In HCV infected Huh7.5.1 cells, the level of miR-122 increased at early time points (at day 19 post-infection), then decreased quickly and reached minimum levels at late time points (at day 32 post-infection)[57]. In a chimpanzee model, inoculation of HCV genotype 1 resulted in increased expression of miR-122 at the first 4 wk (rapidly increasing HCV viral titer), followed by declined levels of miR-122 at 10-14 wk (with elevation of serum ALT)[58].

The above findings provide evidence for reciprocal interactions between miR-122 and HBV/HCV (summarized in Figure 1). It is possible that the dominant virus in HBV/HCV dual infection may affect the level of miR-122 and thereby influence the replication of the other virus, although details of miR-122 and HBV/HCV interactions remain to be further defined.

MIR-122 AS THERAPEUTIC TARGET IN PATIENT WITH HBV/HCV DUAL INFECTION

Since HBV/HCV dual infection is heterogeneous, currently there is no standard therapy for the treatment of HBV-HCV dual infected patients. Although interferon-α (IFN-α) is the only treatment option effective for both viruses, early treatment trials showed that HBV/HCV dual infected patients were less responsive to IFN than HCV mono-infective patients[59]. Therefore, it is very important to identify virological profiles including viral dominances in HBV/HCV dual infection to guide treatment. A previous study report that approximately 70% of dual infected patients have active HCV, whereas 38% of patients have active HBV[60]. Multiple studies have evaluated the efficacy of pegylated interferon-alpha (Peg-IFN-α) and ribavirin on HBV/HCV dual infection with active HCV infection. Potthoff *et al*[61] administered Peg-IFN-α/ribavirin to 19 patients with chronic hepatitis C and positive HBsAg for 24 wk; sustained virologic response (SVR) was achieved in 74% patients (14/19); although HBsAg and HBeAg status remained unchanged, HBV-DNA became negative in two of six HBV-DNA positive patients; however, four of the 13 patients (31%) with HBV-DNA negative patients became positive after clearance of HCV. Liu *et al*[62] conducted a larger multicenter clinical trial and found that HCV SVR was 72.2% in dually infected patients *vs* 77.3% in mono-infected patients with genotype 1 infection. For patients with genotype 2/3, SVR were 82.8% and 84%, respectively. Notably, 11.2% (18/161) of the dually infected patients showed HBsAg clearance and serum HBV-DNA became undetectable in 55.9% (38/68) of patients at the end of the follow-up period. In contrast, 36.3% (28/77) dually infected patients whose pretreatment serum HBV DNA was undetectable showed reappearance of HBV DNA. A separate study has demonstrated the effectiveness of IFN and lamivudin in dually infected patients with active HBV[63]. In that study, eight patients received IFN plus lamivudin for 12 mo and followed by 6 mo of lamivudin alone; HBV DNA and HBeAg clearance were observed in 3/8 patients and serum HCV RNA became negative in 4/8 patients. These results suggest that targeting dominant virus through combined IFN plus ribavirin or IFN plus nucleoside/nucleotide analog might be effective in HBV/HCV dual infection.

Studies have shown that SVR to IFN-based therapy is associated with single-nucleotide polymorphisms near the IL28B gene such as *rs12979860*, *rs12980275* and *rs8099917*. The IL-28B rs12979860 CC,

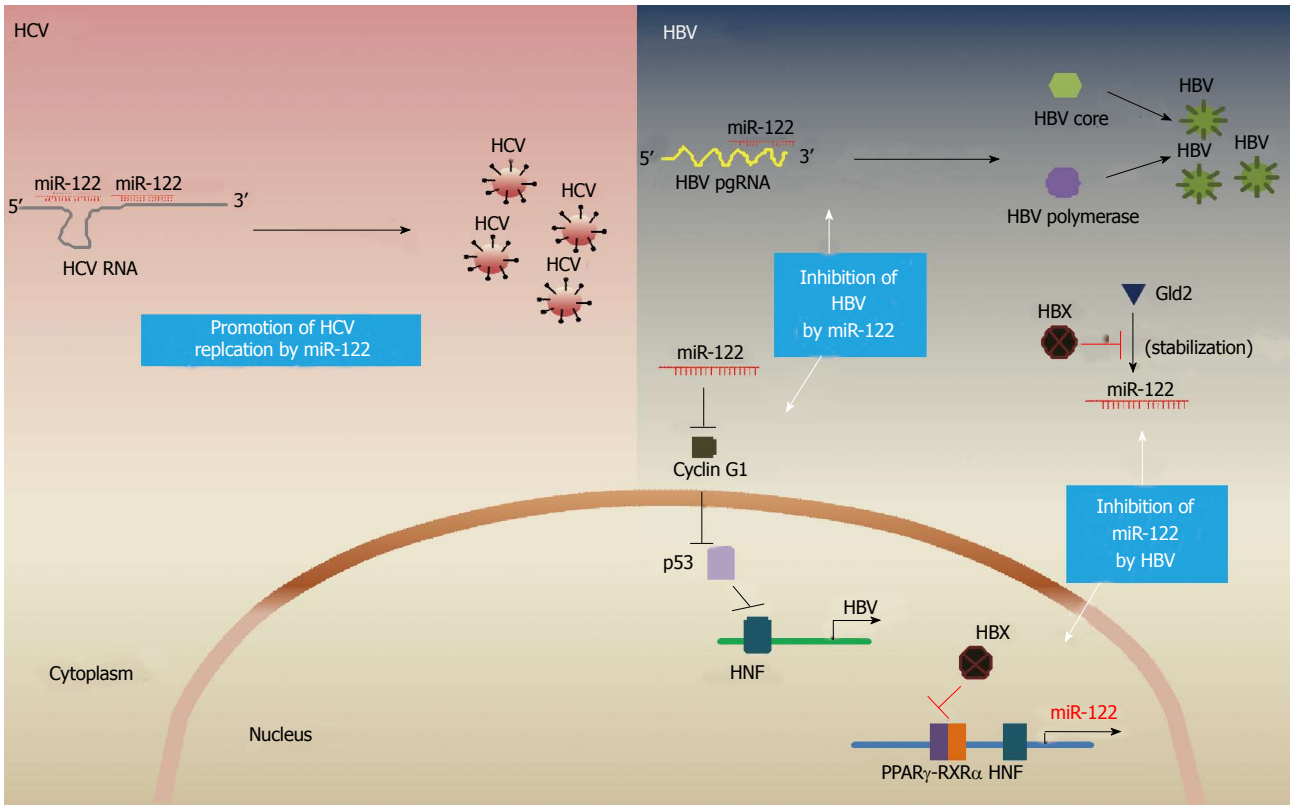

Figure 1 Emerging role of miR-122 in hepatitis B virus and hepatitis C virus infection. Liver specific miR-122 binds to 5'UTR sites of HCV RNA and promotes their translation and replication. In contrast, miR-122 binds to highly conserved HBV pregenomic RNA (pgRNA) and modulating pgRNA stability, leading to inhibition of HBV production. miR-122 also positively regulates p53-mediated inhibition of HBV transcription through direct targeting cyclin G1. HBV infection affects miR-122 expression and stability. Hepatitis B viral X protein (HBX) binds to peroxisome proliferator activated receptor gamma (PPAR-γ) and thereby inhibits miR-122 transcription. In addition, HBX also reduce the miR-122 levels through downregulating Gld2. HBV: Hepatitis B virus; HCV: Hepatitis C virus; miR-122: MicroRNA-122; Gld2: Germline development 2.

rs12980275 AA and rs8099917 TT genotypes are more frequently found in chronic hepatitis C patients (genotype 1) in SVR group than in null virological response (NVR)[64-66]. In case of genotype 2/3, only IL-28B rs12979860 CC genotype is associated with SVR to Peg-INF-α/ribavirin therapy, but not rs12980275 and rs8099917 genotypes[67]. These genotypes are also closely associated with SVR to IFN therapy in patients with HBV infection[68,69]. Consistent with their role in mono-infection, IL-28B genotypes are also associated with SVR to Peg-INF-α/ribavirin therapy in HBV/HCV dual infection. Guo *et al*[70] showed that IL-28B rs12979860 CC and rs8099917 TT genotypes are frequently found in SVR group than NVR in dual infected patients treated with Peg-INF-α/ribavirin. Interestingly, the reactivation rate of HBV DNA was significantly lower in IL28B rs8099917 TT genotypes than their TG + GG genotypes (TT *vs* TG + GG; 13.9% *vs* 41.7%, $P = 0.005$)[70]. Therefore, these results suggest that *IL28B* gene polymorphism can be used to predict HBV reactivation as well as IFN response in HBV/HCV dual infection.

Several recent studies suggest that miR-122 levels might be related to the IL28B genotype. Su *et al*[71] found that IL28B rs8099917 TT genotypes had significantly high pre-treatment miR-122 levels in serum with strong response to treatment than the IL28B GT or GG genotypes. Conversely, low levels of pre-treatment miR-122 have been observed in chronic HCV patients who had no virological response during Peg-IFN-α/ribavirin therapy[71,72]. Hao *et al*[73] showed that IFN-α treatment induced a marked decrease of miR-122 in hepatocyte through sequestration by the IFN-stimulated gene, NT5C3, and thereby negatively affects the anti-HBV efficiency of IFN-α. These findings suggest that the level of miR-122 is regulated by IFN-stimulated genes and is closely related to interferon response in patients with HBV or HCV. Given that Peg-IFN-α/ribavirin is also used as therapeutic drugs for dual infected patients with active HCV, pre- and post-treatment miR-122 levels should be measured in various types of dual infected patients; the data may not only help predict therapeutic response, but also provide insights into viral interaction and reactivation.

As stated in the preceding section, targeting miR-122 using anti-sense nucleic acid is highly effective for the treatment of chronic HCV infection[45-47]. This approach has several advantages, including effectiveness on all HCV genotypes (because of highly conserved miR-122 binding sites across HCV genotypes[74]), no evidence of viral resistance, and lack of significant side effect[46]. However, as mentioned

above, miR-122 has dual function on HBV and HCV replication and it may play a role in reciprocal viral inhibition between these two viruses. Thus, silencing of miR-122 might not be suitable for the treatment of HBV/HCV dual infected patients, even in dual infected patients with dominant HCV, as inhibition of miR-122 may cause reactivation of HBV. Careful testing and screening for HBV is required before initiation of miR-122 inhibitor in patients with positive HCV serology.

On the other hand, evidence also suggests the possibility of miR-122 replacement therapy for the treatment of HBV or even late stage of HCV-associated diseases. Several studies have shown that hepatic level of miR-122 was significantly decreased in the late stage of fibrosis and HCC[75,76], suggesting that miR-122 may inhibit hepatic fibrogenesis and carcinogenesis; these observations argue for miR-122 replacement therapy in late stage of HCV-associated liver disease. Nguyen *et al*[11] found that 83% of HBV-dominant dual infected patients had complete dominance (*i.e.*, with negative HCV RNA). For these cases, it remains to be determined whether miR-122 replacement therapy can be strategized to curtail HBV and improve HBV-associated pathology; should this be attempted, it would be critical to continually monitor HCV replication given the possibility of HCV reactivation. Thus, whether or when to inhibit or enhance miR-122 for therapy requires careful consideration of the context of the viral infections and the stage of the liver diseases.

Given the enormous promise of direct-acting antiviral (DAA) drugs (including NS3/4A protease inhibitors, NS5A and NS5B polymerase inhibitors) for successful interferon-free treatment of HCV across multiple genotypes[77-79], further investigation is warranted to determine whether DAA drugs could be effectively utilized for optimal antiviral therapy in HCV/HBV dual infected patients and whether their antiviral efficacy is dependent upon miR-122.

CONCLUSION

HBV and HCV dual infection is associated with more advanced liver disease than either mono-infection. The modalities and viral dominance are highly variable in patient with HBV-HCV dual infection. Recent clinical and experimental studies have shown reciprocal inhibition between HBV and HCV. However, until now the underlying mechanisms of these inhibitions are poorly understood because of the complicated viral profiles of HBV/HCV dual infection and the lack of optimal experimental models. A better understanding of the dynamic and intricate interactions between HBV and HCV is needed prior to consideration of treatment of HBV/HCV dual infections. The host genes regulated by HBV and HCV viruses have been recognized as important players that mediate the reciprocal inhibition between HBV and HCV and regulate the pathogenesis of viral hepatitis. Recent studies have shown that miR-122 is a crucial host gene that differentially regulates the replication of both HBV and HCV. Conversely, the expression of miR-122 is regulated differently by these two viruses. Although silencing of miR-122 is an attractive therapy against HCV infection, this approach might not be suitable for the treatment of HBV/HCV dual infection, given the potential concern for HBV reactivation. Further studies are needed to better understand the mechanisms for miR-122-mediated hepatotropic viral replication and to devise the optimal regimen for treatment of HBV/HCV dual infection.

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MINIREVIEWS

Cirrhotic cardiomyopathy: Implications for the perioperative management of liver transplant patients

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Abstract

Cirrhotic cardiomyopathy is a disease that has only recently been recognised as a definitive clinical entity. In the setting of liver cirrhosis, it is characterized by a blunted inotropic and chronotropic response to stress, impaired diastolic relaxation of the myocardium and prolongation of the QT interval in the absence of other known cardiac disease. A key pathological feature is the persistent over-activation of the sympathetic nervous system in cirrhosis, which leads to down-regulation and dysfunction of the β-adrenergic receptor. Diagnosis can be made using a combination of echocardiography (resting and stress), tissue Doppler imaging, cardiac magnetic resonance imaging, 12-lead electrocardiogram and measurement of biomarkers. There are significant implications of cirrhotic cardiomyopathy in a number of clinical situations in which there is an increased physiological demand, which can lead to acute cardiac decompensation and heart failure. Prior to transplantation there is an increased risk of hepatorenal syndrome, cardiac failure following transjugular intrahepatic portosystemic shunt insertion and increased risk of arrhythmias during acute gastrointestinal bleeding. Liver transplantation presents the greatest physiological challenge with a further risk of acute cardiac decompensation. Peri-operative management should involve appropriate choice of graft and minimization of large fluctuations in preload and afterload. The avoidance of cardiac failure during this period has important prognostic implications, as there is evidence to suggest a long-term resolution of the abnormalities in cirrhotic cardiomyopathy.

Key words: Cirrhotic cardiomyopathy; Liver transplantation; Diastolic dysfunction; Electrophysiological abnormalities; Perioperative care

Core tip: Cirrhotic cardiomyopathy is characterised by a blunted inotropic and chronotropic response to stress, impaired diastolic relaxation and prolongation of the QT interval. It is only recently that it has been recognised as a definitive clinical entity, and yet it has significant implications in a number of clinical situations in which there is increased physiological demand, which can lead to acute cardiac decompensation and heart failure. Liver transplantation is one such situation, and in this review we discuss criteria for diagnosis, possible methods to limit further deterioration and the perioperative management of these patients.

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INTRODUCTION

Cirrhotic cardiomyopathy (CCM) in a patient with chronic liver disease is a clinical entity characterised by a blunted inotropic and chronotropic response to stress, impaired diastolic relaxation and prolongation of the QT interval. These abnormalities occur in the absence of other known cardiac disease, are independent of liver aetiology, and occur to a variable degree in up to 40%-50% of patients with cirrhosis.

Currently, CCM is often undetected, as it is not widely recognised and is largely asymptomatic at rest, with overt heart failure being rare in cirrhosis. This latent cardiomyopathy classically only manifests itself during periods of physiological or pharmacological stress, but it assumes clinical importance in the setting of events that challenge the cardiovascular system. It is associated with an increased risk of hepatorenal syndrome, particularly following sepsis, and insertion of transjugular intrahepatic portosystemic shunts (TIPS) can precipitate acute cardiac failure following the sudden delivery of an increased volume load to the heart. Liver transplantation presents the greatest physiological challenge, with the significant fluctuations in preload and afterload during the peroperative period. Cardiac decompensation at this stage can lead to graft failure, multi-organ failure and death.

Long term however, there is a reversal of the abnormalities evident in cirrhotic cardiomyopathy, with both structural and functional improvements seen by six to twelve months post-transplantation. It is important therefore to recognise and diagnose this condition of impaired cardiac reserve function to minimise the incidence of decompensation during periods of increased demand. Careful patient selection and additional monitoring for invasive procedures pre-transplantation, followed by appropriate graft allocation and tailoring of both anaesthetic and surgical techniques at the time of transplant may improve postoperative outcomes with improved longer-term survival. The key points are summarised in Table 1.

CLINICAL FEATURES

Cirrhotic cardiomyopathy is characterised by an impaired contractile response to stress, diastolic dysfunction and electrophysiological abnormalities. It is a spectrum of cardiological impairment that has its origin in the haemodynamic changes that accompany end stage liver disease (ESLD). The definition and diagnostic criteria were defined by an international expert consensus committee at the World Congress of Gastroenterology in 2005[1] (Table 2).

Hyperdynamic circulation of cirrhosis

Kowalski *et al*[2] were the first to report the existence of an altered circulation in 1953, describing an increased cardiac output at rest, with an inverse relationship to systemic vascular resistance. In addition, they noted that the increase in cardiac output was not accompanied by a parallel increase in oxygen consumption and that in one third of their study population, the QT interval was prolonged. Their findings were consistently reproduced in subsequent studies[3-7] and the concept of the "hyperdynamic circulation of cirrhosis" was established.

An altered vascular resistance and redistribution of plasma volume has been implicated as the cause of this hyperdynamic circulation[8]. The increase in intrahepatic resistance due to fibrosis causes hypertension in the portal circulation, which stimulates the release of circulating and endothelial vasodilators (both due to a compensatory release and an impaired hepatic degradation). The resulting peripheral arterial vasodilatation and subsequent volume expansion leads to an initially appropriate response of hyperkinesis in the circulatory system.

Over time, with worsening hepatic dysfunction, the increased volume is redistributed to the splanchnic bed. There is a resultant relative reduction in central blood volume (despite an increase in absolute volume), triggering an activation of the SNS and the renin-angiotensin-aldosterone system in an effort to counteract the low arterial blood pressure and volume reduction. Thus patients with cirrhosis exhibit enhanced activity of the SNS with increased circulating catecholamines in direct relation to the severity of the disease[9,10].

These findings of an increased cardiac output at rest and reduced systemic vascular resistance are not present in all patients with ESLD, with the degree of hyperkinesis correlating with worsening hepatocellular insufficiency and portal hypertension[11].

Systolic dysfunction under stress

Left ventricular ejection fraction (LVEF) at rest in the context of chronic liver disease has been reported to be normal[12,13] and often higher than controls[14]. In patients without ascites, the increased pre-load can compensate for cardiac dysfunction, whereas in patients with ascites, the reduced afterload secondary to the systemic arterial vasodilatation compensates for both a decreased preload and contractile dysfunction[15]. Thus, the majority of patients are asymptomatic for heart failure at rest.

When subjected to physiological[5,6,12-14,16] or pharmacological[17,18] stress however, the increase in contractility and cardiac output is significantly blunted in comparison to matched controls. There is an abnormal ventricular response to an increased

Table 1 Cirrhotic cardiomyopathy and liver transplantation

Key points
CCM is a latent cardiac dysfunction that is independent of aetiology and may be unmasked during periods of increased cardiovascular demand
It is characterised by systolic incompetence to stress, diastolic dysfunction and electrophysiological abnormalities
The persistent over-activation of the SNS in cirrhosis leads to down-regulation and dysfunction of the β-adrenergic receptor, a key pathological feature in CCM
Clinical implications include an increased risk of hepatorenal syndrome, cardiac failure following TIPS insertion and increased risk of arrhythmias during acute gastrointestinal bleeding
Diagnosis can be made using a combination of echocardiography (resting and stress), tissue Doppler imaging, cardiac MRI, 12 lead ECG and measurement of biomarkers
Cardiac status should be re-evaluated regularly until liver transplant
Peri-operative management at transplantation should involve careful choice of graft and minimisation of large fluctuations in preload and afterload
Long term there is a resolution of the abnormalities in CCM

CCM: Cirrhotic cardiomyopathy; SNS: Sympathetic nervous system; TIPS: Transjugular intrahepatic portosystemic shunts; ECG: Electrocardiogram.

Table 2 2005 World Congress of Gastroenterology diagnostic and supportive criteria for cirrhotic cardiomyopathy[1]

A working definition of cirrhotic cardiomyopathy
A cardiac dysfunction in patients with cirrhosis characterised by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease
Diagnostic criteria
Systolic dysfunction
Blunted increase in cardiac output on exercise, volume challenge or pharmacological stimuli
Resting LVEF < 55%
Diastolic dysfunction
E/A ratio < 1 (age-corrected)
Prolonged deceleration time (> 200 ms)
Prolonged isovolumetric relaxation time (> 80 ms)
Supportive criteria
Electrophysiological abnormalities
Abnormal chronotropic response
Electromechanical uncoupling
Prolonged QTc interval
Enlarged left atrium
Increased myocardial mass
Increased BNP (brain natriuretic peptide) and pro-BNP
Increased troponin I

BNP: B-type natriuretic peptide; LVEF: Left ventricular ejection fraction.

ventricular filling pressure, which correlates with the severity of liver disease. During certain treatment interventions therefore, such as liver transplantation and TIPS, the volume and pressure load stresses may be significant enough to overcome the "auto-protection" provided by the low systemic vascular resistance and acutely unveil previously asymptomatic latent cardiac disease.

Diastolic dysfunction

Left ventricular diastolic dysfunction (LVDD) is the most prominent characteristic of cirrhotic cardiomyopathy and is thought to precede systolic dysfunction. Its prevalence has been found to be over 50% in cirrhotic patients[19-21] and can be detected at rest using trans-thoracic echocardiography (TTE).

In the early stages of cirrhosis, the expanded blood volume and subsequent increased cardiac preload causes overloading of the left ventricle (LV) and can lead to impaired contractility. There is a resultant increase in LV mass[21] with decreased compliance and relaxation, resulting in abnormal filling of the ventricle. The presence of LVDD has not been found to correlate with the aetiology of liver disease[22], however, the severity of LVDD correlates with worsening liver disease[22,23]. Its prevalence is higher in patients with ascites[19,21,23] and there is evidence to suggest that paracentesis induces an improvement (Figure 1)[24].

The presence and severity of LVDD prior to transplantation has been found to be associated with an increased mortality[22,23,25]. Karagiannakis *et al*[22] evaluated the 2-year probability of patients' survival classified by the presence of diastolic dysfunction and reported survival rates of patients with and without LVDD to be 88.2% *vs* 96.4% at 6 mo; 70.6% *vs* 89.3% at 12 mo; 53% *vs* 85.7% at 18 mo and 47% *vs* 82.1% at 24 mo (Figure 2).

The difference in survival becomes more significant after the first year of follow-up, which is presumably why some shorter studies cannot elucidate a trend. In addition, survival is also related to the severity of LVDD. Ruíz-del-Árbol *et al*[23] reported that after a twelve month follow up period from baseline investigations, survival was 95% in those without LVDD, 79% in those with grade 1 LVDD and 39% in those with grade 2 LVDD.

Electrophysiological abnormalities

Three main electrophysiological abnormalities exist in cirrhotic cardiomyopathy: (1) Prolongation of the QT interval; (2) electromechanical dyssynchrony; and (3) chronotropic incompetence.

A prolonged QTc interval (> 440 ms) is reported to be present in up to 50% of patients with cirrhosis. It is not related to aetiology of liver disease, worsens in parallel with the severity of disease (Figure 3) and may be associated with a reduced survival[26,27]. This abnormality is also frequently prolonged in non-cirrhotic patients with portal hypertension[28].

The QT interval represents length of ventricular electric systole and a prolongation can lead to severe ventricular arrhythmias, syncope and sudden death.

The coupling of electrical depolarisation to ventricular

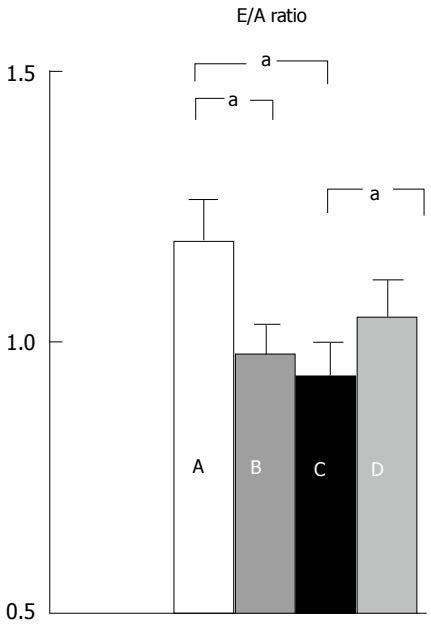

Figure 1 Left ventricular diastolic function in normal subjects (A); cirrhosis (B); cirrhosis with tense ascites (C); cirrhosis with ascites after paracentesis (D). $^{a}P < 0.05$, E/A ratio: (early peak: late peak filling velocities). Ratio declines with worsening LVDD. Pozzi *et al*[24].

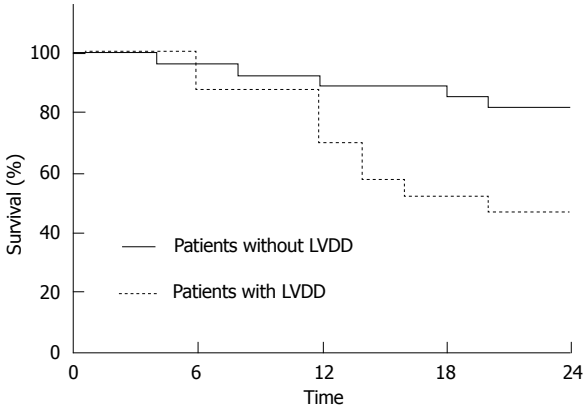

Figure 2 Differences in survival of patients according to the presence of left ventricular diastolic dysfunction. LVDD: Left ventricular diastolic dysfunction. Karagiannakis *et al*[22].

contraction is also abnormal in cirrhosis. Henriksen and colleagues[27] found this electromechanical dyssynchrony to be greater in patients with a prolonged QTc interval, which in turn was related to systemic circulatory dysfunction. The resultant effect of this abnormality is a further impairment of cardiac contractility.

Finally, in CCM there is an impaired ability to increase heart rate in response to activation of the sympathetic nervous system and an increased demand in cardiac output[13,16,29,30]. The decrease in heart rate variability has been found to be associated with increasing severity of cirrhosis and additionally to poor prognosis and increased mortality[31]. It represents an impaired ability to maintain cardiac output at a level that matches cellular demands.

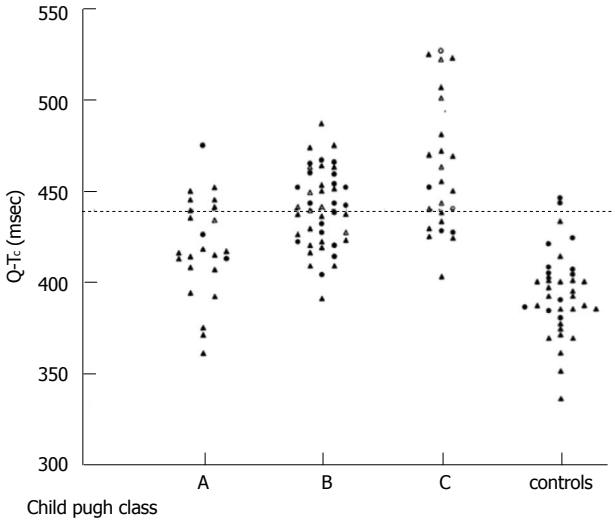

Figure 3 Individual values of QTc interval in patients with cirrhosis (divided according to Child-Pugh classes) and controls. Bernardi *et al*[26].

Cardiac autonomic dysfunction

Autonomic dysfunction is common in cirrhosis and is characterised by an increase in baseline sympathetic nervous system (SNS) activity[10] and decreased baroreflex sensitivity[32]. The baroreceptor and volume receptor stimulation caused by the low arterial blood pressure and blood volume drives this over-activity, causing an increased cellular exposure to noradrenaline and an eventual impairment in β-adrenergic function[9]. These changes play an important role in the development of the hyperdynamic circulation and its adverse effects.

PATHOPHYSIOLOGY

Multiple pathogenic mechanisms have been implicated in the development of cirrhotic cardiomyopathy[33,34]. The diminished inotropic and chronotropic responses to sympathetic stimulation are mainly secondary to a significant down-regulation (reduced density and function) of the β-adrenergic receptors[35] following long-term exposure to persistently high levels of catecholamines[36].

In addition, there is a decrease in the fluidity of the cardiomyocyte plasma membrane caused by an accumulation of cholesterol and bile acid. This adversely affects the receptor-agonist interaction of not only the β-adrenergic receptors[37], but also the other protein receptors embedded in the plasma membrane, ultimately having the effect of compromising cardiomyocyte activation.

There is also increased activity of the cardiac inhibitory systems in cirrhosis, involving substances such as nitric oxide (NO)[38], carbon monoxide (CO)[39] and endogenous cannabinoids (endocannabinoids)[40,41]. The resultant effect is negative inotropy, which further exacerbates the cardiac dysfunction in cirrhotic

cardiomyopathy.

DIAGNOSIS

There is no single diagnostic test that can identify patients with cirrhotic cardiomyopathy and predict who will develop postoperative complications[42,43]. Furthermore, there is no comprehensive data that exists to guide the preoperative cardiac assessment of patients undergoing consideration for liver transplantation.

Diastolic dysfunction

Two-dimensional transthoracic echocardiography: A number of studies to date have used the E/A ratio to determine the presence of diastolic dysfunction, with a ratio < 1 being considered as a positive finding[15,24,44,45]. It represents the pattern of blood flow through the mitral valve. Under normal conditions, early rapid passive filling causes a peak in the transmitral flow profile, known as the E wave, and late rapid filling due to atrial contraction results in a second smaller peak known as the A wave. In the presence of diastolic dysfunction, early passive filling (E wave) is reduced due to an increasingly non-compliant left ventricle, with a greater contribution to filling from atrial contraction during the late phase of diastole (represented by an abnormally large A wave). The overall effect is a reduction in the E wave to A wave ratio.

The E/A ratio may be insufficient as a single parameter to characterise LVDD as it can normalise despite increasing severity of dysfunction. As LV stiffness increases and impairs passive filling, left atrial pressure rises (along with increasing LA dilatation) and eventually drives the filling of the ventricle in early diastole, thereby increasing the E wave. This initially restores the E/A ratio to the normal range, with eventual elevation to supra-normal values. Other features of the TTE are subsequently used to supplement the evaluation of LV relaxation, such as the deceleration time (DT) and isovolumetric relaxation time (IVRT), both of which are initially prolonged, then shortened with increasing severity of dysfunction. Interventricular septal and posterior wall thickness have also been found to be increased in LVDD[14,15,24,46].

In the general population left ventricular stiffness and hence diastolic dysfunction increase with age, so interpretation of TTE findings should be made in context of this variable[47].

Tissue Doppler imaging: The parameters that are measured on conventional two-dimensional (2D) ECHO are dependent on cardiac loading conditions and describe fluid movements rather than tissue dynamics. Tissue Doppler echocardiography has now been suggested as a more accurate modality to assess for diastolic dysfunction[8,47].

Cardiac MRI: Cardiovascular magnetic resonance (CMR) has become the gold standard method for assessing function and morphology in diseases of the myocardium[48], and is of particular use in obese patients where TTE may be suboptimal. It allows repeated evaluation of disease course and detection of subclinical changes prior to dysfunction. The addition of gadolinium contrast (late gadolinium enhancement) has allowed analysis of the intercellular matrix in post-infarct scars, non-ischaemic cardiomyopathies and amyloidosis[48,49]. This ability to analyse myocardial infiltration and fibrosis has diagnostic potential in the early recognition of cirrhotic cardiomyopathy.

Systolic incompetence

Dobutamine and exercise stress echocardiography: In addition to assessing for inducible ischaemia secondary to coronary artery disease, stress echocardiography can be used to detect underlying chronotropic and inotropic incompetence under conditions of increased cardiovascular demand. They are useful screening tools prior to transplantation, but the criteria for undertaking these tests remains varied between institutions.

In a review of 157 pre-transplant dobutamine stress echocardiographys (DSEs), Umphrey *et al*[50] found 37% to be inconclusive due to failure to reach 85% of their maximum predicted heart rate.

Furthermore, the inability to achieve > 82% of this target correlated with an increased risk of having an adverse cardiovascular event up to 4 mo post-OLT. Another group found the incidence of chronotropic incompetence on DSE in cirrhosis to be over 85%[51], when defined by a heart rate reserve of less than 80%. No patient had taken medication that had an effect on haemodynamics until the study was completed. In contrast to this, out of the 58 patients with an inconclusive test in the previous study, 50% had taken a beta-blocker within 12 h of the investigation.

Since beta blockade is responsible for moderation of the heart rate, it raises the question as to whether or not patients undergoing DSE, in part to assess for chronotropic incompetence, should have this drug withheld for 24 h prior to this investigation to enable a more accurate and comparable evaluation of their underlying cardiac reserve function.

STRAIN AND STRAIN RATE IMAGING BY ECHOCARDIOGRAPHY

Strain imaging using either tissue doppler or myocardial speckle tracking and strain rate imaging are becoming increasingly recognised as valuable non-invasive tools for a more comprehensive and reliable assessment of myocardial function[52]. These modalities can detect early changes in systolic function at rest. The long axis sub-endocardial fibres are thought to be more susceptible to damage than the radial-

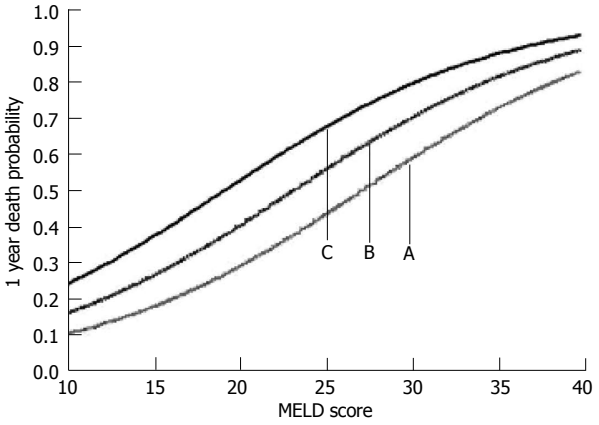

Figure 4 Estimated 1 year probability of dying as a function of: (A) MELD score alone; (B) MELD score and hs-TnT of 4-8 ng/L; and (C) MELD score and hs-TnT of > 8 ng/L. Taken from Wiese *et al*[64].

orientated ones in the midwall[20] so may convey the first manifestation of cardiac impairment. In addition, peak systolic strain rate has been found to be more closely related to contractility than ejection fraction determined by TTE[52].

Electrophysiological abnormalities

Twelve lead Electrocardiogram: Prolongation of the QT interval, when corrected for heart rate (QTc) is commonly quoted to be > 440 ms. In normal subjects, QT interval is also affected by age and gender, with the length being longer in older subjects and females. However, in cirrhosis this gender difference is abolished[53].

Biomarkers

Atrial natriuretic peptide (ANP) is secreted in response to atrial stretch, with the resultant effect of lowering blood pressure and cardiac preload. It is stored as pro-ANP, which is cleaved to ANP and NT-proANP, the latter of which is thought to be a better marker of LV dysfunction[54,55]. ANP levels reflect volume overload and are increased in patients with ascites[46].

B-type natriuretic peptide (BNP) is similarly cleaved from its pro-peptide and released from ventricular myocytes in response to ventricular wall stretch or myocardial ischaemia[56], with the primary purpose of reducing cardiac hypertrophy and fibrosis[57]. Increased concentrations have been detected in cirrhosis, with levels correlating with the severity of liver disease, presence of diastolic dysfunction, myocardial hypertrophy and survival[46,49,58,59]. Preoperative levels of BNPs (BNP and NT-proBNP) are powerful independent predictors of cardiovascular events and mortality in patients undergoing non-cardiac surgery[60,61], with further enhancement of this risk stratification seen with the additional measurement of postoperative levels[62,63].

High sensitivity Troponin T (hs-TnT) is also increased in decompensated cirrhosis with evidence emerging for it to be a strong independent prognostic marker for survival. Wiese *et al*[64] found that the risk of dying within 1 year predicted by the MELD score is increased by a factor of 1.6 if the hsTnT is 4-8 ng/L and by a factor of 2.7 if hsTnT is more than 8 ng/L (Figure 4).

Proinflammatory markers: Inflammatory activation has been shown to aggravate the circulatory failure and worsen the prognosis in cirrhosis. There is evidence to show that levels of soluble urokinase-type plasminogen activator receptor (suPAR) and high-sensitive C-reactive protein (hsCRP) are potential markers of this interaction. Levels have been found to correlate significantly with Child class and haemodynamic derangement[64].

Currently, the commonly used scoring systems for assessment of severity of liver disease such as MELD and Child Pugh do not incorporate the circulatory or inflammatory state. Therefore, these markers may in the future enable a more comprehensive assessment of the liver transplant candidate.

CLINICAL IMPLICATIONS

Pre-transplant

TIPS: Prior to liver transplantation, additional events that take place in the clinical course of a patient with end stage liver disease may be impacted on by the presence of cirrhotic cardiomyopathy. In particular, diastolic dysfunction may become clinically problematic following procedures that involve a sudden volume load to a stiff, non-compliant ventricle.

One such procedure is the insertion of a TIPS, in which a high splanchnic blood flow is rapidly delivered to the systemic circulation, resulting in an almost two fold increase in central pressures and pulmonary capillary wedge pressure[65,66]. This not only aggravates the hyperdynamic circulation, with a sustained rise in cardiac output for up to 1 year post procedure[49], but also several cases of heart failure following TIPS have been reported[67-69]. In addition, there is evidence to suggest that those patients with diastolic dysfunction defined by an E/A ratio $\leqslant$ 1 have a lower post-TIPS ascites clearance and probability of survival at one year than those without[44,45] (Figures 5 and 6).

The non-compliant left ventricle renders the patient less able to adequately increase their cardiac output in response to the sudden volume load which, in addition to the worsening of the vasodilation following the procedure, results in a relative under-filling of the central blood volume and poor clearance of the ascites. The dumping of cardioactive substances from the splanchnic to systemic circulation post-TIPS also causes a further prolongation of the QT interval, which worsens post procedure at 1-3 mo and stays elevated above the baseline value[28].

The above findings suggest that careful patient selection for TIPS is needed and a consideration of

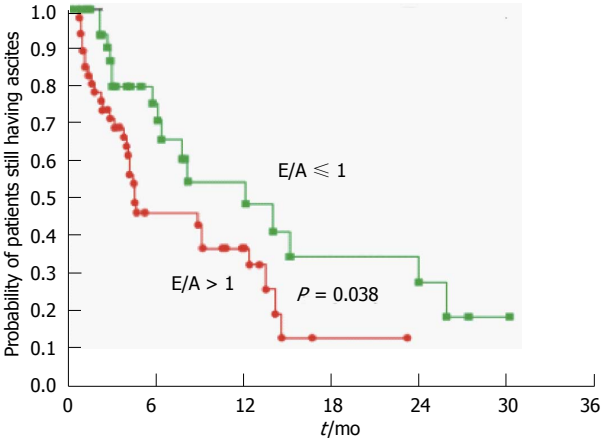

Figure 5 Probability of patients still having ascites after transjugular intrahepatic portosystemic shunts insertion in the group with diastolic dysfunction (E/A ⩽ 1) and the group without (E/A > 1). Rabie *et al*[45].

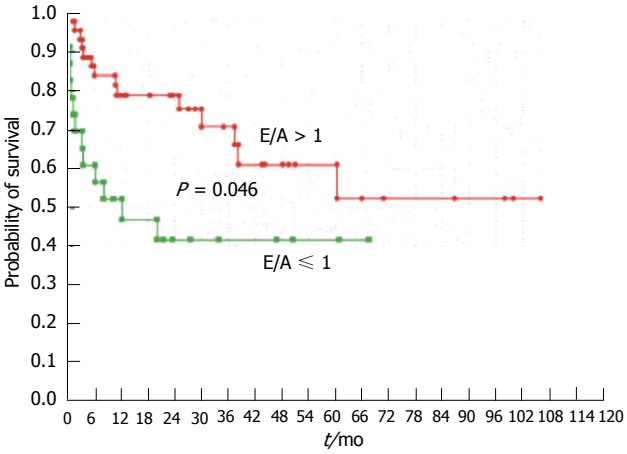

Figure 6 Probability of survival in patients with (E/A ⩽ 1) or without (E/A > 1) diastolic dysfunction. Rabie *et al*[45].

referral directly to transplantation may be warranted in those with diastolic dysfunction. If TIPS is undertaken, increased cardiac monitoring post-procedure may be beneficial in those patients with suspected cirrhotic cardiomyopathy, for early recognition and treatment of potential cardiovascular complications.

Hepatorenal syndrome

This is a functional renal failure that occurs in the presence of liver dysfunction, splanchnic vasodilatation and intense activation of the endogenous vasoconstrictor systems, with important prognostic implications in a patient with decompensated cirrhosis. There is increasing evidence to suggest that inotropic and chronotropic insufficiency in CCM may have a role in the progression to hepatorenal syndrome (HRS). Krag *et al*[70] demonstrated that the number of cirrhotic patients with ascites who developed HRS type 1 within three months of initial assessment was higher in the group with a low cardiac index (< 1.5 L/min per square meter) than in the high cardiac index group (43% *vs* 5% respectively). Furthermore, patients with the lowest cardiac index at baseline had significantly poorer survival at 3, 9 and 12 mo.

Likewise a cardiac output < 6.0 L/min was found to be an independent predictor of HRS in a group of patients with cirrhosis and tense ascites who, at baseline had normal creatinine levels (Figure 7)[71]. The implication is that the patients who are unable to maintain cardiac output in the presence of systemic arterial vasodilatation at a level that maintains a sufficient renal perfusion pressure are predisposed to the development of renal dysfunction and HRS.

Spontaneous bacterial peritonitis

The susceptibility of a patient with a decreased cardiac reserve to further organ impairment becomes more pronounced in the face of systemic infection. In a study of patients with a diagnosis of spontaneous bacterial peritonitis (SBP), those that subsequently developed renal failure had a reduced cardiac output both at the time of infection diagnosis and at infection resolution[72]. If the additional physiological demands caused by the inflammatory mediated vasodilatation and negative inotropy could not be met, a rapidly progressive impairment of systemic haemodynamics followed, with severe renal and hepatic failure, aggravation of portal hypertension, encephalopathy and death.

Gastrointestinal bleeding

QT prolongation in cirrhosis is common and one of the postulated causes is the baseline sympathetic nervous system hyperactivity. Therefore, a sudden increase in this activity caused by acute gastrointestinal bleeding further lengthens this time interval with potential adverse outcomes. Trevisani *et al*[73] showed that in a group of cirrhotic patients with this complication, the QT interval lengthened at the time of bleeding and all those that died had a longer QTc than survivors. In addition, QTc was able to independently predict survival, with a best cut-off value of ⩾ 460 ms.

IMPLICATIONS OF DIAGNOSIS AND MANAGEMENT

When a patient displays features of CCM, this is an indication to perform more frequent cardiovascular reassessment, with regular TTE studies perhaps every three months prior to transplantation. Deterioration can then be detected earlier, enabling prompt management of symptomatic heart failure, appropriate cardiovascular monitoring at the time of TIPs or acute gastrointestinal bleeding and appropriate fluid therapy and circulatory support on diagnosis of SBP.

Positive diagnosis may also affect the decision to proceed with TIPS, as underlying CCM has been associated with not only suboptimal ascites clearance and LV dysfunction, but also increased mortality. In

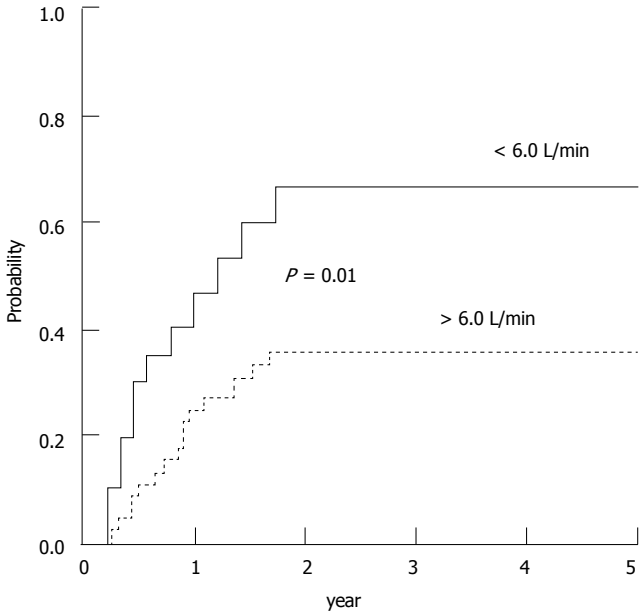

Figure 7 Probability of developing hepatorenal syndrome during follow-up in patients with baseline cardiac output higher and lower than 6 L/min. Ruiz-del-Arbol *et al*[71].

this situation the decision may be to refer straight to transplantation.

At the time of transplantation, the presence of an underlying latent cardiac failure may also affect the choice of graft. Mittal *et al*[74] followed up 970 liver transplant recipients over an average of 5.3 years, and found that those with pre-transplant diastolic dysfunction were significantly more likely than those without to develop acute cellular rejection (58.6% *vs* 31% respectively), graft failure and all-cause mortality, making the initial quality of graft ever more important (Figures 8 and 9).

Management

There is currently no specific treatment for cirrhotic cardiomyopathy. Patients are managed as for heart failure due to other aetiologies, with sodium restriction, diuretics and careful management of their preload and afterload. Vasodilators such as ACE inhibitors are avoided as they exacerbate the reduced systemic vascular resistance.

Many patients with cirrhosis are beta-blocked for the management of portal hypertension. It is still unclear what impact this has on CCM, but the additional negative inotropic and chronotropic effects may have an important role in the development of and outcome from adverse cardiac sequelae during periods of increased physiological demand. On the other hand, both the acute and chronic administration of beta-blockers have been shown to shorten the QT interval in cirrhotic patients who have elevated baseline values[75,76]. Although the improved QT interval potentially has preventative benefits in the context of life-threatening arrhythmias, the deleterious effect of beta blockade on cardiac output and hence clinical outcome remains to be studied.

Long-term aldosterone blockade may also have potential therapeutic benefits. In preliminary studies it has been shown to reduce left ventricular wall thickness and may potentially improve diastolic dysfunction[77].

INTRAOPERATIVE

Maintaining preload and fluid management

During liver transplantation there are significant haemodynamic fluctuations and immense physiological demands on the recipient. The third space losses over the long procedure can be copious with the added effect of substantial blood loss and on-going ascites production[43]. If a patient has cirrhotic cardiomyopathy, an important intraoperative aim should be to minimise excessive fluctuations in preload and afterload, to avoid acute cardiac decompensation.

Surgical technique can greatly influence physiological status, with cross clamping of the inferior vena cava causing the greatest cardiovascular instability. In a patient with cirrhotic cardiomyopathy the use of caval preservation techniques (piggyback), or the addition of veno-venous bypass if caval cross clamp is employed, will minimise the large variations in preload with the maintenance of caval flow during explantation[78,79]. This would decrease the reliance on positive chronotropy to maintain cardiac output in the face of reduced venous return, a compensation mechanism that may be impaired in CCM. It could also attenuate the sudden change in preload on reperfusion of the new liver, which can precipitate acute left ventricular failure and pulmonary oedema if diastolic dysfunction is present.

Reperfusion also involves the release of cold, ischaemic metabolites and vasoactive mediators from the graft that can further exacerbate systemic vasodilatation and cause myocardial depression. An impaired contractile response to stress may render the patient unable to overcome these effects and result in an inadequate cardiac output for tissue perfusion, including the newly implanted liver. Techniques to

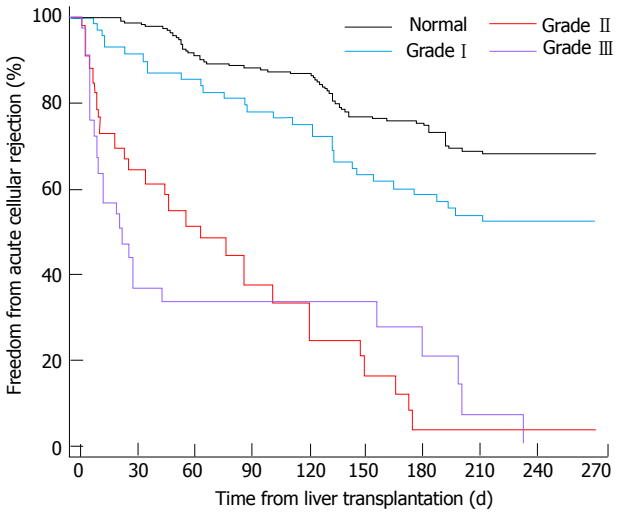

Figure 8 Time to acute rejection for various grades of left ventricular diastolic dysfunction. Mittal *et al*[74].

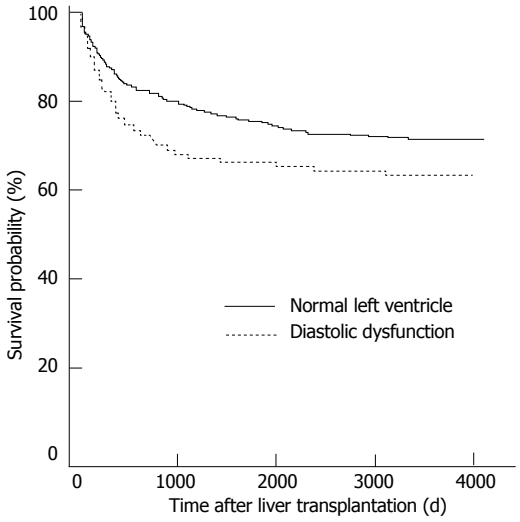

Figure 9 Survival analysis for patients with left ventricular diastolic dysfunction *vs* patients without. Mittal *et al*[74].

minimise reperfusion "hit" are especially important in the context of CCM, including normalising acid base and electrolyte status prior to reperfusion, and the use of as good quality grafts as possible.

Haemodynamic monitoring

In light of the special considerations in CCM during OLT, additional haemodynamic monitoring may be warranted for the early detection of cardiac decompensation and to guide therapy. Both aggressive fluid administration and excessive vasoconstriction can precipitate acute cardiac failure in this group, so careful titration of inotropic support and the avoidance of excess intravenous fluids are necessary.

The use of pulmonary artery flotation catheters (PAFC) enables measurement of continuous cardiac output and the derivation of systemic vascular resistance and hence afterload. In addition many PAFCs allow measurement of right ventricular end diastolic pressures and volume. Other methods of continuous non-invasive cardiac output (CO) monitoring are less accurate, unless recalibrated at times when there are significant changes in CO, *i.e.*, after caval cross clamping and following reperfusion.

Transesophageal echocardiography (TOE) is an imaging modality that is becoming increasingly popular in evaluating intraoperative haemodynamic status during liver transplantation[80]. It provides the added benefit of real time assessment of the response to fluid, direct visualisation of global and regional myocardial performance and detection of air and thromboembolism[81]. In addition, it can allow an earlier visual detection of right ventricular failure, as the substantial compliance of the right ventricle allows significant dilation before any pressure changes that would be detected with a PAFC[82]. It also permits re-evaluation of the recipient's cardiac function at the start of surgery, which would highlight deterioration since previous assessment. A limiting factor over its use however, is the training required for competent scanning and interpretation of images[83]. There is also the small risk of bleeding from oesophageal varices.

Both PAFC and TOE have important advantages in the guidance of therapy for the CCM patient. The choice between the two remains operator dependent and reliant on skill base and experience.

Electrolytes/arrhythmias

During liver transplantation, there can be further prolongation of the QT interval with the associated increased risk of ventricular arrhythmias. One study reported that 54% of recipients showed a marked prolongation of QTc (⩾ 500 msec) during the anhepatic phase, with values remaining prolonged at each stage compared to baseline[84]. 77% of the patients with a pre-operative increased QTc and 36% of the group with a normal starting value showed this abnormality.

Electrolyte imbalance is common during this procedure and can further precipitate cardiac instability. Ionised hypocalcaemia can occur as a result of citrate toxicity following the transfusion of large amounts of citrated blood products causing further depression of myocardial contractility. Reperfusion of the donor liver can acutely raise serum potassium to levels that can potentially result in fatal arrhythmias. Additionally, hypomagnesaemia has been shown to precipitate cardiac arrhythmias that are refractory to conventional treatment and can also lead to hypokalaemia and hypocalcaemia[85]. Careful monitoring of these electrolytes and judicious replacement should be employed to minimise further cardiac instability in a condition with pre-existing limited cardiac reserve.

POST OPERATIVE COURSE

Immediately after liver transplantation there is a significant increase in blood pressure and peripheral vascular resistance with restoration of normal liver

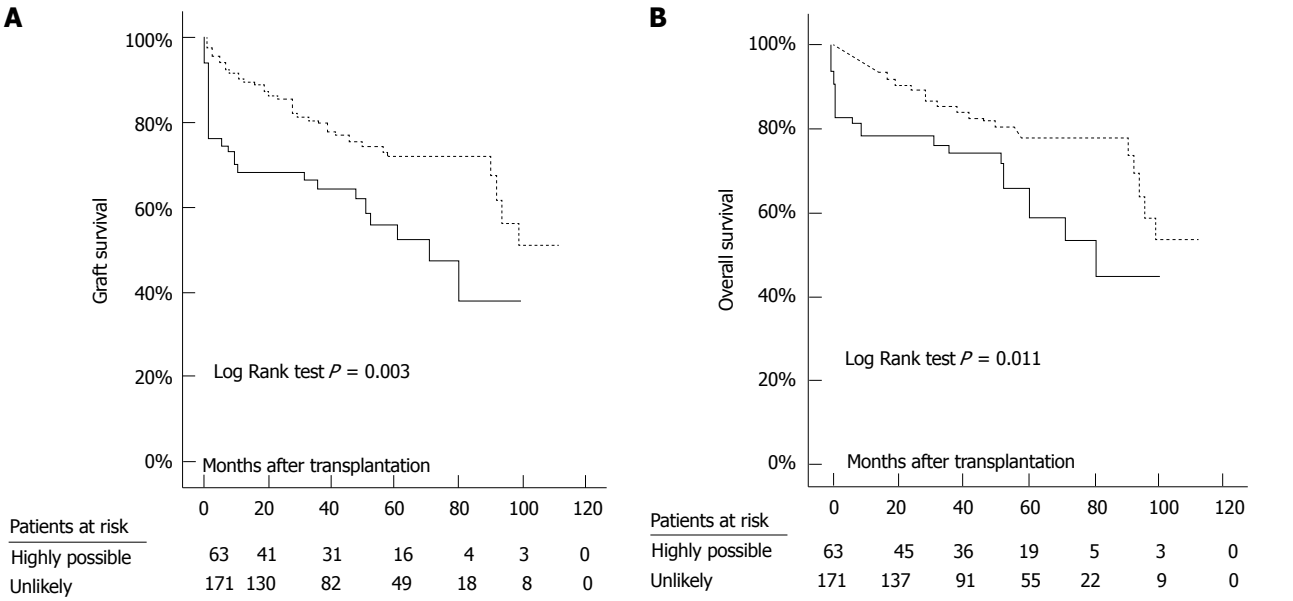

Figure 10 Graft (A) and overall (B) survival in patients with highly possible (continuous line) and those with unlikely (dashed line) peri-transplant heart failure. Josefsson *et al*[25].

function and portal pressure. This increase in preload and afterload can be another stage at which cardiac de-compensation in CCM occurs, typically in the first week after OLT. Various authors have reported cases of pulmonary oedema following this procedure, a complication that can impair gas exchange and stress critical oxygen delivery to the new organ[86,87], with incidences being reported at between 30% and 50%[88-90].

Peri-transplant heart failure and myocardial depression have been found to correlate with both a preoperative impaired cardiac reserve function and increased morbidity and mortality post-operatively[25,91]. Josefsson *et al*[25] found it to be associated with a longer ITU stay (14.5 d *vs* 4 d), a longer inpatient stay (33 d *vs* 21 d), a higher peri-transplant infection rate (42% *vs* 19.9%) and higher long term patient and graft mortalities (Figure 10).

In a search to identify predictors of heart failure after liver transplantation, Dowsley *et al*[92] found that markers of diastolic dysfunction on pre-operative TTE (E/e' > 10 and LA volume index $\geqslant$ 40 mL/m^2) were associated with a 3.4 fold and 2.9 fold increase in risk respectively. Furthermore, Kaplan-Meier analysis of LA volume index below and above 40 mL/m^2 revealed survival differences of 82% *vs* 54% at 1 year and 71% *vs* 50% at 5 years respectively. Therapondos *et al*[93] found elevated baseline BNP levels in those who went on to develop pulmonary oedema in the first 3 mo after transplant. These pre-transplant markers that are associated with an increased risk of heart failure may aid the prediction of post liver transplant outcomes.

LONG TERM POST OLT

After transplantation, there is a triphasic course of myocardial recovery in CCM. The first phase is the aforementioned perioperative cardiac failure. Over the next few weeks, the restoration of the portal circulation and the hypertensive side effects of the calcineurin inhibitors administered for immunosuppression, result in an additional increase in afterload, potentially leading to further decompensation. LV diastolic function (as measured by E/A and IVRT) has been shown to deteriorate for up to 3 mo post transplant[93].

In the final phase, there appears to be a recovery of functional, structural and electrophysiological abnormalities. Whereas the hyperdynamic syndrome normalizes in some patients over time[94], it persists in others[95]. There is a significant improvement in myocardial performance, with normalization of the systolic response to stress, improvement of diastolic dysfunction and regression of ventricular wall thickness between 6-12 mo after transplantation[30,49]. In addition, there is an improvement of QT prolongation in approximately 50% of patients from as early as three months[96-98].

CONCLUSION

Cirrhotic cardiomyopathy describes a condition of impaired cardiac reserve function. It is characterised by a systolic incompetence to stress, diastolic dysfunction and electrophysiological abnormalities. Although mostly asymptomatic at rest, it can be unveiled during periods of physiological or pharmacological stress and predisposes the patient to complications such as hepatorenal syndrome, acute cardiac failure and life-threatening arrhythmias. Regular re-evaluation of cardiac status should be undertaken to minimise the risk of decompensation. Management at the time of liver transplantation should involve careful selection of donor grafts and tailoring of the anaesthetic and

surgical techniques to avoid large fluctuations in preload and afterload. If cardiac failure in the peri-operative period can be avoided, there is potentially a good outcome with long term reversal of the cardiovascular abnormalities.

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Recommendations for the use of chemoembolization in patients with hepatocellular carcinoma: Usefulness of scoring system?

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Abstract

Several hepatocellular carcinoma (HCC) staging systems have been established, and a variety of country-specific treatment strategies are also proposed. The barcelona - clinic liver cancer (BCLC) system is the most widely used in Europe. The Hong Kong liver Cancer is a new prognostic staging system; it might become the reference system in Asia. Transarterial chemoembolization (TACE) is the most widely used treatment for HCC worldwide; but it showed a benefit only for intermediate stage HCC (BCLC B), and there is still no consensus concerning treatment methods and treatment strategies. In view of the highly diverse nature of HCC and practices, a scoring system designed to assist with decision making before the first TACE is performed or prior to repeating the procedure would be highly useful.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Barcelona Clinic Liver Cancer; Prognostic scoring systems

Core tip: Despite its widespread use in hepatocellular carcinoma, the indications for Transarterial chemoembolization are still debated. There are no rules about the treatment modalities or strategy to follow. To overcome these difficulties, a simple scoring system, including prognostic variables, designed as a decision support, would be useful. Deciding when we have to move from a loco-regional treatment to a systemic option is matter of significant interest, particularly since sorafenib now provides us with a solution.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths[1]. It generally develops secondarily to chronic liver disease, mainly after a hepatitis B or C viral infection[2]. It is therefore particularly prevalent in sub-Saharan Africa and South East Asia. It is increasingly common in Europe and the Unites States of America[3]. Nonalcoholic steatohepatitis is a frequently reported risk factor[4,5]. HCC is a complex disease because, in addition to the characteristics of the tumour (its dimensions, the number of lesions present, vascular invasion and extrahepatic spread) and the patient's performance status, therapeutic decision-making also takes into account other parameters such as liver function, since both underlying cirrhosis and portal hypertension (PHT) complicate the treatment of HCC and limit the available curative options which are also impacted by delays in diagnosing the cancer[6]. Several HCC staging systems integrating these prognostic variables have been established as a guide to treatment practices, and a variety of country-specific treatment strategies are also proposed[7]. However, no "universally" recognised classification exists, leading to wide variations in treatment practices, particularly where patients who are not eligible for curative treatment are concerned. Several Asian countries have their own staging system[8]. In Europe and the United States, the barcelona-clinic liver cancer (BCLC) system is the most widely used and is approved by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases[9]. This staging system has demonstrated superiority over other systems and is based on randomised clinical trials. The BCLC divides HCC into four groups as a function of the number of nodules present, their size, the Child-Pugh score, the presence of PHT, performance status (PS), the presence of symptoms, vascular invasion and extrahepatic spread. It has the distinct advantage of proposing an evidence-based treatment strategy for different stages of the disease (Figure 1). Based on local clinical experience and expert opinions, the Asian guidelines recommend different treatment methods including external radiotherapy and intra-arterial hepatic chemotherapy. The Hong Kong liver Cancer (HKLC) is a new prognostic staging system established using the prognostic factors of 3856 patients most of whom presented with HBV[10]. The HKLC might become the reference system in Asia. It includes variables comparable to those in the BCLC system but also takes into account the diffuse nature of HCC. It divides HCC into 9 sub-groups and also puts forward a treatment strategy (Figure 2). We recently validated this scoring system in a European cohort consisting of 665 patients most of whom had alcoholic cirrhosis- or hepatitis C virus-related HCC; however, unlike the results published by Yau *et al*[10], we found that the HKLC and BCLC classifications were similar in their discriminatory ability for the prediction of survival[11].

A preliminary intermediate analysis of the international Bridge study showed that transarterial chemoembolization (TACE) is the most widely used treatment for HCC worldwide, ahead of both surgical removal and systemic treatments[12]. TACE is designed to induce necrosis of the hyperarterialised tumour with the aim of achieving local tumour control whilst preserving liver function. Its use has been recommended since the publication of two positive randomised studies in 2002[13,14]. The technique has since been improved with the use of calibrated drug-eluting beads, making it possible to standardise the procedure and reduce the systemic passage of the cytotoxic substances used[15], with comparable outcomes to those of conventional TACE in terms of tumour control (PRECISION V trial)[16].

RECOMMENDATIONS FOR THE USE OF CHEMOEMBOLIZATION IN PATIENTS WITH HCC

Despite its widespread use, the indications for TACE are still debated. The two reference randomised trials included 112 and 80 patients, the survival benefit was limited (4 mo)[17] and the results of the meta-analyses are contradictory. Unlike the earlier meta-analyses[17,18], Oliveri *et al*[19] did not find any benefit for TACE, but the analysis was criticised because it included inappropriate studies[20,21]. In Europe, TACE is recommended for intermediate stage HCC (BCLC B), but this group includes a broad spectrum of tumours (encapsulated or infiltrating, unifocal or multifocal) and patients with different degrees of liver function and consequently the survival benefit is not the same, for instance, for patients classified Child-Pugh A and B[22,23]. Careful patient selection is therefore necessary, particularly since sorafenib now provides us with a solution for cases in which chemoembolization is contraindicated or ineffective[24]. Based on evidence and experts' opinion, the general consensus is that TACE is appropriate for large nonresectable (> 50 mm) or multinodular, asymptomatic tumours without vascular invasion or extrahepatic spread and when the Child-Pugh class is A or B7[25]. In parallel, Raoul *et al*[26] listed the cases of intermediate stage HCC in which TACE was contraindicated. The factors identified included age, comorbidities, liver function and tumour characteristics, especially diffuse HCC and tumours measuring in excess of 100 mm in diameter (Figure 3).

However, no consensus exists for treatment methods, the drugs to be used, the type of beads, the number of courses to be administered, the interval to be allowed between sessions and the objectives (complete response, disease stabilization). In the randomised study by Lo *et al*[14], cisplatin-based TACE was performed every 2 to 3 mo until disease progression, depending on individual tolerance; the patients treated received a mean of 4.5 sessions. In

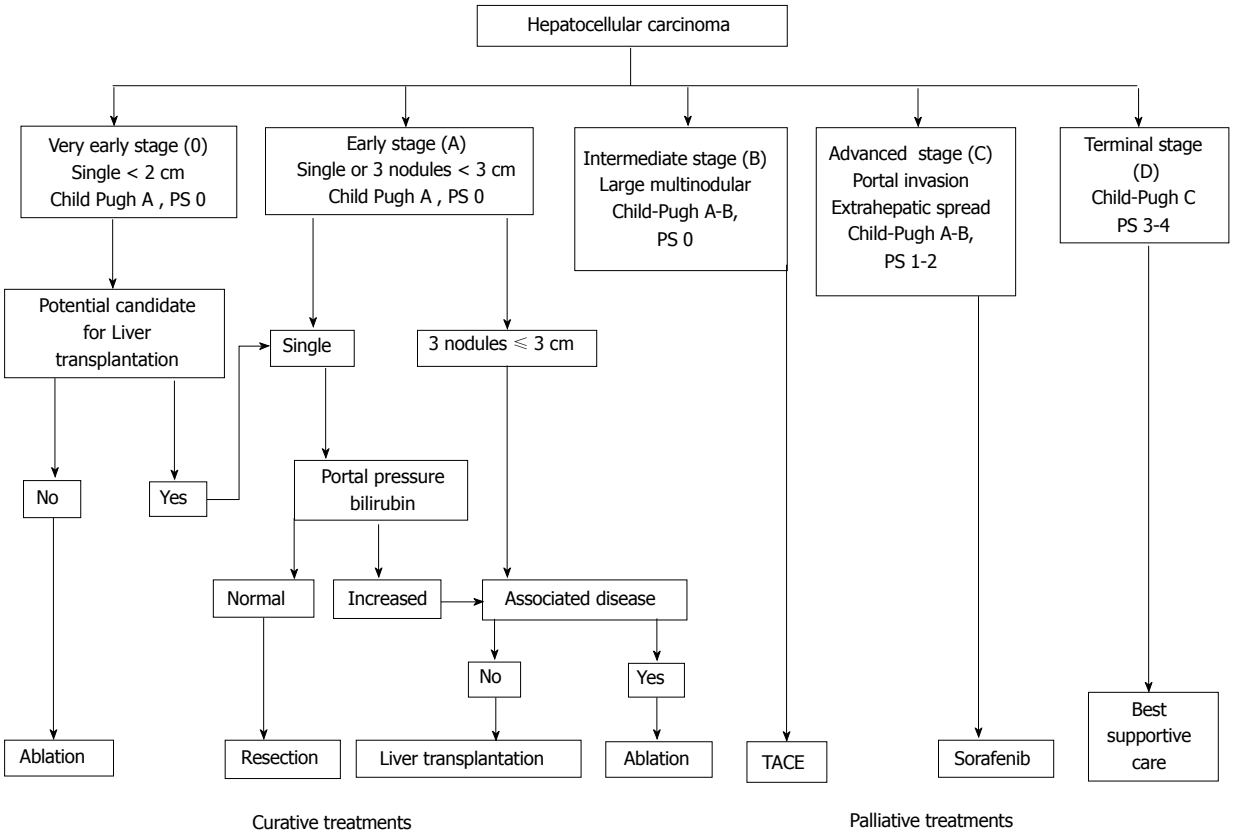

Figure 1 Barcelona Clinic Liver Cancer staging and treatment strategy[9]. BCLC: Barcelona Clinic Liver Cancer; PS: Performance status; TACE: Transarterial chemoembolization.

the randomised study by Llovet *et al*[13], doxorubicin-based TACE or arterial embolization alone were performed 2 and then 6 mo after the initial session, and then potentially every 6 mo thereafter until progression (vascular invasion or extrahepatic spread), depending on individual tolerance. These patients received a mean of 3.08 embolization sessions and 2.8 of TACE. There is currently no standardised treatment timescale: the sessions can be given at regular intervals or on an "as needed" basis, as a function of the radiologic response. TACE is a potentially toxic treatment and its efficacy can be outweighed by its toxicity particularly since many of the patients treated have underlying cirrhosis[27,28]. Two recently published studies (on demand or selective sequential TACE) report comparable results in terms of survival[22,23]. Antoch *et al*[22] treated 124 patients with HCC graded BCLC A (32%), B (39%) and C (29%) with 4 (± 3) selective TACE sessions, repeated every 4 wk. There was a 4.7% major complications rate, including 1 death. The 1 year survival rate was 79%, dropping to 51% at the 2 year time point. In a study by Terzi *et al*[23], 151 patients with BCLC A (51.5%), B (40%) and C (6%) HCC were treated with on demand TACE. The complete response rate after the first TACE was 48% for the 151 patients treated; it was 57% for the 60 patients undergoing a 2nd session and 55% for the 22 patients treated a third time. The median time to progression was 8.5 mo between the first two TACE, and 8 mo between the second and third sessions. The 1 year survival rate was 70%, dropping to 52% at the 2 year time point. There are no firm data demonstrating the superiority of one strategy over another, but performing TACE guided by the radiologic response and individual tolerance appears to be the most logical option.

Raoul *et al*[26] proposed a treatment strategy based on the radiologic response observed after two TACE (Figure 4). A partial response or a disease stabilization is considered sufficient justification to interrupt treatment, since TACE is regarded as a palliative treatment option for locally advanced disease[26]. Other authors believe that a response must be obtained before treatment can be repeated and equate stability with treatment failure[29,30]. Evaluation of response is therefore a major challenge. It is not possible to appreciate tumor response after TACE using the conventional dimension criteria Response Evaluation Criteria In Solid Tumor (RECIST); a beneficial effect is not always associated with tumor reduction and requires for its assessment the advantages of functional imaging[31]. Contrast uptake criteria - both EASL and mRECIST - provide a more accurate evaluation of response after TACE. While the EASL and mRECIST criteria differ in terms of target lesions (respectively all *vs* ⩽ 2) and calculation methods (bidimensional *vs* unidimensional), they are comparable and correlated with survival. In the

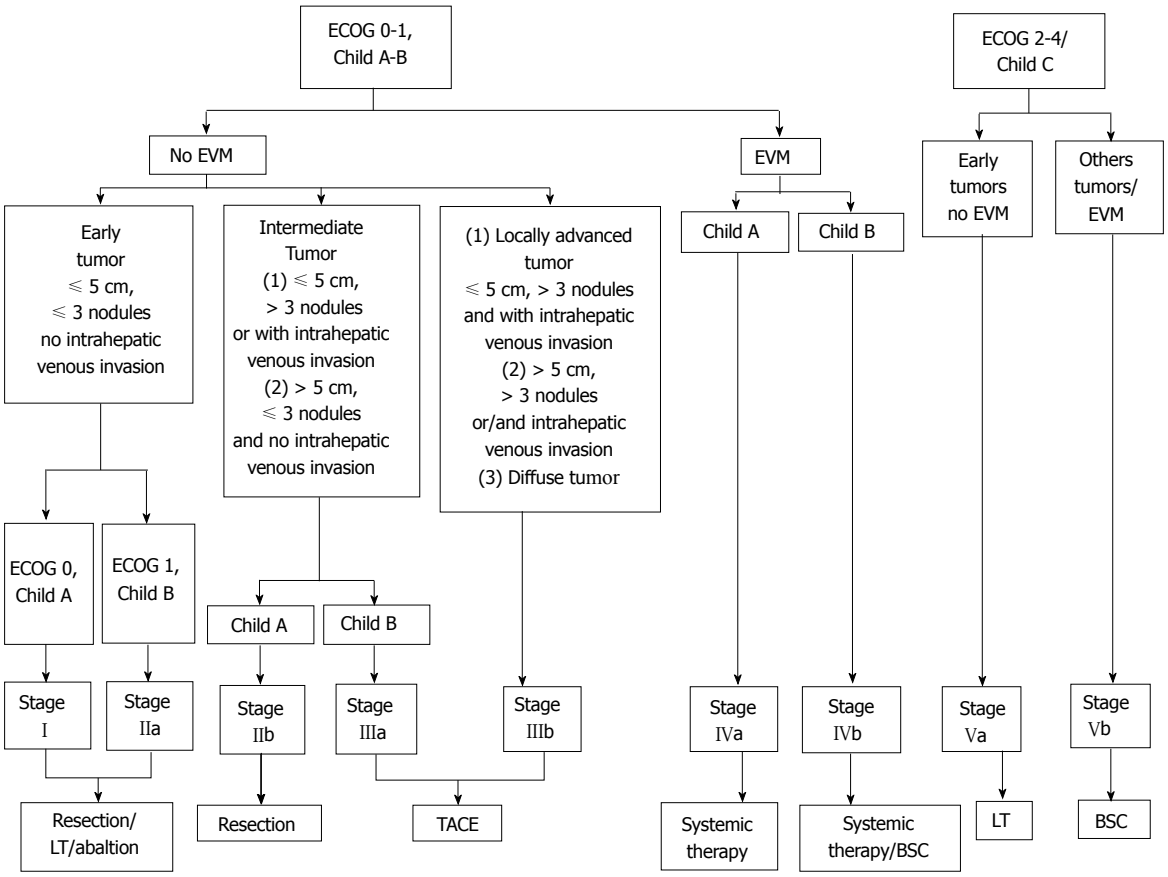

Figure 2 Hong Kong Liver Cancer prognostic classification scheme[10]. HKLC: Hong Kong Liver Cancer; LT: Liver transplantation; OS: Overall survival; PS: Performance status; TACE: Transarterial chemoembolization; EVM: Extrahepatic vascular invasion/metastasis.

Absolute contraindications
- Decompensated cirrhosis (Child-Pugh B ⩾ 8) including:
  - Jaundice
  - Clinical encephalopathy
  - Refractory ascites
  - Hepatorenal syndrome
- Extensive tumor with massive replacement of both entire lobes
- Severely reduced portal vein flow (*e.g.*, non tumor portal vein occlusion or hepatopetal blood flow)
- Technical contraindications to hepatic intra-arterial treatment, *e.g.*, untreatable arteriovenous fistula
- Renal insufficiency (creatinin ⩾ 2 mg/dL or creatinine clearance < 30 mL/min)

Relative contraindications
- Tumor size ⩾ 10 cm
- Comorbidities involving compromised organ function:
  - Active cardiovascular disease
  - Active lung disease
- Untreated varices at high risk of bleeding
- Bile-duct occlusion or incompetent papilla due to stent or surgery

Figure 3 Contraindications for conventional transarterial chemoembolisation[26].

studies by Gillmore *et al*[32] and Kim *et al*[33], radiologic response according to the EASL and mRECIST criteria was found to be independent prognostic factor for survival. However, these criteria are not applicable for all types of HCC[34].

There is no consensus concerning the rules for discontinuing treatment. It appears logical that TACE should not be pursued in cases of "obvious" tumour progression, which Bruix *et al*[29] referred to as "Untreatable progression", *i.e.,* massive liver involvement, extrahepatic spread and vascular invasion. Other contraindications include a significant deterioration in liver function after the first session and failure to achieve an objective response after two sessions (Figure 5). In such cases, a different treatment option will be offered if permitted by the patient's performance status and liver function.

In routine practice, TACE has applications beyond intermediate stage HCC. It is a therapeutic option for certain patients with BCLC A HCC who are not eligible for curative treatment. In a study by Burrel *et al*[35], median survival in selected BCLC A patients treated with microbeads was 54.2 mo *vs* 47.7 mo in patients with intermediate stage HCC. Some authors also

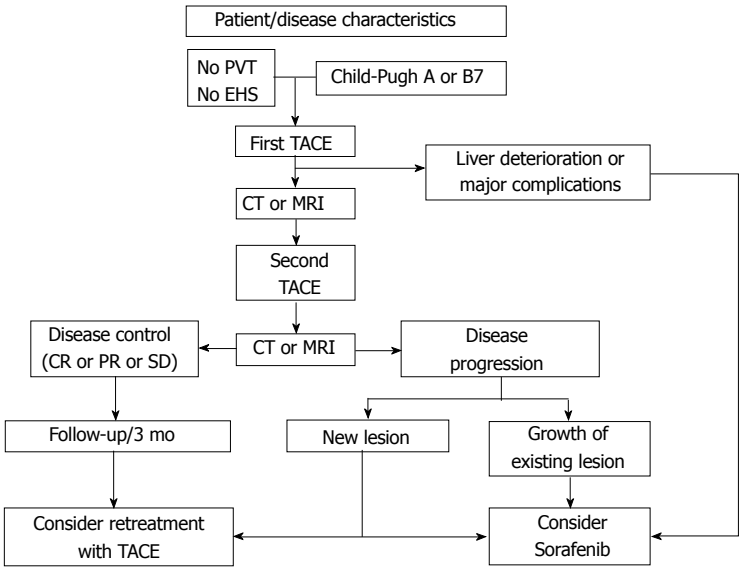

Figure 4 Proposed treatment algorithm for the repetition of conventional transarterial chemoembolization in patients with intermediate-stage hepatocellular carcinoma. Response defined according to modified RECIST criteria. CR: Complete response; CT: Computed tomography; cTACE: Conventional TACE; EHS: Extrahepatic spread; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; PD: Progressive disease; PR: Partial response; PVT: Portal vein thrombosis; RECIST: Response Evaluation Criteria In Solid Tumours; SD: Stable disease; TACE: Transarterial chemoembolization.

consider that TACE can be used to treat advanced HCC, since progression to metastatic disease is rare[36]. In the randomised study by Lo *et al*[14], about 20% of the patients treated with TACE presented with segmental portal vein thrombosis but no significant difference in survival was detected amongst these patients whether they were treated with TACE or not. In both the Asian Pacific Association for the Study of the Liver (APASL) guidelines and HKLC scoring system, TACE is a possible treatment option for patients with HCC and limited portal vein thrombosis[8,10] (Figure 6). A number of Asian studies and a meta-analysis of eight trials (including five retrospective studies) have shown a survival benefit *vs* untreated control arms, on condition that the portal vein thrombosis is limited[37-40]. However, it is not possible to validate the use of TACE in this indication on the basis of these results. In addition to the risks related to embolization, the recurrence rate is relatively high in patients with vascular invasion; segmental portal vein thrombosis was the independent prognostic factor with the greatest impact on survival in a Japanese cohort of 8510 patients treated with TACE[41]. A combination of sorafenib and TACE has therefore become a viable treatment option for patients with locally advanced HCC. A retrospective study conducted in Austria found comparable survival results with sorafenib and TACE in patients with locally advanced HCC and vascular invasion (BCLC C)[42]. However, there are no studies providing a definitive response for such patients at the present time. The sorafenib-TACE combination has been explored in two recently published meta-analyses, each including six studies. However, there are large variations in the designs of the studies included (randomised and retrospective cohorts), the populations enrolled (intermediate and advanced HCC) and the treatment methods[43,44]. Zhao *et al*[45] suggest using the sorafenib-TACE combination in certain BCLC C patients as a function of a score calculated from four independent prognostic variables: vascular invasion, Child-Pugh class A or B, number of nodules 1-2 or $\geqslant$ 3, and ECOG (Eastern Cooperative Oncology Group) PS 0 or $\geqslant$ 1. These results have yet to be confirmed and validated in a prospective study before they can be more widely applied.

USEFULNESS OF SCORING SYSTEM?

We do not have any guidelines concerning the number of TACE to be performed before switching to another treatment strategy. It is evident that, in view of the highly diverse nature of HCC and practices and the numerous therapeutic options now available, a scoring system designed to assist with decision making before the first TACE is performed or prior to repeating the procedure would be highly useful. Several prognostic indices designed to help practitioners select appropriate candidates for an initial or repeat conventional TACE have been put forward in the past but none has been formally enshrined in the guidelines since they are difficult to implement or insufficiently discriminatory and are limited to conventional TACE[46,47]. The potentially useful staging systems published recently include: the hepatoma arterial-embolization prognostic (HAP) score published by Kadalayil *et al*[48] in 2013 which was also designed as an aid to selecting appropriate candidates for TACE. In this system, patients are awarded 1 point for each of the following four variables if present: albumin < 36 g/L, bilirubin > 17 mcmol/L, AFP > 400 ng/mL and tumour > 7 cm. The patients were then divided into four median survival groups on the basis of their HAP scores: HAP A (0 points) 27.6 mo, HAP B (1 point) 18.35 mo, HAP C (2 points) 9.0 mo and HAP D (> 2 points) 3.6 mo. This scoring system was developed using the prognostic variables generated by a cohort of 114 BCLC A (35%), B (31%), C (31%) and D (4%) patients included over a period of more than 10 years . It was validated in 167 patients considered to be comparable, but more of whom presented with segmental portal vein

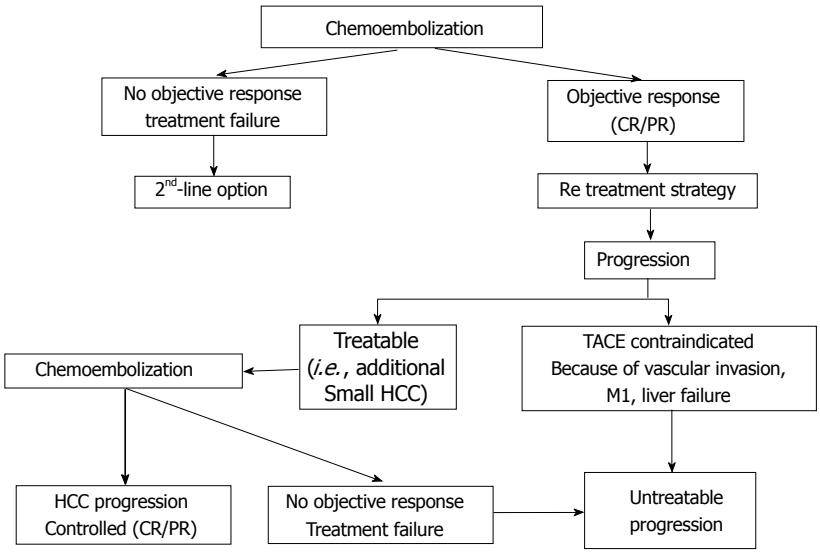

Figure 5 Diagram to define untreatable tumor progression[29]. CR: Complete response; PR: Partial response; HCC: Hepatocellular carcinoma.

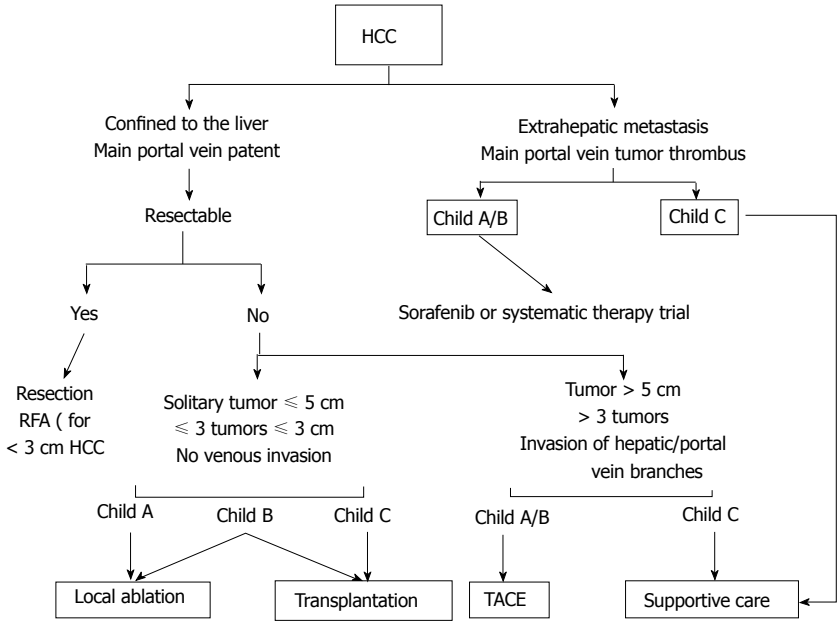

Figure 6 APASL guideline on the treatment algorithm for hepatocellular carcinoma[8]. RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization.

thrombosis (28% *vs* 6%, respectively). These authors suggest that a strategy other than TACE is more appropriate for C and D score patients.

The assessment for retreatment with TACE (ART) score published by Sieghart *et al*[30] in 2013 is calculated before performing a second TACE. It is based on three parameters (increase of ASAT by > 25%, increase in Child-Pugh score from baseline and tumor response). Increase (+ 25%) in ASAT was the parameter associated with the most powerful coefficient (4 points), the lowest was allocated to the radiological response (1 point)[30]. Patients are divided into two groups on the basis of the resulting scores (0-1.5 drop in score or drop of 2.5 points and over) with a different prognosis: 23.7 *vs* 6.6 mo. This system was developed using a regression model in a cohort of 107 patients enrolled over 10 years, most of whom presented with alcoholic cirrhosis and were BCLC B HCC. The authors suggest continuing TACE until the score changes from 0 to 1.5. This score is also applicable to subsequent courses[49].

We performed a retrospective analysis of the HAP score in a cohort of 153 Child- Pugh A (91%) or B (9%) cirrhotic patients with BCLC A (17%) (not suitable for curative treatment), BCLC B (69%) and BCLC C (14%) (owing to segmental portal vein thrombosis) HCC[50]. These patients underwent a mean of 2.75 conventional TACE sessions. The response rate (EASL criteria) was 61%. Mean follow-up was 19 mo [17-23]. The HAP score divided the patients into three groups each with a different survival time (Table 1); median survival was the same for the HAP A and HAP B groups (31 mo). The patients in the HAP C and HAP D groups were considered to be poorer candidates for TACE and had a median survival of 22 mo and 18 mo, respectively, which is higher than the figures reported for the Kadalayil *et al*[48] cohort (9.0 mo and 3.6 mo, respectively). It should be noted that the risk of death

Table 1 Overall survival in a cohort of 153 patients treated by TACE using the HAP score with a cut-off value of: 0 (HAP A) *vs* 1 (HAP B) *vs* 2 (HAP C) *vs* > 2 (HAP D)

HAP	HAP A (*n* = 46)	HAP B (*n* = 43)	HAP C (*n* = 49)	HAP D (*n* = 15)
Median-survival, mo (95%CI)	31 (25-37)	31 (20-51)	22 (17-25)	18 (6-32)
P value		0.0454		

HAP: Hepatoma arterial-embolization prognostic; TACE: Transarterial chemoembolization.

in the HAP B and HAP D groups was not significantly different from that in the HAP A reference group [HR = 0.88 (0.52-1.50), *P* = 0.640; HR = 1.56 (0.81-2.99), *P* = 0.1820, respectively]. Only the patients in the HAP C group were at significantly higher risk of death *vs* the HAP A reference group [HR = 1.69 (1.02-2.80); *P* = 0.0436]. In total, the ability of the score to identify good candidates for TACE appears limited, since each variable is allocated the same number of points (1 point), for example, albumin < 36 g/L and AFP > 400 ng/mL; and only HCC exceeding 70 mm are taken into account although the success of TACE is partially dependent on the size of the tumour (generally less than 50 mm) and the number of lesions present[51].

According to Kudo *et al*[52], the ART score is only applicable to a minority of patients in Japan, since the interval between two sessions exceeds 3 mo; the treated tumours being smaller as a result of a best screening in the country. In the patients treated twice consecutively (only 9.6% of the population), the ART score did not highlight a significant difference in survival between the two groups. As our TACE method was similar to that of the Viennese team, we carried out a retrospective analysis of ART scores before the second TACE in a total of 321 French patients with viral and/or alcoholic HCC enrolled in two cohorts in Marseilles and one in Nancy[53]. In order to create a population similar to that of Sieghart *et al*[30], we selected patients who had undergone at least two successive conventional TACEs without any other treatments, excluding patients undergoing pre-transplant TACE or presenting with severe cirrhosis (Child-Pugh ⩾ 9). Our patients included BCLC B HCC, BCLC A HCC who were not eligible for curative treatment and unlike the study by Sieghart *et al*[30], cases of BCLC C HCC with sectoral portal vein thrombosis, as these patients were treated with TACE in routine practice before the advent of sorafenib (Table 2). Radiologic response was assessed using the EASL scoring system.

Our findings differed from those of Kudo *et al*[52] since, in our three cohorts, the ART score clearly divided the patients into two groups with different median survival times for an aggravation of 0-1.5 *vs* ⩾ 2.5. However, changes in the score were not correlated with the prognosis in these three cohorts. Median survival was lower in patients with an ART score of 1, *i.e.,* not showing a radiologic response, *vs* those with an ART score of 4, *i.e.,* an increase in AST > 25% (Tables 3-5). Fifty-six percent (56%), 23% and 38% of the patients with an ART score of 4 in each of the cohorts, respectively, showed a radiologic response. Unlike the results reported by Sieghart *et al*[30], an increase in AST > 25% was not an independent prognostic factor in the Marseilles cohorts. We analysed and compared the patients showing a partial and complete radiologic response in the ART 0-1.5 and ART ⩾ 2.5 groups (in cohorts 1 and 2) (Table 6). The median survival times were similar, but the patients were offered a different treatment strategy as per the approach recommended by Sieghart *et al*[30].

Table 2 Baseline patients and disease characteristics in three sets (%)

Characteristics	Cohort 1 (*n* = 139)	Cohort 2 (*n* = 82)	Cohort 3 (*n* = 100)
Age, median, yr (95%CI)	67 (65-68)	63 (60-69)	68.5 (66-71)
Sex, M/F	84/16	90/10	88/12
Cirrhosis or advanced fibrosis (F3)	100	100	94
Aetiology: Virus/alcohol/virus + alcohol/ NASH	47/35/6/10	49/29/9/7	27/46/6/8
Child-Pugh score: A/B	69/31	75/25	95/5
BCLC A/B/C	47/34/19	34/46/20	10/81/9
Infiltrative tumours	17	22	2
Segmental portal vein thrombosis	15	19.5	9
AFP < 200 ng/mL	78	60	77
AFP ⩾ 200 ng/mL	22	40	23
Diagnosis based on: Imaging/ biopsy	85/15	77/23	80/20
Incidental/screening/symptoms	17/70/13	31/53/16	19/66/15
Previous treatments (surgery, RFA)	15	15	18

NASH: Non-alcoholic steatohepatitis; BCLC: Barcelona Clinic Liver Cancer; AFP: α-fetoprotein; RFA: Radiofrequency ablation.

We assessed the ART score before the 3rd TACE in 126 cirrhotic patients with virally induced (57%) or alcohol-induced HCC (33%); these patients were BCLC A (45%), BCLC B (45%) and C (10%), and had undergone an average of 4 TACE[54]. The score also distinguished two groups with a significantly different median survival time, but changes in the score were not correlated with prognosis. Once again, median survival was lower in the patients with an ART score of less than 1 than those whose score was evaluated at 4 (Table 7).

In view of these results, the ART score calculated before the 2nd and 3rd TACE cannot be used to define the treatment strategy for all patients, particularly those whose ART score is evaluated to be 1 and 4, poorly distributed. A prospective study is required to further explore and establish the prognostic value of this score.

CONCLUSION

TACE is the most widely used treatment for HCC

Table 3 Overall survival in the first cohort of patients using the ART score calculated before the second transarterial chemoembolisation with a cut-off value of: 0-1.5 *vs* ⩾ 2.5

ART (*n* = 139)	ART [0] (*n* = 67)	ART [1] (*n* = 11)	ART [1.5] (*n* = 18)	ART [2.5] (*n* = 3)	ART [3] (*n* = 2)	ART [4] (*n* = 16)	ART [5] (*n* = 5)	ART [5.5] (*n* = 5)	ART [6.5] (*n* = 3)	ART [7] (*n* = 2)	ART [8] (*n* = 7)
Median-survival, mo (95%CI)	37 (31-42)	9 (7-14) 34 (28-38)	28 (25-40)	10 (5-27)	17 (12-21)	28 (7-36)	14 (12-16) 13 (10-16)	13 (6-15)	5 (3-5)	22 (8-36)	5 (4-11)
P value ART (0, 1.5) *vs* ART ⩾ 2.5						< 0.0001					

TACE: Transarterial chemoembolization; ART: Assessment for retreatment with TACE.

Table 4 Overall survival in the second cohort of patients using the ART score calculated before the second TACE with a cut-off value of: 0-1.5 *vs* ⩾ 2.5

ART (*n* = 82)	ART [0] (*n* = 39)	ART [1] (*n* = 14)	ART [1.5] (*n* = 5)	ART [2.5] (*n* = 1)	ART [3] (*n* = 3)	ART [4] (*n* = 5)	ART [5] (*n* = 10)	ART [5.5] (*n* = 1)	ART [8] (*n* = 4)
Median-survival, mo (95%CI)	27 (22-38)	11 (7-18) 22 (15-27)	15 (11-50)	N/A	10 (3-31)	31 (8-31)	8 (7-12) 10 (8-23)	N/A	8 (4-23)
P value ART (0, 1.5) *vs* ART ⩾ 2.5					0.07				

TACE: Transarterial chemoembolization; ART: Assessment for retreatment with TACE.

Table 5 Overallsurvival in the third cohort of patients using the ART score calculated before the second TACE with a cut-off value of: 0-1.5 *vs* ⩾ 2.5

ART (*n* = 100)	ART [0] (*n* = 38)	ART [1] (*n* = 30)	ART [1.5] (*n* = 3)	ART [2.5] (*n* = 8)	ART [4] (*n* = 10)	ART [5] (*n* = 8)	ART [6.5] (*n* = 2)	ART [8] (*n* = 1)
Median-survival, mo (95%CI)	49 (36-63)	21 (17-26) 27.4 (24.7-37.8)	23 (21-23)	13 (6-15)	24 (19-35)	19 (9-20) 15.5 (13.0-23.7)	14 (13-15)	9 (-)
P value ART (0, 1.5) *vs* ART ⩾ 2.5				0.0001				

TACE: Transarterial chemoembolization; ART: Assessment for retreatment with TACE.

Table 6 Overall survival of patients using the ART score calculated before the third TACE with a cut-off value of: 0-1.5 *vs* ⩾ 2.5

ART (*n* = 126)	ART [0] (*n* = 73)	ART [1] (*n* = 12)	ART [1.5] (*n* = 6)	ART [2.5] (*n* = 4)	ART [4] (*n* = 21)	ART [5] (*n* = 2)	ART [6.5] (*n* = 4)	ART [7] (*n* = 2)	ART [8] (*n* = 2)
Median-survival, mo (95%CI)	35 (30-37)	12 (10-18) 31 (27-36)	34 (27-38)	13 (8-24)	28 (19-41)	21 (9-32) 21 (13-28)	8 (5-9)	28 (25-31)	6 (4-8)
P value ART (0, 1.5) *vs* ART ⩾ 2.5					0.004				

TACE: Transarterial chemoembolization; ART: Assessment for retreatment with TACE.

but its efficacy has mainly been demonstrated in a selected population of patients with intermediate stage HCC, *i.e.,* with large (> 50 mm) or multinodular nonoperable tumours, without vascular invasion or extrahepatic spread and a Child-Pugh class of A or B7. Other "good" indications probably exist, including some forms of limited HCC which are not eligible for curative treatment (radiofrequency ablation or surgery), and even some cases of advanced HCC in combination with other treatments, but this can only be confirmed by randomised studies. While there is still no consensus concerning treatment methods, including the use of DC beads *vs* conventional TACE, the indications and contraindications of TACE and the strategy to be employed are better defined than previously, as are the rules for discontinuation-for example, the

Table 7 Characteristics, median survival, comparative study of patients (first and second cohorts) with an objective radiologic response in both ART 'groups before the second TACE (%)

Patients with radiologic response	ART (0-1.5) (*n* = 113)	ART ⩾ 2.5 (*n* = 28)	*P* value
AFP < 200 ng/mL	81	82	1.00
AFP ⩾ 200 ng/mL	19	18	
Child-Pugh A/B	77/23	61/39	0.05
BCLC A/B/C	55/41/4	50/42/8	0.14
Median TACE sessions (95%CI)	3 (3-4)	2 (1-5)	0.17
Median-survival, mo (95%CI)	33 (27-38)	28 (13-35)	0.04
Median follow-up, mo (95%CI)	25 (22-29)	21 (13-31)	0.42

BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization.

"untreatable progression" defined by Bruix *et al*[29]. The classification systems do not as yet take into account the histological criteria or biomarkers correlated with survival nor do they integrate all the patients whose profiles differ depending on their geographical location. Consequently, a score that combines different prognostic markers could be a useful aid when deciding to perform a first or repeat TACE and also help standardise current strategies, especially since new treatment options are now available (biotherapies, radioembolization in clinical trials). However, none of the recently published scores (ART, HAP) replaces the rigorous selection of patients on the basis of their liver function, underlying conditions, tumour characteristics and EASL or m RECIST radiologic response correlated with post-TACE survival times.

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MINIREVIEWS

Hepatitis C virus reinfection after liver transplant: New chances and new challenges in the era of direct-acting antiviral agents

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Abstract

The first interferon-free regimens have been approved for the treatment of patients with chronic hepatitis C virus (HCV). In the liver transplant (LT) setting, these regimens are expected to have an important effect, because graft loss due to HCV recurrence is a serious problem after LT. The response to the hitherto conventional treatment with pegylated interferon and ribavirin is poor. The significantly better response rates achieved with boceprevir-based and telaprevir-based triple therapy have led to better graft and patient survival rates, but severe drug interactions with immunosuppressants limit the feasibility of this therapy for LT patients. With the approval of sofosbuvir in January 2014, of simeprevir in May 2014, and of daclatasvir in August 2014, three antiviral agents are now available and promise to be applicable without relevant adverse effects or negative interactions with immunosuppressants. Thus, 2014 marks the beginning of a new era of treatment options for HCV recurrence after LT. Although safety and efficacy studies of several interferon-free regimens for patients with HCV recurrence after LT have achieved good preliminary results, reports of clinical experiences with LT patients are scarce. The lack of randomized studies, the small number of enrolled and carefully selected patients, and the heterogeneity of these studies make the results questionable. Real-life experiences are eagerly awaited so that clinicians can estimate the usefulness and the pitfalls of these new regimens. Additionally, the high costs of these agents may limit their accessibility for many patients. The aim of this review is to summarize the current experience with and the expectations of the new direct-acting antiviral agents for LT patients.

Key words: Hepatitis C virus; Liver transplant; Interferon; Sofosbuvir; Simeprevir; Daclatasvir

Core tip: In the liver transplant (LT) setting, graft loss due to hepatitis C virus (HCV) recurrence is a serious problem after LT. The former conventional treatment with pegylated interferon and ribavirin is unsatisfying, due to poor response rates and tolerability. With the first interferon-free regimens that are currently being approved for the treatment of patients with chronic HCV, 2014 marks the beginning of a new era

of treatment options for HCV recurrence after LT. This review summarizes the current experience with and the expectations of the new direct-acting antiviral agents in the setting of LT.

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LIVER TRANSPLANT IN THE SETTING OF CHRONIC HCV INFECTION

Chronic hepatitis C virus (HCV)-induced end-stage liver disease, with or without hepatocellular carcinoma, is still the leading indication for liver transplant (LT), and reinfection of grafts by HCV is the main cause of allograft loss[1,2]. Most patients experience recurrence of HCV infection after LT, and such recurrence can be associated with substantially accelerated cirrhosis of the graft in as many as 30% of patients[3,4]. A subgroup of patients experience fibrotic cholestatic hepatitis (FCH), a severe and extremely aggressive form of HCV recurrence characterized by rapid progression to graft failure and death. Once cirrhosis develops, the annual risk of hepatic decompensation is approximately 40%, and 10% to 25% of patients will die or require retransplantation within 5 years after the first LT[5]. Unfortunately, the outcome of patients undergoing re-transplantation is poor, and most transplant centers are reluctant to offer a second LT for patients with cirrhosis of the graft due to HCV reinfection[6,7].

The shortage of donor organs, in conjunction with the accelerated progression of HCV in LT patients, emphasizes the need for effective clinical strategies aimed at treating or preventing HCV recurrence after transplant. Three approaches have been described, according to the timing of treatment: antiviral therapy before LT, which is appropriate only for patients with compensated cirrhosis; preemptive treatment after LT[8]; and treatment of an established reinfection[9]. Thus, after transplant, HCV patients can be treated either immediately with a preemptive approach or with a recurrence-based approach when liver damage is diagnosed. The advantages of preemptive or early treatment after transplant are low serum HCV-RNA levels and no substantial damage to the graft, as determined by histologic studies[10]. Although these factors are positive predictors of a favorable outcome, this therapeutic approach has been difficult to manage because the combination of pegylated interferon (PegIFN) and ribavirin (RBV) is associated with poor tolerability and reduced efficacy[5]. Therefore, the preferred approach to date has been to delay antiviral treatment until histological evidence establishes a diagnosis of HCV-related chronic hepatitis after transplant.

It has been reported that the presence of substantial portal tract fibrosis or of portal hypertension one year after LT are predictors of a higher risk of clinical decompensation and death; therefore, these characteristics help determine which patients urgently need treatment[11]. For patients with FCH, meaning a severe recurrence of HCV early after transplantation, antiviral therapy would be life-saving; however, previous treatment options were unable to eradicate HCV in most cases, with the deleterious consequences of graft loss and death.

PREVIOUS THERAPEUTIC STRATEGIES

Since the discovery of the HCV in 1989[12], the development of effective therapeutic strategies has been hampered by the unavailability of cell-culture and small-animal models for investigating the virus. During the last decades, therapeutic approaches remained limited to unspecific IFN-based regimens with insufficient efficacy. Early trials of IFN monotherapy achieved sustained virologic response (SVR) in fewer than 10% of cases[13]. The introduction of combination therapy with RBV and IFN and the modification of IFN to PegIFN, which can be administered weekly and is associated with improved pharmacokinetics (PK), resulted in higher SVR rates[14]. The PegIFN and RBV dual combination treatment was the standard of care for all HCV genotypes for about 10 years. For many chronically infected patients, this treatment regimen fails to eradicate HCV and is associated with additional adverse effects, and it is even less efficacious for LT patients. The overall rates of SVR with PegIFN plus RBV are low, ranging from 30% to 40% across various reports[5,15]. These poor virologic response rates were mainly due to a high frequency of treatment discontinuation and also dose reduction which became necessary because of poor tolerance or adverse effects[16]. Moreover, as LT recipients are susceptible to hematologic toxicities, especially anemia, RBV dose reductions and the use of erythropoietin are common. Hematologic toxicity necessitates a dose reduction for nearly 70% of patients and early discontinuation of treatment for nearly 30%[16-18]. Moreover, some reports indicate that antiviral therapy may increase the risk of acute graft rejection[19]. The risk of rejection for LT patients ranges from 5% to 10%[20]. However, the probability of survival for patients with SVR after LT is clearly better than that for patients who do not respond to therapy[21].

In May 2011, the first-generation protease inhibitors (PIs), boceprevir (BOC) and telaprevir (TLV), broke this paradigm. The United States Food and Drug Administration approved these drugs for use in association with PegIFN and RBV[16,22]. Both PIs inhibit the same viral protein (NS3/4A) that is crucial for viral replication, and both are active against GT 1

but not against other HCV genotypes. For the other HCV genotypes, PegIFN plus RBV remained the standard of care. Several studies have evaluated the feasibility of these regimens for several hundred LT patients with HCV recurrence[23-26]. About one-third of the patients received BOC and the majority was treated with TLV. Most patients had advanced-stage fibrosis, and approximately half had received at least one previous course of antiviral treatment. The reports described rapid virologic response rates from 53% to 67%, and SVR rates 12 wk after the end of therapy from 48% to 62%[27,28]. While these results were quite encouraging in terms of efficacy, the administration of direct-acting antiviral agents (DAAs) after LT was associated with serious concerns about tolerability and the risk of severe adverse events[24-26]. Indeed, the bone marrow-suppressive effect of TLV and BCV could amplify the anemia, neutropenia, and thrombocytopenia induced by RBV and PegIFN[29]. In addition, TLV and BCV cause severe dermatologic effects, such as generalized pruritus and anorectal disorders[30].

In addition, drug-drug interactions are a serious obstacle of the new antiviral agents. First-generation PIs (TLV, BCV) are not only processed by but also inhibit the CYP3A4 isoenzyme, which is involved in the metabolism of most drugs, including the calcineurin inhibitors (CNIs) cyclosporin A (CSA) and tacrolimus (TAC). BCV has been shown to cause a 2.7-fold increase in the area under the curve (AUC) of CSA and a 17-fold increase in the AUC of TAC, whereas TLV causes a 4.6-fold increase in the AUC of CSA and a 70-fold increase in the AUC of TAC[31,32]. Considering the narrow therapeutic range of CSA and TAC, dose adjustments are of imminent importance, and these drugs must be very closely monitored when they are combined with PIs[27,28,33].

Although the first-generation PIs achieved a substantial improvement in terms of efficacy, their described disadvantages and the fact that IFN is still necessary limit the patient population for which this treatment strategy is appropriate. For particular groups of patients, IFN-based regimens are contraindicated or not applicable or repeatedly failed. Those patients depend on the development of IFN-free regimens.

In this respect, the recent introduction of second-generation DAAs, including PIs, polymerase inhibitors, and nonstructural protein inhibitors has initiated a new era of HCV treatment.

FUTURE THERAPEUTIC STRATEGIES: NEW DAAS AND IFN-FREE REGIMENS AFTER LT

For decades, HCV has successfully escaped from all efforts to generate more efficient drugs, although research efforts have been intense[34]. Viral replication *in vitro* or in small-animal models could not be achieved, and functional studies were limited to chimpanzees[35-37], what caused an important drawback to DAA development. The ultimate breakthrough for HCV drug development may be dated to establishment of the HCV replicon system, what was not earlier that 1999[34,38]. HCV subgenomes, which compose the non-structural proteins NS3-NS5 linked to a selectable marker, can efficiently replicate *in vitro*. A few years later, a full-length isolate of HCV became available which can produce infectious viral particles *in vitro*[34,39]. The resulting improvement in the understanding of the viral life cycle opened the doors for the development of the first-generation DAAs. Drug development was further supported by structural biology, which has provided high-resolution images of the structures of the virus, revealing additional crucial drug targets, such as NS3, NS5A, and NS5B. These images have allowed modelling of interactions between specific replication inhibitors and their targets[34,40,41].

With the advent of the NS5B polymerase inhibitor sofosbuvir (SFV)[42], the NS3 PI simeprevir (SMV)[43], and the NS5A replication inhibitor daclatasvir (DCV)[44], three "second-wave" DAAs are now available and promise to be appropriate for LT patients, without severe adverse effects or negative interactions with immunosuppressants.

However, reports of trials of IFN-free DAA combinations in patients after LT are still scarce. The combination of SFV and RBV was the first IFN-free regimen to be tested for treating HCV recurrence in a compassionate use program[45]. Preliminary results of the use of this combination for 24 wk with recurrent HCV hepatitis after LT report a high overall SVR rate of almost 80%. The treatment is not only well tolerated but did also achieve a significant improvement in liver function tests and encephalopathy as well as decompensation[16,46]. Importantly, no clinically significant interactions with common immunosuppressants were observed and no episodes of rejection occurred. Overall, the preliminary analysis of experiences with patients in these programs indicate that a SFV-based regimen can inhibit HCV replication in most patients. This impairement of viral load goes in line with an improvement in clinical parameters and condition in the majority of those patients. However, although these results are already very encouraging, longer follow-up periods and a larger number of patients are needed to assess the impact on disease progression[46]. In addition, SFV and RBV have been successfully used to treat FCH[47,48].

In a phase 2, open-label study, 61 patients who were on the waiting list for liver transplant due to HCV cirrhosis were treated with SFV and RBV for up to 48 wk. At the time of LT, 43 patients had HCV RNA below detection levels and 30 patients (70%) had still a negative viral load 12 wk after LT. The most frequently reported adverse events were fatigue, headache and anemia[49].

To date, only a few reports reflect experiences

with the use of other IFN-free regimens other than SFV and RBV for LT patients. Fontana *et al* reported the first patients who were successfully treated with a combination of DCV and IFN or DCV and SFV for 24 wk combatting a severe HCV recurrence after LT[50,51]. In the meantime, several multicentric clinical trials are ongoing to assess the safety and efficacy of several oral DAA combinations for patients with HCV recurrence: (1) ABT450/ABT267/ABT333/RBV for 24 wk (NCT01782495); (2) SMV/DCV for 24 wk (NCT01938625); (3) SFV/RBV for 24 wk (NCT01779518); and (4) SFV/LDV/RBV for 12 or 24 wk (NCT01938430).

It is expected that the approval of these combinations for the use after LT will dramatically change the management and outcome of LT patients[16]. First summary reports implement suggestions for IFN-free treatment regimens for LT patients[52,53]. However, there remain several challenges and uncertainties for the use of IFN-free regimens to treat patients with very aggressive forms of hepatitis C (such as FCH), which occurs very early after transplantation. The pitfall may be the early setting while patients are still taking high doses of immunosuppressants[16]. Therefore, this period bears the risk of opportunistic infections[54]. Moreover, patients are during that period are often recovering from or being treated for surgical complications.

Indeed, the potential interaction of DAAs with CSA, TAC, and other immunosuppressants is an important issue for LT patients. Fortunately, most anti-HCV therapeutics which currently in phase 3 development have been successfully tested for potential interactions with CSA and TAC, at least in healthy volunteers. Co-administration studies in healthy volunteers found no clinically significant interactions with CSA or TAC[16,51].

Another common feature of LT patients is renal failure. Most patients exhibit a low glomerular filtration rate (GFR) because of previous renal damage that is aggravated by the long-term use of CSA or TAC[55]. In some cases, dose adjustments may be necessary and some compounds like SFV may be excluded from application if the GFR is lower than 30 mL/min.

A further issue that requires particular attention is the usually high viral load in patients who underwent LT, most likely due to the immunosuppression[56]. Exorbitantly high viral loads may well be a prerequisite for the selection of drug-resistant strains that may result in a virologic relapse if the appropriate combination of DAAs is not used. Therefore, after LT, resistance testing may become a necessary tool in the choice of the appropriate antiviral combination for the benefit of treatment efficacy and patient outcome[57].

TREATMENT BEFORE OR AFTER LIVER TRANSPLANT?

Treatment of patients while before liver transplant or while they are awaiting LT, respectively, may have several advantages. From the experiences with successful therapy of Hepatitis B, improvement in liver function may also be awaited for HCV clearance, and LT may become unnecessary in some cases. However, safety data and pharmakokinetics are not available for all compounds when administered to patients with cirrhosis classified as Child-Pugh B or C. Early reports suggest that deterioration of liver function is slightly accelerated after the administration of SFV/DCV to patients with decompensated cirrhosis after LT[58]. These observations suggest that treatment immediately after LT may be the better strategy for decompensated and severely sick patients.

Currently still a problem concerning patients awaiting LT is the uncertainty of treatment duration, because the length of time that a patient must remain on the waiting list cannot be predicted[16]. Though we can anticipate that, in the near future, all patients awaiting LT will receive successful treatment with the opportunity to receive LT after clearance of the virus, given the historical course of HBV.

Concerning treatment of HCV infection after LT, a few issues remain to be solved. Safety data as well as PK analyses are needed for this special patient population, particularly for those patients with advanced graft damage.

As well, drug-drug interaction studies are crucial because of the metabolism of CSA and TAC and a therapeutic range which is considerably narrow. This accounts not only for interactions with immunosuppressants but also with other commonly used drugs. Last but not least, a high barrier to resistance is also relevant for the use of direct-acting antivirals, particularly when high serum levels of HCV-RNA are observed[16].

CONCLUSION

Liver transplant due to HCV is a yet unmet challenge and a public health burden. Current developments predict a fundamental change of this situation: a large patient population for whom IFN-based treatment regiments are contraindicated, will now achieve access to potent antiviral therapies. While the use of novel DAA-based regimens in sufficient time before LT will prevent reinfection of the graft with HCV and avoid the need for retransplantation, the successful treatment of already recurred graft infection and damage in immunosuppressed patients after LT will pave the way to make a retransplant feasible. Most importantly, an early enough treatment of HCV patients on the waiting list will stabilize liver function with the consequence that LT will be dispensable in those individuals and HCV-related end stage liver disease can be expected to disappear from the transplant waiting list in the near future.

However, the efficacy of DAAs applied after LT in terms of SVR cannot yet be quantified, nor has their

adverse-event profile been ascertained for patients who have undergone LT. In addition, the potential predictors of SVR have not yet been identified. However, the absence of drug- drug interactions between CNIs and DCV, SMV, and SOF, in combination with the so far reported significantly improved SVR rate achieved with these DAAs, offers a promising perspective. Given the potential clinical benefits, more extensive and reliable clinical data about the effects of these new potent HCV inhibitors on patients with recurrence of HCV infection after LT are urgently needed.

One of the remaining difficulties with these new regimens is the huge increase in treatment costs[42]. Affordability could be the pacemaker to set up strategies for personalization of treatment in areas of the world with economic limitations and also in selected patient populations. Some old but in certain cases sufficiently effective regimens using IFN-based regimens may find a niche in those patients with a history of several failed DAA regiments or who harbor multiple resistance-associated variants. While we experience the dusk of IFNs, these substances might stay advantageous for HCV therapy in consideration of features like absence of viral resistances, comparatively low costs and avoidance of drug-drug interactions in patients who are reliant on various concomitant medications.

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MINIREVIEWS

Hepatitis B and immunosuppressive therapies for chronic inflammatory diseases: When and how to apply prophylaxis, with a special focus on corticosteroid therapy

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Abstract

Currently immunosuppressive and biological agents are used in a more extensive and earlier way in patients with inflammatory bowel disease, rheumatic or dermatologic diseases. Although these drugs have shown a significant clinical benefit, the safety of these treatments is a challenge. Hepatitis B virus (HBV) reactivations have been reported widely, even including liver failure and death, and it represents a deep concern in these patients. Current guidelines recommend to pre-emptive therapy in patients with immunosuppressants in general, but preventive measures focused in patients with corticosteroids and inflammatory diseases are scarce. Screening for HBV infection should be done at diagnosis. The patients who test positive for hepatitis B surface antigen, but do not meet criteria for antiviral treatment must receive prophylaxis before undergoing immunosuppression, including corticosteroids at higher doses than prednisone 20 mg/d during more than two weeks. Tenofovir and entecavir are preferred than lamivudine because of their better resistance profile in long-term immunosuppressant treatments. There is not a strong evidence, to make a general recommendation on the necessity of prophylaxis therapy in patients with inflammatory diseases that are taking low doses of corticosteroids in short term basis or low systemic bioavailability corticosteroids such as budesonide or beclomethasone dipropionate. In these cases regularly HBV DNA monitoring is recommended, starting early antiviral therapy if DNA levels begin to rise. In patients with occult or resolved hepatitis the risk of reactivation is much lower, and excepting for Rituximab treatment, the prophylaxis is not necessary.

Key words: Hepatitis B virus; Inflammatory bowel disease; Rheumatic disease. Dermatologic diseases; Corticosteroids; Anti-tumor necrosis factor; Prophylaxis; Immunosuppressants

Core tip: Few reviews have been published including data of the three more common inflammatory diseases that require immunosuppressive therapy: inflammatory bowel disease, rheumatic and dermatologic diseases. This paper is focused on the risk of reactivation of hepatitis B virus under immunosuppressants, and particularly corticosteroids. Although most of the guidelines do not specify the necessity of prophylaxis in case of monotherapy with corticosteroids, the specialists responsible of these patients are usually concerned about this issue. Moreover, the risk with low systemic bioavailability new corticosteroids has not been evaluated in previous reviews. This work summarizes the evidence of VHB reactivation in patients with inflammatory diseases: when and how to apply prophylaxis, with a special focus on "new" and "old" steroids.

López-Serrano P, de la Fuente Briongos E, Carrera-Alonso E, Pérez-Calle JL, Fernández Rodríguez C. Hepatitis B and immunosuppressive therapies for chronic inflammatory diseases: When and how to apply prophylaxis, with a special focus on corticosteroid therapy. *World J Hepatol* 2015; 7(3): 539-547 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i3/539.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i3.539

INTRODUCTION

Hepatitis B virus (HBV) is a preventable viral infection, but it is estimated that 2 billion persons worldwide are infected, and a significant number of case reports and clinical studies have pointed the risk of reactivation of this infection in patients on immunosuppressive therapies[1,2] Immunosuppressive and biological treatments are use more and sooner, during long periods of time in patients with inflammatory diseases (ID), including inflammatory bowel disease (IBD), rheumatic or dermatologic diseases. Therefore, the safety of these treatments is a deep concern among gastroenterologist, rheumatologist, and dermatologist

PREVALENCE OF HBV INFECTION IN PATIENTS WITH INFLAMMATORY DISEASES

The prevalence of the hepatitis B infection varies significantly worldwidely, from 1%-2% in the Western countries, to more than 8% in Asia and Africa[3-5].

Among patients with inflammatory diseases, those with IBD are assumed to have a higher risk of HBV infection because of the potential nosocomial transmission[6]. Biancone *et al*[7] found that IBD population had higher prevalence of hepatitis B surface antibody (HBsAb) than controls[7], and studies conducted in endemic areas have reported a rate of present and past HBV infection of 40%[8]. Conversely, recent researches in Spain and France describe exposure rates similar to the general population[9,10], while Kim *et al*[11], in South Korea, cannot find IBD as a risk factor for HBV, with a reported prevalence of hepatitis B surface antigen (HBsAg) of 3.7% in IBD *vs* 4.4% in the control group[11].

In rheumatic diseases, the HBV status has also been evaluated. Irish investigators identified in a cohort of 200 rheumatoid arthritis (RA) patients only 4 cases with positive hepatitis B core antibody (HBcAb) and 11 with positive HBsAb, with no cases of positive HBsAg[12]. The prevalence of concurrent chronic hepatitis B infection in a cohort of RA patients in China was 11.2%, consistent with the prevalence in the general population[13], although the reported rate of HBsAg in ankylosing spondylitis (AS) by Zheng *et al*[14] was 25.4%, higher than in the general population or patients with other spondyloarthropathies and RA. In Japan, where approximately 20% Japanese individuals are infected with HBV, HBsAg and HBcAb positive occurred in 0.7% and 25.6% of patients with RA[15].

There are very few studies to determinate the prevalence of HBV infection in psoriasis and no significant differences between these patients and general population have been found[16].

Therefore, we cannot say that ID patients are a great risk population for HBV infection, at least according to the more recent research. The Table 1 summarizes some of the more relevant studies that have evaluated the prevalence of HBV in patients with inflammatory diseases.

DEFINITIONS OF HEPATITIS B INFECTION AND REACTIVATION

The exposure to HBV can be divided broadly by the viral load and the liver biopsy into three categories: (1) active chronic HBV, characterized by an elevated serum alanine transaminase (ALT) (usually more than twice the upper limit of normal) and DNA levels above 2000 IU/mL; (2) inactive hepatitis B carrier, defined by low HBV DNA levels, typically < 2000 IU/mL, and normal ALT. There is not a significant necroinflammatory activity on the liver biopsy; and (3) resolved HBV infection. These patients are characterized by negative HBsAg and positive HBsAb. Patients with occult HBV infection (OBI) test positive only for HBcAb[17].

HBV reactivation is the reappearance of active necroinflammatory disease, marked by a 1.5-2-fold increase in ALT levels and DNA viral load > 2000 IU/mL in an inactive hepatitis B carrier, or a positivization from a previously undetectable DNA in an individual with a resolved hepatitis B[18,19].

EFFECT OF IMMUNOSUPPRESSIVE THERAPY ON HBV INFECTION

The HBV-induced liver inflammation is predominantly immune mediated: the host immune response

Table 1 Studies evaluating the prevalence of hepatitis B virus infection in patients with inflammatory diseases

Ref.	Disease	Country	Publication year	Number patients	HBcAb	HBsAg
Biancone *et al*[7]	IBD	Italy	2001	494	UC 11.5% CD 11%	UC 0.64% CD 2.1%
Loras *et al*[9]	IBD	Spain	2009	2056	UC 8% CD 7.1%	UC 0.8% CD 0.6%
Chevaux *et al*[10]	IBD	France	2010	315	UC 1.6% CD 2.8%	UC 1.59% CD 0.79%
Huang *et al*[3]	IBD	China	2014	714	UC 41.6% CD 39.8%	UC 5.7% CD 5.3%
Kim *et al*[11]	IBD	South Korea	2014	513		UC 4.1% CD 3.3%
Zou *et al*[13]	RA	China	2013	223	11.2%	
Watanabe *et al*[15]	RA	Japan	2014	7650	25.6%	0.7%
Conway *et al*[12]	RA	Ireland	2014	200	2%	0%
Zheng *et al*[14]	AS	China	2011	439		25.4%
Cohen *et al*[16]	Psoriasis	Israel	2010	12502		0.74%

IBD: Inflammatory bowel disease; HBcAb: Hepatitis B core antibody; HBsAg: Hepatitis B surface antigen; RA: Rheumatoid arthritis; AS: Ankylosing spondylitis.

causes a hepatocellular damage following the HBV replication, which can result in an acute or chronic liver necroinflammation.

Immunosuppressants lead to an increase in DNA viral due to both a effect on the host immune response, as to a stimulatory effect of these drugs on hepatitis B virus[20]. The corticosteroids may increase the expression of HBV through a glucocorticoid-responsive element, which has been detected in viral genoma, and stimulates viral replication in patients under these treatments[21].

On the other hand, tumoral necrosis factor (TNF) α and interferon gamma (IFNγ) are important in the clearance of HBV from infected hepatocytes, so the use of anti-TNF drugs in patients with chronic HBV infections may result in an increase in viral replication[22,23].

Despite the increase in viral replication, the major damage hardly ever appears at the time of maximal immunosuppression and usually occurs once the immunosuppressive therapy is withdrawn, during the phase of immune reconstitution, when the immune system is able to destroy the hepatitis B-infected hepatocytes, producing the liver disease[5,24]. Clinically these exacerbations can vary, ranging from a subclinical or asymptomatic course to a severe acute hepatitis and even death[25].

HBV REACTIVATIONS IN PATIENTS WITH INFLAMMATORY DISEASES

In ID patients the risk of hepatitis B reactivation is highest with the use of monoclonal antibodies anti-CD20 (rituximab)[26], but it may also be fatal in inactive hepatitis B carriers patients undergoing other immunosuppressant treatments[27]. Cases of HBV reactivation have been reported in RA patients treated with methotrexate (MTX) generally at doses lower than 10 mg/wk[28], and anti-TNF agents, specially with IFX, but also with adalimumab and etanercept[29-31].

Oshima *et al*[32] measured the risk of hepatitis B with anti rheumatic drugs and found a significant association between them and the occurrence of hepatitis exacerbation {corticosteroids [OR = 2.3 (1.3-4)], MTX: [4.9 (3.9-6.0)] and rituximab: [7.2 (5.3-9.9)]}[32]. Lee *et al*[33] and Nakamura *et al*[34] reported an incidence of viral reactivations of 5.3% and 12% in the patients under immunosuppressive treatment and inactive viral infection[33,34].

Regarding IBD, reported cases of reactivation of HBV with IFX have been extensively reviewed elsewhere[35-38]. Most of them have occurred in patients with co-treatment with immunomodulators such as azathioprine or MTX. The Spanish REPENTINA study showed that no single drug was specifically involved, and the risk seems to be associated with the intensity of immunosuppression[39,40].

Case reports of HBV exacerbation in severe psoriasis patients have also been published[41]. In a recent review in patients with rheumatic, digestive, and dermatologic autoimmune diseases, treated with any of the anti-TNF inhibitors, by Pérez-Alvarez *et al*[42] HBV reactivation was observed in 39% of hepatitis B carriers in hepatitis B carriers. Reactivations were more frequent in patients previously treated with other immunosuppressive agents (96% *vs* 70%, $P = 0.033$) and less in those who received antiviral prophylaxis (23% *vs* 62%, $P = 0.003$).

Although anti-TNFs are the most common biological therapy in patients with ID, human IgG1κ monoclonal antibody of interleukin-12/23 (ustekinumab) has become an emerging therapy, especially in chronic psoriasis, but also in IBD. There are scarce data about the safety of ustekinumab and the relationship between IL-12 or IL-23 and HBV, but some cases of reactivations have been described[2,43,44].

Regarding patients with resolved HBV or OBI, the risk is much lower. Reactivations following chemotherapy or potent immunosuppressive drugs such as Rituximab have been reported specially on the onco-haematological field[13,45], and although some cases have also been published with anti-TNF therapy, the rate is not relevant[46]. Tamori *et al*[47] described the reactivation risk in 50 patients, with positive results for hepatitis B core antibody, treated with immunosuppresants for rheumatic diseases: the reactivation was 10 times more likely in those with HBsAg positive than in the HBsAg negative grupo (20% *vs* 2%)[47].

Finally, Cassano *et al*[48] analyzed 62 psoriatic patients with occult viral infection, treated with anti-TNFs agents. There were no signs of HBV activation after a period of 4 years, which supports the safety of the use of immunosuppressant drugs (others than

Table 2 Hepatitis B virus reactivations in patients treated with steroids

Ref.	Disease	Study	Patients (*n*)	HBV status (*n*)	Pre-emptive therapy	HBV reactivations (*n*)
Cheng *et al*[58]	Autoimmune diseases	Case report	2	CHB (1) RS (1)	No	2
Nakanishi *et al*[57]	Polymyositis	Case report	1	CHB	No	1
Zanati *et al*[59]	Mixed connective tissue disease	Case report	1	CHB	No	1
Bae *et al*[60]	Rheumatoid arthritis	Case report	1	CHB	No	1
Li *et al*[61]	Idiopathic nephrotic syndrome	Prospective	41	CHB (41)	No	21
Yang *et al*[62]	Connective tissue disease	Retrospective	98	CHB (21) Not applied (77)	No	4
Loras *et al*[39]	IBD	Retrospective	25	CHB	No	6

Adapted from Xuan *et al*[63]. CHB: Chronic hepatitis B, inactive carriers included; RS: Resolved infection; HBV: Hepatitis B virus.

rituximab) in this scenario[48].

CORTICOSTEROIDS AND IMMUNOSUPPRESSION

The risk of infections with corticosteroids are proved to be increased with the dose[49]. Although it is not clearly defined, a dose equivalent to either 2 mg/kg of body weight or a total of 20 mg/d of prednisone are accepted to suppress significatively the immune system in treatments longer than two weeks[50-52].

The Consortium of Rheumatology Researchers of North America evaluated the risk for all infectious events, which were increased with prednisone 10 mg/d (incidence rate ratio relative:1.30; 95%CI: 1.11-1.53) whilst any dose increased the risk of opportunistic infections[53].

Concerning IBD population, there are no precise data on the dose of corticosteroids associated with the increased risk, but Rahier *et al*[25] in the *Second* European evidence-based Consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease, indicate that doses greater than 20 mg/d of prednisolone in adults are immunusuppressive and increase the percentage of events[25].

Beyond that, the combination of two or three of immunosuppressant drugs enhances the probability of infections[54,55].

Corticosteroids and HBV reactivation

First reactivation was described by Wands *et al*[56] in 1975, in 20 patients with lymphoproliferative and myeloproliferative disorders receiving chemotherapy.

Subsequently, other cases were communicated in rheumatic and autoimmune diseases: Nakanishi *et al*[57] and Cheng *et al*[58] reported HBV reactivations after high dose of steroids in monotherapy. None of the cases had received prophylaxis for HBV, and developed clinical disease in 4, 5 and 9 mo after the beginning of the treatment. Zanati *et al*[59] and Bae *et al*[60] have also described two fatal cases of HBV reactivations in patients with connective tissue diseases treated with steroids and chloroquine.

Another prospective study, carried in 41 Chinese adults with idiopatic nephrotic syndrome and inactive hepatitis B, compared standard doses of prednisone *vs* lower doses of prednisone plus mycophenolate mofetil (MMF)[61]. Without pre-emptive therapy, the risk of exacerbation was lower in MMF-prednisone regimen than the group with prednisone in monotherapy, reflecting the major effect of higher doses on the viral reactivation.

Yang *et al*[62] identified in a retrospective study, four cases of viral hepatitis flares in HBV carriers, who received at least 6 mo of high doses of systemic corticosteroid for connective tissue diseases[62], and resulted in a mortality of fifty percent.

Finally, Xuan *et al*[63] have reviewed 30 cases of reactivation after steroids, in monotherapy or combined with other therapies, in 144 patients with rheumatic diseases. The mean time to reactivations was 9.8 mo. Although the findings indicate that the risk of hepatitis reactivation mostly relies on the prednisone doses, as some cases of HBV reactivation have occurred with low-dose-prednisone therapies, caution is advisable[60].

In IBD there have also been reports of reactivation in patients under corticosteroids, with or without azathioprine or anti-TNF therapy, even resulting in severe acute hepatitis[6,64,65]. The Spanish REPENTINA study describes 6 cases of HBV reactivation in IBD patients during conventional immunosuppressive therapy, two of them with prednisone in monotherapy. Fifty percent of the cases resulted in liver failure and one of them required a liver transplantation[39]. The Table 2 reflects the cases of HBV reactivation with steroid therapy.

Reactivation of HBV and "new" corticosteroids

The topically acting oral steroids are agents characterized by a low systemic bioavailability due to an important first-pass liver metabolism. So, the typical adverse effects of steroids are partially avoided because of a lower concentration of the drug in plasma[66]. The most representative are beclometasone dipropionate (BDP) and budesonide. Many studies have reported their efficacy compared to placebo,

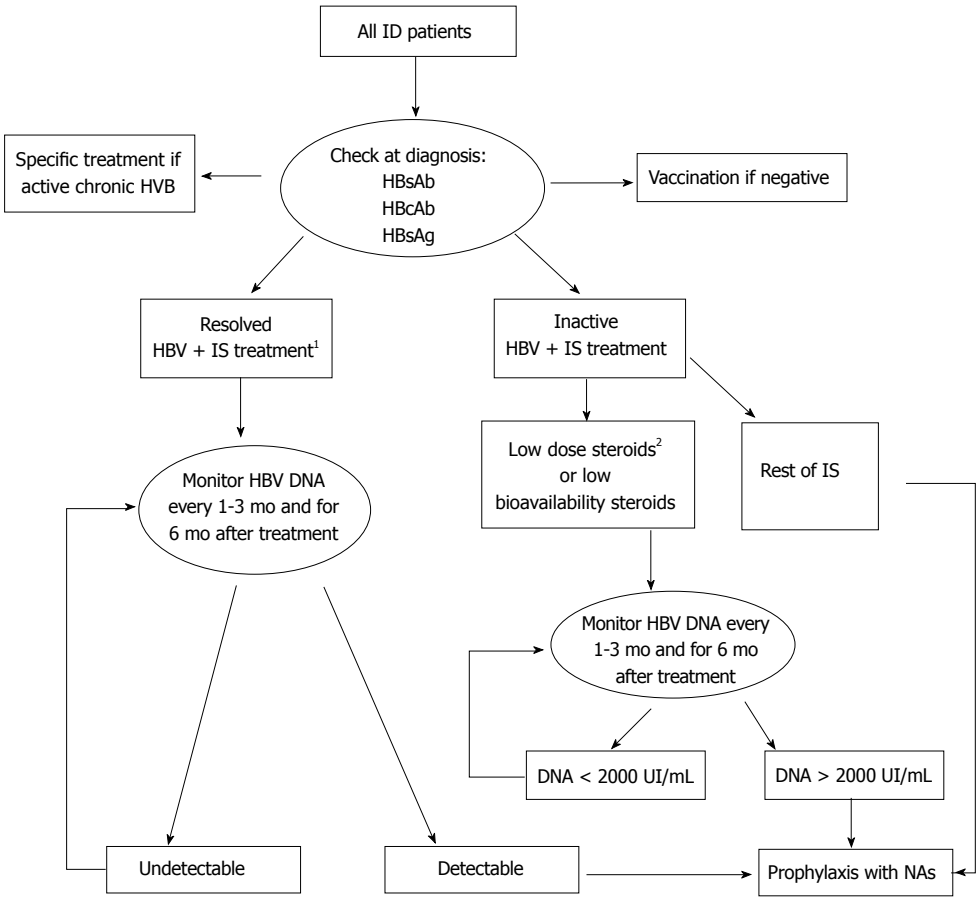

Figure 1 Algorithm suggested for the management of patients with inflammatory diseases and hepatitis B virus infection. [1]Except Rituximab, in which antiviral prophylaxis is desirable; [2]Low dose steroids ⩽ 20 mg/d prednisone. Adapted from Lopez-Serrano *et al*[17]. ID: Inflammatory diseases; IS: Immunosuppressants (steroids, thiopurines, methotrexate and biologics); NAs: Nucleoside/nucleotide analogues; HBcAb: Hepatitis B core antibody; HBsAg: Hepatitis B surface antigen; Low biodisponibility steroids: Budesonide or beclometasone dipropionate; HBV: Hepatitis B virus.

conventional steroids and 5-ASA[67-70]. Nunes *et al*[71] reported the Spanish experience of oral BDP in a retrospective and multicenter study that included more than four hundred patients with active UC. Mild secondary effects were described in 7.6% of the cases, but no serious events either cases of HBV reactivations were identified.

Lichtenstein *et al*[72] reviewed five trials evaluating budesonide for up to 1 year for mild-to-moderate CD compared to placebo. Budesonide 6 mg/d was found to be significantly associated with mild infections (P = 0.023), but clinically important events were rare, and no HBV reactivations were observed.

PROPHYLAXIS AGAINST HEPATITIS B REACTIVATION

The aim of the diverse guidelines is to provide clear recommendations for clinical practice. Before 2005 there were no recommendations for HVB and HCV screening in IBD or rheumatic patients requiring immunosuppression[73]. The appropriate time to do hepatitis B serologic tests is at diagnosis, better than the moment of considering immunosuppression[74-77]. The tests must include HBsAb, HBsAg, and HBcAb, in order to detect also OBI, and HBV vaccination or HBV-DNA quantification must be done depending on the results[25].

Patients with active chronic HBV should receive the antiviral treatment applicable to immunocompetent patients, but this is not the goal of this review.

Prophylaxis in hepatitis B resolved infection

Excepting rituximab, there is no evidence for systematic anti-viral prophylaxis in resolved or OBI in patients on immunosuppresants, taking into account that the HBV reactivation occurs rarely[78]. These patients must be followed closely during therapy (every 1-3 mo, and for 6 mo after stopping treatment), and considered for prophylactic therapy according to DNA levels[5] (Figure 1).

Prophylaxis in inactive hepatitis B carriers

The American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommend the early introduction of pre-emptive therapy in those HBsAg carriers which are going to start immunosuppressive therapy,

including immunomodulators, biologic therapy and corticosteroids[74,75]. As we have commented previously, a dose of prednisone higher than 20 mg/d appears to be sufficiently immunosuppressive, in treatments longer than two weeks, so that prophylaxis must be considered. It must be introduced 1-3 wk before therapy and continue for 6 mo to 1 year after withdrawal [77,79,80].

Apart from cases reviews, there is no strong evidence to make this recommendation in patients with ID and low doses of corticosteroids in short term basis. More conservative management advises to monitor regularly HBV DNA and to start early antiviral therapy if DNA level arises[6,81,82]. The same recommendations can be made in the case of the low systemic bioavailability steroids (budesonide and BPD).

Which antiviral drug must we choose?

Lamivudine has been the most frequent agent used agent in this scenario, having proved to reduce the reactivation risk and the associated mortality and morbidity. However, Lamivudine resistance develops in 53%-76% of patients after 3 years of treatment, therefore, this agent is only appropriate when a short course of therapy is needed. As immunosuppressants for ID usually are used for long term, nucleoside/nucleotide analogues (NAs) with a lower rate of resistance must be considered. Tenofovir and entecavir have a higher barrier to resistance, and should be used if treatments longer than 12 mo are planned[6,76,77,82,83].

In those patients with OBI with a high risk of reactivation, lamivudine may still have a role, because of its low cost, and the low or absent HBV viremia in these cases[76,78]. Alternative antiviral medications for lamivudine would be adefovir and telbivudine[20]. In all cases, but more closely if lamivudine, adefovir or telbivudine are used, serum AST/ALT levels and hepatitis B viral load must be monitored every 3 or 6 mo.

In conclusion, HBV reactivations are not uncommon in inactive HBV patients treated with immunosuppressive therapy for inflammatory diseases. Current guidelines highly recommend prophylaxis in case of immunosuppressive therapy, including patients receiving steroids in monotherapy. However, steroids at low doses, treatments shorter than two weeks and low biodisponibility steroids are unlikely to need prophylaxis, although studies are lacking in this setting. These patients and those with occult or resolved HBV precise regularly HBV DNA monitoring during immunosuppressant therapy in order to detect reactivations. Entecavir or tenofovir are recommended as the optimal agents against HBV reactivation.

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Hepatitis C in hemodialysis patients

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Abstract

Despite reduction of hepatitis C prevalence after recognition of the virus and testing of blood products, hemodialysis (HD) patients still comprise a high risk group. The natural history of hepatitis C virus (HCV) infection in dialysis is not fully understood while the clinical outcome differs from that of the general population. HD patients show a milder liver disease with lower aminotransferase and viral levels depicted by milder histological features on liver biopsy. Furthermore, the "silent" clinical course is consistent with a slower disease progression and a lower frequency of cirrhosis and hepatocellular carcinoma. Potential explanations for the "beneficial" impact of uremia and hemodialysis on chronic HCV infection are impaired immunosurveillance leading to a less aggressive host response to the virus and intradialytic release of "hepatoprotective" cytokines such as interferon (IFN)-α and hepatocyte growth factor. However, chronic hepatitis C is associated with a higher liver disease related cardiovascular and all-cause mortality of HD patients. Therapy is indicated in selected patients groups including younger patients with low comorbidity burden and especially renal transplant candidates, preferably after performance of a liver biopsy. According to current recommendations, choice of treatment is IFN or pegylated interferon with a reported sustained viral response at 30%-40% and a withdrawal rate ranging from 17% to 30%. New data regarding combination therapy with low doses of ribavirin which provide higher standard variable rates and good safety results, offer another therapeutic option. The new protease inhibitors may be the future for HCV infected HD patients, though data are still lacking.

Key words: Hemodialysis; Hepatitis C infection; Interferon; Ribavirin; Protease inhibitors

Core tip: Despite reduction of hepatitis C prevalence, hemodialysis (HD) patients still comprise a high risk group. HD individuals with chronic hepatitis C virus infection have lower aminotransferase and viral levels, milder histological features and a lower frequency of cirrhosis and hepatocellular carcinoma. However, liver disease is related to higher cardiovascular and all-cause mortality in this patient population. According to current recommendations, choice of treatment is interferon or pegylated interferon, whilst low doses of ribavirin also seem to have promising results. Data regarding new protease inhibitors are still lacking.

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EPIDEMIOLOGY OF HEPATITIS C: GENERAL POPULATION *VS* HAEMODIALYSIS PATIENTS

The prevalence of hepatitis C virus (HCV) infection worldwide is 3% and the infected people are estimated at 170 millions. Prevalence rates in Africa, America, Europe and South-East Asia are under 2.5%. In the Western Pacific regions the average ranges between 2.5% and 4.9% while in some parts of the Middle East HCV prevalence reaches 13%[1-3]. In Greece, a survey among 7016 hemodialysis (HD) patients conducted by the Hellenic Center for Infectious Diseases Control and the Hellenic Society of Nephrology in 2003, revealed a mean anti-HCV prevalence of 7.5%. Before 1990, the main routes of HCV transmission were blood product transfusions, intravenous drug use and unsafe medical procedures. Since the systematic screening of blood products, the risk of HCV infection related to transfusions is extremely low (1/20000000)[4]. Currently, the main sources of HCV infection are intravenous drug use, unsafe medical procedures, mother-to-child transmission and the use of unsterilized materials in activities such as acupuncture and tattooing. Household and sexual transmission is extremely low. The dialysis-related risk is estimated at 2% per year. There is a wide range in the prevalence of HCV infection among HD patients in different parts of the world, varying from 1% to 90%. In northern Europe the prevalence rate is less than 5%, in southern Europe and the United States around 10% and in many countries of northern Africa, Asia and South America ranges between 10%-70%[2]. With the systematic screening of blood products and the use of erythropoiesis-stimulating agents, the risk of transfusion related HCV infection in dialysis patients has dramatically decreased; however, they continue to comprise a "high-risk" group In several studies, the prevalence of HCV infection correlated strongly with time on dialysis, independently of the burden of transfusions and it was higher in HD than in peritoneal dialysis or home HD patients. These data strongly suggest that nosocomial transmission plays a crucial role[5]. Therefore, the Kidney Disease Improving Global Outcomes (KDIGO) workgroup for the prevention of HCV transmission in dialysis patients focused on the implementation of hygienic precautions concerning the staff of HD units and the sterilization of the dialysis machines. Of major importance is the fact that isolation of HCV infected patients does not seem to protect against HCV transmission in HD units and therefore it is not recommended[2].

HCV GENOTYPE DISTRIBUTION

A total of 6 different genotypes and multiple subtypes of HCV, each with a different geographic distribution have been identified. Genotype 1 is the most prevalent genotype worldwide. Subtype 1b is more frequent in Europe and Japan while subtype 1a in the United States. Genotype 2 is prevalent in North America, Europe, Japan (Subtypes a and b) and in northern Italy (subtype c). Genotype 3a is frequently seen in India and in European and American drug abusers while genotype 4 is encountered in North Africa, Middle East and among European drug abusers. Genotype 5 has been found in South Africa, genotype 6 in Hong Kong, genotypes 7, 8, 9 in Vietnam and genotypes 10 and 11 in Indonesia[6-8].

There are no firm data concerning the distribution of HCV genotype among HD patients. In studies conducted in the Netherlands, France, Morocco, Mexico and Turkey, there was a predominance of genotype 1b among patients on HD[9,10]. In a study from the United States, subtype 1a was the most frequent among dialysis patients while in Italian HD patients subtypes 2a and 3a predominated. Some of these studies showed a different genotype distribution in dialysis patients than in the general population, some others did not. In general, subtype 1a seems to be more frequent among HD patients than in the general population[9].

An interesting point is that dialysis patients are susceptible to mixed genotype infections attributed to multiple exposures in the dialysis environment. Mixed infections are not often identified due to their short duration and to the lack of sensitivity of the molecular techniques. When more sensitive techniques were applied, 13% of HCV infected HD patients were diagnosed with a mixed infection. In these patients, one of the transmitted subtypes usually prevails in the course of the disease and it is in general subtype 1a[9,11].

NATURAL HISTORY OF HEPATITIS C

In the general population, acute HCV infection affects 1/100000 subjects per year. In 50%-90% of the cases the infection is asymptomatic. In 30% of cases of acute hepatitis the resolution is spontaneous. Acute hepatitis resolves in 20%-30% of the cases spontaneously while in the vast majority it progresses to chronic hepatitis manifesting with variable, usually mild degrees of inflammation and fibrosis[12,13]. Liver damage is thought to be mediated by HCV-induced host cellular immune response rather by the cytopathic effect of the virus *per se*[3]. About 10%-40% (average 20%) of patients with chronic HCV infection will end up to cirrhosis in the second or third decade after infection while 1%-23% will develop hepatocellular carcinoma (HCC)[3,12,13]. The incidence of HCC in cirrhotic patients is 3% per year while the incidence

of death due to complications of cirrhosis is 4% per year. Factors that contribute to fibrosis progression are alcohol consumption, smoking, metabolic syndrome, co-infection with HIV or other hepatotropic viruses while older age at infection is considered as a major negative prognostic factor[14]. The role of viral load and genotype, as risk factors of progression, is debatable[3,12,13]. Chronic active HCV infection may also have extrahepatic impact, such as cryoglobulinemia-associated vasculitis, cutaneous manifestations, ocular lesions, sialadenitis and B-cell lymphoma[3].

The natural history of HCV in dialysis patients is difficult to assess due to inherent disadvantages. The infection in HD patients is usually asymptomatic and serum aminotransferase and Gamma-Glutamyl Transpeptidase levels are typically within the normal range. Moreover, there are concerns about performing liver biopsy in this group of patients because of platelet dysfunction and higher bleeding risk. Most of the studies show a milder HCV-associated liver disease in dialysis patients compared to the general population. In the study by Okuda *et al*[15], none of the dialysis patients with chronic HCV infection progressed to cirrhosis in contrast to more than ¼ of the infected patients in general population. In the study by Ishida *et al*[16], including patients from 314 hemodialysis units in Japan, the incidence rates of HCC and cirrhosis (1.8% and 8.6% respectively) in HCV (+) dialysis patients were much lower than in HCV (+) patients without renal disease (15%-20% for cirrhosis and 5%-28% for HCC). In the prospective study by Nakayama *et al*[17] among 1470 HD patients with follow up of 6 years, the incidence rate of HCC in dialysis patients was 0.6%, whereas in non-dialyzed patients it reached per year 1.2%. Interestingly, there is an inverse correlation with dialysis duration. In the study by Ishida *et al*[16], the incidence of cirrhosis and HCC in patients who were less than 10 years on dialysis, was higher than for those with dialysis duration of more than 10 years.

Risk factors for progression of liver disease in HCV-positive dialysis patients

Several factors have been associated with a more rapid progression of liver disease in the HD population. These include alcohol abuse, tobacco consumption, older age of HCV acquisition, duration of infection as well as co-infection with HIV or other hepatotropic viruses[18]. There is no universal agreement among authors regarding the role of genotype (especially 1b), viral load, metabolic syndrome and AST levels as risk factors for progression of HCV-related liver disease. On the other hand, male gender and liver siderosis, which are established risk factors for hepatitis progression in HCV (+) non uremic patients, did not correlate with a more severe liver disease in HD patients[8,19,20].

In some studies, genotype 1b correlated with a higher rate of evolution to chronicity, a more severe liver disease and a more aggressive course. Some others suggest that genotype 1b is just a marker of more severe disease reflecting a longer duration of infection, since patients affected with genotype 1b are usually older[8].

DIAGNOSIS OF HEPATITIS C IN CHRONIC KIDNEY DISEASE AND HEMODIALYSIS

Initial testing for HCV

According to the KDIGO hepatitis C guidelilnes of 2009, it is recommended that "patients on haemodialysis should be tested when they first start haemodialysis or when they transfer from another haemodialysis facility" while for predialysis patients with chronic kidney disease the recommendation to test for hepatitis C is weak. Especially dialysis patients who are candidates for kidney transplantation should be screened, evaluated and if necessary treated for hepatitis C before entering the waiting list[21].

Which is the preferable method for initial HCV testing in dialysis patients?

According to the KDIGO guidelines, either initial testing with enzyme immunoassay (EIA) or with nucleic acid testing (NAT) is suggested, depending on the low or high prevalence of the virus in the country and in the particular haemodialysis unit. Currently, 3rd generation EIA is used as the preferable immunologic assay and has proven high sensitivity also in dialysis patients[22]. As in the general population, detection of the HCV antibody by EIA in HD patients may be indicative of acute, chronic or even resolved infection. So, it is reasonable to perform initial screening for hepatitis C in HD patients with 3rd generation EIA while the use of recombinant immunoblotting assay (RIBA), as the confirmatory assay in case of positive EIA, has been largely replaced by NAT[23]. On the other hand, an unknown but substantial number of hemodialysis patients will test negative for anti-HCV antibodies while having detectable HCV viraemia. In a large multicentre German study by Hinrichsen *et al*[24] including 2796 dialysis patients, 0.8% of the entire study population was HCV-RNA positive but anti-HCV negative.There is no doubt that detection of HCV-RNA by RT-PCR is the most sensitive and specific assay for HCV detection. Given the proportion of false negative results and the higher prevalence of the virus in dialysis patients, the preferential test for initial screening is NAT, but the final decision is up to every dialysis unit and depends on the prevalence of HCV infection and the financial status of the particular country[21].

Retesting for HCV in dialysis patients

In initially anti-HCV negative HD patients, retesting with EIA should be considered every 6-12 mo. Retesting with NAT is suggested in patients with unexplained elevation of aminotransferase levels, if there is suspicion of an outbreak of HCV in a HD unit,

in patients on the waiting list for transplantation and for the monitoring of therapy in those who are treated[21].

Liver biopsy

Of major importance in clinical practice is the identification of CHC severity in all patients settings including patients on dialysis.

Besides liver biopsy, novel non-invasive techniques are used to validate hepatic fibrosis. Transient elastography (TE, Fibroscan) evaluates the degree of fibrosis by liver stiffness measurement. It has been used in non-uraemic patients with CHC with good results[25]. The other non-invasive method is the AST-to-platelet-index (APRI). In HD patients, despite promising results reported in one study[26], TE needs to be validated in larger cohorts, whereas APRI has shown low diagnostic accuracy[27]. Thus, liver biopsy still remains the gold standard in the evaluation of CHC and, besides concerns regarding higher bleeding risk due to uremic platelet dysfunction, it has been shown that it can be safely performed also in HD patients[28].

The question concerning the indications of liver biopsy performance remains largely unanswered. Ideally, liver biopsy would be useful, at least initially, in all HCV positive HD patients with sustained viremia in order to evaluate the severity of liver disease, the necessity of therapy and the long term prognosis. In fact, according to the KDIGO guidelines, the only clearly defined subgroup of HD patients in whom a baseline liver biopsy is recommended is this of kidney transplant candidates[2]. The rationale for this recommendation is that a substantial percentage of renal transplant candidates (up to 25%) may have subclinical pre-cirrhotic disease which precludes kidney transplantation. Moreover, kidney transplant candidates with persistent viremia should be treated in order to achieve sustained virological response (SVR) or at least low viremic load before transplantation. Within this context liver biopsy may guide therapy and determine prognosis.

PRINCIPAL DIFFERENCES IN CLINICAL, LABORATORY AND HISTOLOGICAL PARAMETERS IN HEMODIALYSIS PATIENTS WITH HCV INFECTION

Lower viral load in dialysis patients

Based on literature data, HCV load in HD patients is usually low. However, in a few studies similar or even higher viral loads were found compared to non-uremic patients[29-31] while fluctuation of HCV-RNA as well as intermittent viremia have also been reported[32].

Two prospective trials with intermediate to long term follow up have both shown a decrease in HCV-RNA levels and even clearance of the virus in some instances over time in HD patients but not in non-uremic controls[15,33].

Impact of hemodialysis procedure on viral load: A number of studies have investigated HCV viral kinetics before, during and after a regular 4-h hemodialysis session. Most studies revealed a significant decrease in HCV viral load during HD session with a return to basal levels after 48 h, prior to the next dialysis session[33,34].

When the effect of the type of dialysis membrane on viral load kinetics was examined, there was a decrease with hemophan and polysulfone membranes but not with cuprophan[35]. The main mechanisms for the explanation of the intradialytic reduction of HCV are the following.

Passage of the virus through the membrane into the dialysate or the ultrafiltrate

This mechanism seems rather insufficient to explain viral load reduction during HD, since HCV virions are larger (30-40 nm) than the pores of the dialysis membrane (10-20 nm). Three studies failed to detect HCV-RNA in the dialysis ultrafiltrate[36-38].

Adsorption of the virus or viral particles by the dialysis membrane

In vitro and *in vivo* studies evaluating this potential mechanism of HCV reduction have revealed rather conflicting results. The negative findings from *in vitro* investigations render this mechanism unlikely[39,40].

HCV reduction via host-mediated factors

The basis of this interesting concept is that the contact of patients blood with extracorporeal circulation leads to the release of pro-inflammatory cytokines such as IL-6 and TNF-α as well as IFN-α and hepatocyte growth factor (HGF), factors with potentially antiviral properties[34].

Nevertheless, the impact of the dialysis membrane or the dialysis procedure (hemofiltration, hemodiafiltration) on HCV viral kinetics requires further investigation.

Lower aminotransferase levels

Several studies have shown that aminotransferase (AST, ALT) levels are low in patients on dialysis and this reduction appears to occur already in patients with advanced chronic kidney disease even before the initiation of renal replacement treatment[41,42]. HD patients with CHC have serum aminotransferase levels which are at the upper limit but still within the normal range, although higher compared to HCV (-) HD patients.

The diminished values of liver enzymes restrict their diagnostic significance while their use as a tool for hepatitis surveillance and follow-up is unreliable. There is no definite explanation regarding the lower transaminase levels observed in HD patients, although several aetiologies have been postulated.

Vitamin B6 (co-factor for aminotransferase syn-

Table 1 Histological features in hepatitis C virus infected hemodialysis patients

Ref.	Study design/ country	No. of patients	Control group	Histological features	Milder histology in HD as conclusion
Barril[50]	Multicenter observational/ Spain	*n* = 218 HD pts Rebiopsy after 7 yr (*n* = 181)	None	70% chronic hepatitis 3% steatosis 15% cirrhosis 74% stable disease 11% progression	(+/-)
Trevizoli *et al*[48]	Case-control study/Brasil	*n* = 36 aHCV(+) HD pts	*n* = 37 aHCV(+) with normal renal function	HD pts *vs* control group: Hepatic fibrosis 47.2% *vs* 73% ($P < 0.025$) Inflammatory activity 27.7% *vs* 59.5 % ($P = 0.003$)	(++)
Mysorekar *et al*[51]	Observational/ India	*n* = 45 aHCV(+) HD pts	None	67% (*n* = 30/45) mild inflammatory activity+mild fibrosis (stage 0, 1, 2)	(+/-)
Sterling *et al*[31]	Prospective case-contrrol study/United States	*n* = 50 aHCV(+) HD pts (transplant candidates)	Two A.Normal renal function, normal ALT (*n* = 43) B.Normal renal function, ↑ ALT (*n* = 43)	Advanced liver disease (bridging fibrosis/ cirrhosis) in 22% of HD pts *vs* 12% in group A (NS) and 48% in group B ($P < 0.001$) Mild hepatic inflammation in 62% of HD pts (score1-3) *vs* 36% in control groups A and B ($P < 0.0001$)	(++)
Cotler *et al*[30]	Case-control study/United States	*n* = 46 aHCV(+) HD pts	*n* = 46 aHCV (+) with normal renal function	HD pts *vs* control group: Less inflammatory activity ($P < 0.001$) Less bridging fibrosis/cirrhosis (13% *vs* 30%, $P = 0.043$)	(++)
Aslinia *et al*[49]	Cross sectional/ United States	*n* = 156 aHCV(+) HD pts	*n* = 138 aHCV(+) with normal renal function	HD pts *vs* control group: Less necroinflammation ($P < 0.05$) Less fibrosis ($P < 0.0001$)	(++)
Becker *et al*[19]	Brasil	*n* = 216 aHCV(+) HD pts	None	77% absence of septal fibrosis (F0, F1) 12% F2 7% F3	(+)
Sakellariou *et al*[18]	Comparative analysis/Greece	*n* = 61 aHCV(+) HD pts	*n* = 326 non-HD, aHCV(+) pts	4% cirrhosis HD pts vs control group: Milder stage ($P = 0.033$) Lower grade (periportal activity, portal inflammation, lobular activity) ($P < 0.001$) Lower frequency of: Lymphoid aggregates (10.2% *vs* 50%, $P < 0.001$) Bile duct lesions (1.7% *vs* 22.1%, $P < 0.001$) Less extent of steatosis in HD pts ($P = 0.022$)	(++)

HD: Hemodialysis; HCV: Hepatitis C virus; ALT: Aminotransferase.

thesis) deficiency could not be verified as a causative factor due to controversial and limited data[42-44].

In the study by Huang *et al*[45] it was hypothesized that AST levels reflect the high metabolic activity of homocysteine due to its high values observed in HD patients and their correlation with low AST.

The contribution of hemodilution in the decrease of aminotransferases has also been examined by several investigators. Yasuda *et al*[42] observed a 15-35% increase in serum ALT/AST after dialysis compared to the pre-dialysis values. Sombolos *et al*[46] showed that in patients who underwent euvolemic HD, there were no differences in ALT/AST levels prior to and after the procedure. On the other hand, when HD with fluid removal or isolated ultrafiltration was performed, there was an increase in aminotransferase levels after the procedure, indicating that the aminotransferase reduction during dialysis could not be attributed to removal of enzyme inhibitors.

Low aminotransferase levels may also be ascribed to factors related with dialysis procedure, and to the impact of dialysis on disease severity through reduction of viremia, increased production of HGF and IFN-α and lymphocyte activation[47].

Milder histology

There are only a few studies referring to histological data of CHC in HD patients. Mild CHC has been recorded in the majority of previous studies of HCV-infected HD patients with a low incidence of severe fibrosis and cirrhosis (5.5%-13%)[19,48,49]. In a recent comparative study of our group, hepatitis activity (including portal inflammation, interface hepatitis and lobular activity) was minimal or mild in 90% of HD patients. In the same series, fibrosis was usually minimal/mild (60%) or absent (26%) while severe fibrosis and cirrhosis was found in 5% and 3.5% of the cases respectively. A significant lower frequency compared to non-uremic patients was also demonstrated regarding the specific CHC features such as lymphoid aggregates (10.2% *vs* 50%), bile duct lesions (1.7% *vs* 22.1%) and steatosis[18] (Table 1).

PATHOGENETIC BACKGROUND OF CHRONIC HEPATITIS C IN HEMODIALYSIS PATIENTS

As already mentioned, in HD patients setting HCV-induced liver disease runs a more benign course showing a lower incidence of cirrhosis compared to non-uremic patients[16].

Histological findings are strongly supportive of a particularly mild chronic hepatitis type while the demonstration of minimal and mild necroinflammatory activity and lack of immune-related specific features such as lymphoid aggregates and "hepatitic" bile duct lesions, speak in favor of a deficient immune reaction.

Although the factors that "protect" uremic patients from immune mediated liver injury are not very well known, there are some interesting theories for this apparently paradox. Alterations in acquired immunity leading to impaired immune response against pathogens and to inadequate response to vaccinations have been recorded in HD patients[52]. This deficient immune response seems to be multifactorial. There is a defective function of antigen presenting cells (APC's) in means of co-stimulation *via* B7-2(CD86) on monocytes leading to ineffective T-cell proliferation and activation[53]. Most studies dealing with uremia and dialysis have focused on T-cell immunity, however B-cell function is also affected in patients with end-stage renal disease. Altered B-cell subpopulations with an imbalance between immature and memory B-cells as well as reduced expression and signaling *via* the BAFF receptor resulting in reduced B-cell survival and differentiation, have been reported[54,55]. This status of "reduced immunosurveillance" rather than overt "immunosuppression" seems to exert an anti-inflammatory protective effect on dialysis patients with chronic HCV infection.

Another possible pathogenetic mechanism explaining the milder disease profile of hepatitis C in HD patients is that hemodialysis procedure increases the levels of HGF. HGF is a potent mitogen for hepatocytes that promotes liver regeneration and restitution of liver cell loss[56,57]. In a study by Rampino *et al*[58] in 1999, a marked and sustained release of HGF was observed in HD compared to non-HD patients and this was further associated with milder histological findings and a lower degree of fibrosis. The low viral load as triggering factor of a weak immune reaction has also to be taken into consideration.

IMPACT OF HCV INFECTION ON SURVIVAL OF HD PATIENTS

Impact on overall survival

HCV-associated mortality in HD patients is attributed to cirrhosis complications, the development of HCC or to the additive effect of uremia and HCV predisposing to infections and sepsis. The true impact of HCV infection on survival is difficult to assess in HD population, so that longitudinal studies with sufficient numbers of patients, meta-analyses and multicenter cohorts or national surveys are needed. It has to be stressed that HD patients have a 4 to 5 times higher age-adjusted death rate compared to non-uremic patients and a 30-fold increase in cardiovascular deaths, whereas liver-related mortality is usually low[59,60]. This means that HD patients may die from other causes, mainly cardiovascular, before developing cirrhosis or HCC. However, cumulative literature data in this field point towards an increased mortality of anti-HCV(+) HD patients compared to their anti-HCV (-) counterparts.

In a meta-analysis by Fabrizi *et al*[61], that included four studies with a total of 2341 patients and a mean follow up of 37-96 mo, the summary estimate for adjusted relative risk (aRR) of all-cause mortality in HCV(+) HD patients was 1.57 (95%CI: 1.33-1.86) compared to their non-infected counterparts.

As shown in Table 2, data from four national surveys confirm the higher HCV-associated mortality in HD patients[62-65].

Impact of HCV infection on cardiovascular mortality

An increased cardiovascular risk among HD patients with HCV infection has been reported in several studies. Kalantar-Zadeh *et al*[66] studied 2.778 HCV (+) dialysis patients and found that hazard ratio (HR) for cardiovascular death was 1.48 (95%CI: 1.05-2.08, P = 0.02) and persisted after adjustment for several case-mix parameters and for available surrogates of the "malnutrition-inflammation syndrome".

It has been observed that HCV infected patients on dialysis have a greater prevalence of hypoalbuminemia when compared to non-infected patients. In the study of Kalantar-Zadeh *et al*[67] serum levels of albumin were significantly lower in HCV-positive dialysis patients (3.68 ± 0.45 *vs* 3.76 ± 0.41 g/dL). The authors suggested that the impact of HCV infection on nutritional status and inflammation may be a main cause of cardiovascular mortality in the dialysis population. In another study conducted by Petit *et al*[68], HCV infection was associated with liver steatosis, insulin resistance and hypoadiponectinemia, factors related to the metabolic syndrome as well as independent risk factors for cardiovascular mortality. Oyake *et al*[69] evaluated 94 dialysis patients with measurements of aortic stiffness by carotid-femoral pulse wave velocity (PWV). They found that mean blood pressure and HCV viremia were significantly and independently associated with high PWV. Cerebrovascular and cardiovascular event rates were significantly higher in HCV-positive dialysis patients. The authors suggested that HCV infection plays an atherogenic role through aggravation of the metabolic syndrome and dyslipidemia.

In a meta-analysis of fourteen observational studies, the adjusted relative risk for cardiovascular mortality in HCV infected dialysis patients was 1.26 (95%CI: 1.10-1.45)[70]. In a national wide survey

Table 2 Hepatitis C virus-associated mortality in hemodialysis patients: National surveys

National survey	Ref.	No. of HD patients	Outcome	Relative risk
Australia New Zealand Dialysis and Transplant Registry	Scott *et al*[62]	23046 (10-yr follow up)	Independent and significant association between a HCV(+) and all-cause mortality	HR = 1.25 (95%CI: 1.07-1.46, P = 0.004)
National or regional dialysis registries of 10 Asia-Pacific countries/areas (Australia, New Zealand, Japan, China, Taiwan, Korea, Thailand, Hong-Kong, Malaysia, India)	Johnson *et al*[63]	173788	Data available for Australia, New Zealand and Japan	HR = 1.25 (95%CI: 1.07-1.46, P = 0.004)
Dialysis Outcomes and Practice Patterns Study (DOPPS) from the United States (in three continents: Europe, Japan, United States)	Goodkin *et al*[64]	206134	a HCV(+) is an independent predictor of all-cause mortality	RR = 1.17 (P < 0.0159)
The Japanese cohort	Japanese Society of Dialysis[65]	76201	a HCV(+) is an independent predictor of all-cause mortality	RR = 1.37 (95%CI: 1.15-1.62, P = 0.003)

HD: Hemodialysis; HCV: Hepatitis C virus.

Table 3 Meta-analyses of trials with convetional and pegylated interferon-α in hepatitis C virus infected hemodialysis patients

Conventional IFN/Peg IFN therapy	Ref.	No. of patients	Dose	Duration	SVR (%)	Withdrawal rate (%)
Two metaanalysis studies (IFN-α)	Fabrizi *et al*[73]	269	1.5-6.0 MU/d to 3 times per week	24-48 wk	37	17
IFN-α	Russo *et al*[74]	213	3.0-5.0 MU/d to 3 times per week	24-48 wk	33	30
Two head-to-head comparisons (IFN-α *vs* PegIFN-α)	Fabrizi *et al*[75]	645	1.0-6.0 MU/d to 3 times per week (n = 529)	8-48 wk	39	19
			135-180 μg/wk (a-2a) or 0.5-1.0 μg/kg per week (a-2b) (n = 116)	48 wk	31	27
IFN-α *vs* PegIFNα + ribavirin	Gordon *et al*[76]	546	1.0-6.0 MU/d to 3 times per week (n = 459)	16-48 wk	41	26
			135-180 μg/wk (a-2a) or 0.5-1.0 μg/kg per week (a-2b) (n = 87)	24-48 wk	37	28
PegIFN-α	Fabrizi *et al*[77]	254	135-180 μg/wk (a-2a) or 0.5-1.1 μg/kg per week (a-2b)	24-48 wk	33	23

HD: Hemodialysis; HCV: Hepatitis C virus; IFN-α: Interferon-α; PegIFN-α: Pegylated interferon-α; RBV: Ribavirin.

from Autralia-New Zealand within a total of 23046 HD patients, a significantly increased cardiovascular risk was found among HCV infected dialysis patients which persisted after adjustment for age and pre-existing cardiovascular disease (HR = 1.34, 95%CI: 1.08-1.67, P = 0.007)[62].

TREATMENT OF HEPATITIS C IN HD PATIENT

Although difficult to establish, there is a true impact of HCV infection on mortality in HD patients. The principal goal of HCV treatment is to decrease liver related mortality. In most studies surrogate endpoint of therapeutic success is virological response. Among virological responses, the most important is SVR defined as undetectable HCV-RNA, measured by a sensitive assay after the completion of 24 wk of therapy.

The currently recommended therapy for CHC in the general population is a combination of pegylated interferon-α (PegIFN-α) and ribavirin (RBV) for 24 wk (genotypes 2 and 3) and 48 wk (genotype1) with achievement of SVR in about 50% of patients[71].

Unfortunately, treatment-related toxicity with IFN-α (influenza-like symptoms, fever, anemia, neutropenia, neuropsychiatric disorders) and with RBV (severe hemolysis) represent a major barrier to successful therapy. Even in the general population a need for dose reduction has been reported in 35%-42% of patients and treatment discontinuation in about 30%[72].

In end stage renal disease patients, although SVR is even slightly better than for non-uremic patients, all treatment options are associated with increased toxicity and higher withdrawal rates[73]. On the other hand, given the enhanced treatment-related toxicity, the high comorbidity and the slow progression of liver disease, there is no rationale to treat all HD patients with HCV viremia. According to the KDIGO Guidelines, the ERBP Work Group considers that "treatment should be mainly considered for younger patients without major comorbidities and that "the decision to treat should be thoroughly discussed with the patients, particularly if they are infected with genotype 1". They also suggest to "try to clear HCV in transplant candidates by appropriate treatment".

Treatment options are the same as for the general population. Conventional IFN-α, pegylated IFN-α (α-2α or α-2β) or combination of IFN with RBV. Given the altered pharmacokinetics in HD and the high treatment related toxicity, the recommended doses in HD are: conventional IFN (2α or 2β) 3MU three times

Table 4 Studies with combined Ribavirin plus Interferon-α therapy in hepatitis C virus infected hemodialysis patients (n > 10)

Combination therapy	Ref.	No. of patients	Dose	Duration	SVR (%)	Withdrawal rate (%)
IFN-α + RBV	Mousa *et al*[78]	20	3 MU (IFN) + 200 mg (RBV) 3 times per week	24 wk (n = 9)	67	0
			3 MU (IFN) + 200 mg (RBV) 3 times per week	48 wk (n = 11)	36	0
PegIFN-α + RBV	Rendina *et al*[79]	35	135 μg/wk (Peg-IFN-a-2a) + 200 mg/qd (RBV)	48 wk (gtp 1) 24 wk (non-gtp 1)	97	14
PegIFN-α + RBV	Carriero *et al*[80]	14	135 μg/wk (Peg-IFN-a-2a) + 200 mg/qd(RBV)	48 wk	29	71
PegIFN-α + RBV	Hakim *et al*[81]	15	135 μg/wk (Peg-IFN-a-2a) + 200 mg/wk to 3 times per week (RBV)	48 wk	7	33
PegIFN-α + RBV	Liu *et al*[82]	35	135 μg/wk (Peg-IFN-a-2a) + 200 mg/qd (RBV)	48 wk (gtp 1) 24 wk (non-gtp 1)	60	17

IFN-α: Interferon-α; PegIFN-α: Pegylated interferon-α; RBV: Ribavirin.

weekly or reduced doses of pegIFN2α 135 μg/wk or 2β 1 μg/kg weekly whereas RBV is not recommended, but it has been used in clinical trials under strict monitoring of anemia in combination with high doses of erythropoietin. Results from five meta-analyses with IFN therapy and from studies with more than 10 patients which received combined therapy with RBV, are summarized in Table 3. Conventional IFN treatment monotherapy resulted in SVR in 33%-45% of HD patients, though with a withdrawal rate from 17%-30%. As shown in Table 3 the response to PegIFN monotherapy is comparable to slightly better than to conventional IFN with a reported discontinuation rate of 23%-28% in three studies and 0%-20% in one. Combination therapy leads to SVR in 40%-50% of patients with genotype 1 and in 80% of patients with genotypes 2 and 3 while the discontinuation rate is about 33% (Tables 3 and 4)[73-82].

A better understanding of the viral cycle of HCV has resulted in the development of new antiviral drugs, targeting specific enzymes of HCV as protease inhibitors (telaprevir and borceprevir) entry inhibitors, nucleosides and non-nucleosides polymerase inhibitors.

The results of phase Ⅱ and Ⅲ trials of the first generation of protease inhibitors (telaprevir and borceprevir) in combination with standard therapy in the general population of HCV infected patients have been very promising including relapsers and partial or null responders[83,84].

In patients with renal failure and hemodialysis, data regarding these new oral antiviral drugs are not available, though they apparently not require dose adjustments to renal function.

Trials with combinations of the new oral antivirals should be the next step in the improvement of care in hemodialysis patients with HCV.

CONCLUSION

HCV infection remains the major cause of chronic liver disease in HD patients. Although it presents a histologically and clinically milder hepatitis than in the general population -probably related to immunocompromised status and HD procedure- it negatively impacts survival on dialysis. Therapy is indicated in selected cases with acceptable tolerability and clearance of the virus in about one third of the treated patients.

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MINIREVIEWS

Probiotics as a complementary therapeutic approach in nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is currently recognized as one of the most common causes of chronic liver disease. It involves a spectrum of conditions that include pure steatosis without inflammation, steatohepatitis, fibrosis and cirrhosis. The key factor in the pathophysiology of NAFLD is insulin resistance that determines lipid accumulation in the hepatocytes and, thus, oxidative stress, which is followed by inflammatory response. However, NAFLD pathogenesis is still largely unknown and has been extensively investigated. Although life style modification with the aim of losing weight has been advocated to treat this disorder, its effectiveness is limited; additionally, there is no specific pharmacologic treatment until nowadays. Recent evidence suggests that the gut microbiota may play a role in the development of insulin resistance, hepatic steatosis, necroinflammation and fibrosis. Differences in gut microbiota between NAFLD patients and lean individuals as well as presence of small intestinal bacterial overgrowth in NAFLD subjects have been demonstrated. Furthermore, some data indicate that the immunoregulatory effects of probiotics may be beneficial in NAFLD treatment as they modulate the intestinal microbiota; improve epithelial barrier function and strengthen the intestinal wall decreasing its permeability; reduce bacterial translocation and endotoxemia; improve intestinal inflammation; and reduce oxidative and inflammatory liver damage. In this article, we review the clinical trials on the use of probiotics in the treatment of NAFLD and discuss the effects of these agents and their efficacy as an emerging therapeutic resource to treat NAFLD patients.

Key words: Fatty liver; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Probiotic; Intestinal microbiota

Core tip: Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver disease. Its pathogenesis is largely unknown, and until nowadays

there is no effective treatment for this disorder. Recent evidence from animal and human studies suggests that gut microbiota may play a role in the development of NAFLD. Furthermore, some data indicate that probiotics may be beneficial in NAFLD treatment. In this context, we conducted a systematic review on the use of probiotics in the treatment of NAFLD and discuss the effects of these agents and their efficacy as an emerging therapeutic resource to treat NAFLD patients.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has been recognized as the most common chronic liver disease in the western world[1]. NAFLD defines a spectrum of liver disosrders that can progress from simple steatosis (nonalcoholic fat liver) to nonalcoholic steatohepatitis (NASH) with or without hepatic fibrosis/cirrhosis. This condition is also a risk factor for hepatocellular carcinoma[2]. NAFLD is a multifactorial disease with a complex pathogenesis involving mechanisms not fully elucidated. It is well known that this condition is strongly associated with insulin resistance (IR), visceral obesity and dyslipidemia. NAFLD prevalence is increasing rapidly due to the current epidemics of obesity and type 2 diabetes[1,2]. There are several clinical trials[3-8] on pharmacologic treatment of NAFLD/NASH; however, no specific pharmacologic treatment has been established until nowadays[1].

Although NAFLD pathogenesis has not been fully elucidated, it has been proposed the "two-hit" theory to explain its development. The "first hit" involves hepatic lipid accumulation due to IR[9]; and, the "second hit" is characterized by oxidative stress followed by lipid peroxidation, secretion of proinflammatory cytokines [*e.g.*, tumor necrosis factor (TNF)-α] and adipokines, and mitochondrial dysfunction, which lead to the progression from simple steatosis to NASH[9,10]. Currently, some authors have considered NAFLD a pathogenetically "multiple-hit" disease[11].

Some evidence suggests that the gut-liver axis could be a point of attack to treat NAFLD[12-19]. Gut microbiota consists of about 10^4 microorganisms that live in a symbiotic relationship with the host, and is influenced by several factors such as diet, age, antibiotic therapy, hygienic habit and infection. These intestinal bacteria produce endotoxin that reach the liver and is phagocyted by the Kupffer cells. Therefore, the liver is constantly exposed to gut-derived lipopolysaccharide (LPS), lipopeptides, unmethylated DNA and double-stranded RNA, which may evoke intense inflammatory reaction[20] that contributes to the progression from steatosis to NASH.

Probiotics are live microbes able to modulate the intestinal microflora[20]. This article aimed to review the clinical trials on the use of probiotics in the treatment of NAFLD and to discuss the effects of these agents and their efficacy as an emerging therapeutic resource to treat NAFLD patients.

LITERATURE RESEARCH

The systematic review was conducted in the PubMed and Medline databases using the following terms: "(NAFLD or NASH or "nonalcoholic steatohepatitis" or "nonalcoholic fatty liver disease" or "fatty liver") and (probiotic or prebiotic or synbiotic or microbiota)". From the 305 articles, we restricted the search to clinical trials performed in humans and written in English. Five clinical trials were initially considered for the present review article. Then, we also searched the reference lists of each selected study by hand, and at the end, we included a total of 8 clinical trials and 1 meta-analysis.

Thus, there were selected all controlled clinical trials in which probiotics or synbiotics were used to treat NAFLD or NASH diagnosed by imaging methods and/or histological evaluation, regardless of the age, sex and ethnic origin of the participants. The trials in which the hepatic function and/or the metabolic and inflammatory parameters were not evaluated before and after the use of the probiotic/synbiotic were excluded from this review.

CLINICAL EVIDENCE OF PROBIOTIC EFFICACY IN THE TREATMENT OF NAFLD IN HUMANS

Loguercio *et al*[12] provided the first evidence that treatment with probiotics could improve some parameters of liver damage and function in patients with different types of chronic liver diseases including 10 biopsy-proven NAFLD males. The patients were given a mixture containing different species of bacteria [*Lactobacillus acidophilus* (*L. acidophilus*), *Bifidobacterium bifidum* (*B. bifidum*), *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus salivarius* (*L. salivarius*), *Lactobacillus bulgaricus* (*L. bulgaricus*), *Lactobacillus casei* (*L. casei*), *Bifidobacterium lactis* (*B. lactis*) and *Bifidobacterium breve* (*B. breve*)] associated with fructooligosaccharides (FOS), and vitamins (B6, B2, B9, B12, D3, C, K) and minerals (zinc and iron) supplementation during 2 mo, followed by a 1 month wash out period. Treatment was followed by a reduction in the serum levels of the aminotransferases, markers of oxidative stress (malondialdehyde and 4-hydroxynonenal) and TNF-α in the NAFLD patients.

Three years late, the same group published

another study in a larger population (22 biopsy-proven NAFLD patients) which received the probiotic VSL≠3, a mixture containing 450 billion bacteria of different species (*Streptococcus thermophilus, B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. casei* and *L. bulgaricus*) at a dose of 2 sachets twice daily during 3 mo. The results corroborate the findings of the previous study as the authors observed improvement in the serum levels of the lipid peroxidation markers malondialdehyde and 4-hydroxynonenal, and also S-nitrosothiols[13].

In 2008, another clinical trial using VSL≠3 in NAFLD patients was published. Four patients received one sachet of the probiotic compound daily during 4 mo. The outcome was chiefly evaluated by the investigation of liver fat by proton magnetic resonance spectroscopy (MRS), which was performed at baseline, after 4 mo of treatment, and at month 7; *i.e.*, after a 3-mo washout. A comprehensive metabolic panel, protime, glycosylated hemoglobin, TNF-α, interleukin (IL)-6, IL-1β and interferon-γ were also evaluated in blood, monthly. All subjects experienced a significant increase in steatosis, and 3 of the 4 patients presented a meaningful change with more than 3% increase in liver fat. After washout, 3 of the 4 participants presented values of liver fat similar to those observed at baseline. There were no significant changes in any of the blood parameters[14].

A double-blind, placebo-controlled pilot study, including 20 obese children (mean age 10.7 ± 2.1 years) with persisting hypertransaminasemia and ultrasonographic (US) bright liver who were non-compliant with lifestyle interventions, was conducted with the aim of evaluating the effects of an 8-wk probiotic treatment. Ten individuals received daily, 12 billion colony forming units (CFU) of *L. rhamnosus*, strain GG; and 10 children received placebo. Evaluation at baseline included: US hepatorenal ratio, standard liver function tests, oral glucose tolerance test, serum TNF-α and antipeptidoglycan-polysaccharide polymers antibodies, and the glucose hydrogen breath test. After the probiotic treatment, it was observed a significant decrease in alanine aminotransferase (ALT) and antipeptidoglycan-polysaccharide antibodies levels; however, there were no alterations in the body mass index (BMI) Z score and visceral fat. Additionally, TNF-α and US bright liver parameters remained stable at the end of the treatment[15].

In a double-blind clinical trial performed in 28 adults with biopsy-proven NAFLD, Aller *et al*[16] evaluated the effects of an acute treatment with a mixture of probiotics. The patients were randomized to receive *L. bulgaricus* and *S. thermophilus* (1 tablet daily containing 500 million CFU) during 3 mo or 1 placebo tablet (120 mg of starch). In the probiotic group, the serum levels of ALT, aspartate aminotransferase (AST) and gamma-glutamyl transpeptidade (GGT) decreased following treatment. In the placebo group, all liver biochemical parameters remained unchanged. The anthropometric parameters and cardiovascular risk factors remained unchanged in both groups.

In 2012, it was published the first and only study in which liver histology was assessed before and after synbiotic treatment of a group of 66 NASH patients. The subjects were randomly and equally separated into 2 groups receiving *B. longum* plus FOS associated with lifestyle modification *vs* lifestyle modification alone. The serum parameters were assessed 4 wk before the beginning of the dietary period, and at weeks 0 (randomization), 6, 12, 18, and 24. Liver biopsies were performed at entry and repeated after 24 wk of treatment. A significant reduction in the serum levels of TNF-α, C-reactive protein (CRP), AST, homeostasis model assessment of insulin resistance (HOMA-IR), and endotoxin was observed in the *B. longum* plus FOS and lifestyle modification group when compared to the individuals who underwent lifestyle modification alone. Likewise, steatosis and the NASH activity index showed a significant improvement[17].

Unlike the earlier results reported by Solga *et al*[14], a chinese group[18] demonstrated in a sample of 20 patients with histological-proven NASH, that probiotic treatment was superior in reducing liver fat, measured by MRS, when compared to the usual care. The subjects were randomized to receive Lepicol probiotic formula (*L. plantarum*, *L. deslbrueckii, L. acidophilus, L. rhamnosus* and *B. bifidum*) (n = 10) or usual care (n = 10) during 6 mo. The results showed a reduction in intrahepatic triglycerides (IHTG) from 22.6% ± 8.2% to 14.9% ± 7.0% in the probiotic group (P = 0.034) and no changes in the usual care group. Furthermore, in most patients (n = 6) of the probiotic group, the IHTG reduced by more than 30% from baseline, whereas the same reduction was observed in only 2 subjects of the usual care group (P = 0.170). The probiotic group also presented a higher reduction in the serum levels of AST (P = 0.008). However, there were no significant changes in BMI, waist circumference, and glucose and lipid serum levels.

Very recently, a novel randomized, double-blind, placebo-controlled clinical trial conducted as a pilot study in 52 NAFLD patients was published. The subjects were supplemented twice daily, for 28 wk, with either a synbiotic (n = 26) or a placebo capsule (n = 26) to evaluate the effects on hepatic fibrosis score (determined by transient elastography), and serum levels of liver enzymes and inflammatory markers. Both groups were advised to follow an energy-balanced diet and physical activity recommendations. At the end of the study, the synbiotic group presented improvement in all the following markers: AST, ALT, GGT, high-sensitivity CRP, TNF-α, total nuclear factor k-B and fibrosis score with significant differences, when compared to the placebo group[19].

According to a review article published in 2011 high-quality preclinical studies and few randomized controlled trials support the therapeutic use of probiotics in liver diseases[20]. The only meta-analysis[21] performed

Table 1 Summary of clinical trials using probiotics to treat nonalcoholic fatty liver disease patients

Ref.	Sample size and underlying hepatic disorder	Design	Intervention	Duration	Results after treatment
Loguercio *et al*[12]	12 chronic HCV infection 10 alcoholic cirrhosis 10 NASH (biopsy-proven)	Prospective, single-center, nonrandomized, noncontrolled pilot study	*Lactobacillus acidophilus, Bifidobacterium bifidum, Lactobacillus. rhamnosus, Lactobacillus. plantarum, Lactobacillus. salivarius, Lactobacillus. bulgaricus, Lactobacillus. casei, Bifidobacterium. lactis* and *Bifidobacterium. breveassociated* plus FOS, vitamins and minerals	2 mo	NASH patients: decreased serum ALT, GGT, MDA, 4-HNE and TNF-α
Loguercio *et al*[13]	22 NAFLD (biopsy-proven) 20 alcoholic cirrhosis 20 chronic HCV infection 16 liver cirrhosis (without any other information)	Prospective, single-center, nonrandomized, noncontrolled	VSL#3 - 900 billion/2 × d	3 mo	NAFLD and alcoholic cirrhosis groups, improved MDA, 4-HNE. All groups improved S-NO plasma levels
Solga *et al*[14]		Open label pilot trial	VSL ≠3 - 450 billion/d	4 mo	All increase steatosis. No significant changes in metabolic panel, protime, glycosylated hemoglobin, TNF-α, IL-6 and 1β, and interferon-γ
Vajro *et al*[15]	20 NAFLD children (10/10) (US + increased aminotransferases)	Randomized, double-blind, placebo-controlled trial	*Lactobacillus rhamnosus* GG (12 billion CFU/d) *vs* placebo	8 wk	Improvement of ALT antipeptidoglycan-polysaccharide antibodies levels. No alterations in BMI z score, visceral fat, TNF-α levels and in US bright liver parameters
Aller *et al*[16]	28 (14/14) NAFLD (biopsy-proven)	Randomized, double-blind, placebo-controlled trial	*L. bulgaricus* and *Streptococcus thermophiles* (500 million CFU/d) *vs* placebo	3 mo	Improvement of AST, ALT and GGT levels No changes in anthropometric parameters and cardiovascular risk factors
Malaguarnera *et al*[17]	66 (34/32) NAFLD (biopsy-proven)	Randomized, double-blind, placebo-controlled trial	*Bifidobacterium longum* + FOS *vs* placebo	24 wk	Improvement of TNF-α, CRP, AST, HOMA-IR and endotoxin levels, steatosis, and the NASH activity index
Wong *et al*[18]	20 (10/10) NAFLD (biopsy-proven)	Randomized, double-blind	Lepicol probiotic formula *vs* nothing	6 mo	Improvement of AST levels and IHTG No differences in BMI, waist circumference, glucose and lipid levels
Eslamparast *et al*[19]	52 NAFLD (US, liver enzymes and fibroScan)	Randomized, double-blind, placebo-controlled trial	Synbiotic (200 million of 7 strain + FOS + probiotic cultures [magnesium stearate (source: mineral and vegetable) + vegetable capsule (hydroxypropyl methyl cellulose *vs* placebo	28 wk	Improvement of ALT, AST, GGT, CRP, TNF-α and total nuclear factor k-B levels, and fibrosis score

NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatites; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase; MDA: Malondialdehyde; 4-HNE: 4-hydroxynonenal; TNF-α: Tumor necrosis factor; FOS: Fructooligosaccharides; VSL#3: Mixture of probiotics; S-NO, snitrosothiols; CFU: Colony-forming unit; BMI: Body mass index; US: Ultrasound; AST: Aspartate aminotransferases; CRP: C reactive protein; HOMA-IR: Homeostasis model assessment of insulin resistance; IHTG: Intrahepatic triglycerides; US: Ultrasound.

to investigate the effects of probiotics in NAFLD was recently published, and its authors concluded that probiotic therapy can reduce liver aminotransferases, total-cholesterol, TNF-α, and improve IR in this condition. The authors recommend modulation of the gut microbiota as new treatment for NAFLD. The summary of the clinical trial described above is presented in Table 1.

RESEARCH

Given the burden of NAFLD at present[22], the difficulty in maintaining lifestyle interventions, and the lack of effective treatment of this disorder, the prognosis of NASH is not optimistic[23]. The promising results of most clinical trials in which the use of probiotics were evaluated in humans, strongly suggest a benefit[12,13,15-19] of these agents in the treatment of NAFLD.

In 2001, the World Health Organization defined probiotic as a live commensal microorganism that, when consumed in adequate quantities, confers health benefit to the host. Lactobacilli and bifidobacteria are normal constituents of the human gastrointestinal microbiota[20], and they represent the main probiotics used in the clinical trials presented above[12,19]. Probiotic treatment aimed at modifying the colonic flora; and, modulate the enteric flora using probiotics are thought to produce benefit for several reasons: (1)

the intestinal bacterial flora favors the digestion and absorption of nutrients; (2) gut microbiota is related to overall immunity of the host[24,25]; and (3) microbiome may alter the synthesis of intestinal hormones such as glucagon-like peptide 1, and influences the host metabolism[24,26].

It is well known that the liver and the gut communicate through the portal venous system; therefore, intestinal-microorganism products may reach and affect the liver. Miele *et al*[27] demonstrated a high prevalence of small bowel bacterial overgrowth (SIBO) and increased gut permeability in NAFLD patients; furthermore, both findings were associated with steatosis severity. Disruption of the intercellular tight junctions was suggested by those authors as the mechanism of the increased intestinal permeability[27]. This increased intestinal permeability associated with the SIBO favors bacterial translocation, exposing the liver to gut-derived bacterial fractions and metabolites constantly[20]. Zhu *et al*[24] characterized the gut microbiomes in NASH subjects. According to their findings, there are increased abundance of alcohol-producing microbiota in these patients as well as elevated blood-ethanol concentration leading to increased oxidative stress and liver inflammation due to the alcohol metabolism.

Indeed, in addition to the increased production of ethanol, the intestinal bacterial microbiota also synthetizes LPS that promotes release of the pro-inflammatory cytokine TNF-α and IL-6 from the hepatic macrophages, which damage the liver, disrupt normal hepatocyte function, lead to mitochondrial oxidative stress, and reduce the clearance of toxins by the hepatocytes[17]. The only clinical trial in which serum endotoxin was measured demonstrated a decrease in their concentrations after the probiotic use[17]. Likewise, in 4 of the 6 clinical trials in NAFLD patients in which the serum markers of oxidative stress were evaluated, the authors observed a reduction in their levels after treatment with probiotics[12,13,17,19]. Serum concentrations of ALT and AST are well-recognized clinical markers of liver damage, and most clinical trials showed a decrease in at least one of his parameters, at the end of the probiotic use[12,13,15-19]. These findings suggest that colonization of the gastrointestinal tract by probiotics is followed by modification of gut flora with subsequent reduction in pro-inflammatory species and, then, improvement of the liver damage.

IR plays a central role in the development and progression of NAFLD. As a consequence of IR, subjects with NAFLD have decreased muscle glucose uptake, impairment in suppression of hepatic endogenous glucose production stimulated by insulin[28,29], and increased lipolytic effect in the adipose tissue resulting in high fatty free acids release[30]. All together these mechanisms can lead to hepatic steatosis. Additionally, the increase in the adipose tissue, especially the visceral fat, has been related to inflammation, decreased release of insulin-sensitizing and anti-inflammatory cytokines, and high expression of pro-inflammatory molecules[31]. Gut microbiota impacts on energy metabolism participating in the homeostatic control and insulin sensitivity[32]. Bäckhed *et al*[33] demonstrated in *germ-free* mice infected with the intestinal bacteria from conventionally raised mice, increase in body fat content even with low food intake (standard chow), IR, and glucose intolerance, within 14 d of the infection. Modulation of gut bacterial flora using probiotics did not result in better glycemic control in 2 clinical trial, evidenced by no changes in glycosylated hemoglobin[14] and glucose serum levels[18]. However, Malaguarnera *et al*[17], observed improvement in HOMA-IR after 24 wk of treatment with a synbiotic. In this situation, the FOS present in the synbiotic supplement may have helped to improve the glycemic control. The assays of intrahepatic fat content also presented different results: Solga *et al*[14] verified increased steatosis measured by MRS after probiotics; Varjo *et al*[15] did not observed any differences in bright liver parameters using US; and Malaguarnera *et al*[17], using histological evaluation, described a decrease in steatosis and NASH activity index at the end of probiotic treatment.

It has been described differences between distal gut microbiota of obese and lean humans. Obese people were shown to have lower Bacteroidetes and more Firmicutes in their distal gut compared to lean control. When obese individuals lose weight on either a fat- or a carbohydrate-restricted low-calorie diet, the prevalence of Bacteroidetes increases in their gut[34]. This finding suggests that microbiota plays a role in increasing the capacity of hosts to harvest energy from their diet. However, no differences in BMI, waist circumference and visceral fat were demonstrated in all clinical trials that evaluated the effect of probiotics in NAFLD patients[15,16,18].

The clinical trials discussed above have some limitations, which may be summarized as followed: (1) differences among the studies regarding the pharmaceutical formulations. In some studies, the mixture contained, in addition to the probiotic, other constituents such as oligoelements, vitamins and prebiotic, which may have influenced the results; (2) great variety in the probiotic doses, bacterial species and duration of treatment; (3) in the large majority, the response to probiotic use was not evaluated by liver biopsy; and (4) a small number of subjects in most studies. However, despite these limitations, the small number of clinical trials, and considering that probiotics are low cost, present good tolerability, and are safe, they should be considered a complementary therapeutic approach in NAFLD patients.

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MINIREVIEWS

Treatment of hepatocellular carcinoma: Steps forward but still a long way to go

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third cause of tumor associated deaths worldwide. HCC incidence rates are increasing in many parts of the world including developing and developed countries. Potentially curative treatments for HCC are resection and liver transplantation, but these are only suitable for patients with small tumors, meeting strict pre-defined criteria, or well-compensated liver disease. Early diagnosis of HCC can be achieved by surveillance of at-risk populations. For patients with non-resectable disease treatments modalities include loco-ablative and systemic therapies. In this review we focus on treatment options in HCC and their allocation. Although significant research is in progress, to this date, the results are unsatisfactory with limited long-term survival. In the fight against this deadly disease, there is still a long way to go.

Key words: Hepatocellular carcinoma; Liver resection; Liver transplantation; Loco-ablative therapies; Sorafenib

Core tip: We chose to focus on the aspect of treatment modalities of hepatocellular carcinoma (HCC) and discuss the benefits and disadvantages of each modality. We report on the diversity of treatments and the allocation of patients with HCC to the different modalities according to the Barcelona-Clinic Liver Cancer. Moreover, we discuss novel treatments currently under investigation and not yet recommended by acceptable guidelines.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths[1]. The highest HCC rates are found in East and South-East Asia and in Middle and sub-Saharan Africa (age-standardized incidence rates

35.5/100000 in men and 12.7/100000 in women and 16.6/100000 in men and 8/100000 in women respectively), whereas rates are low in Northern and Eastern Europe and United States (3.8/100000 in men and 1.6/100000 in women). Among primary liver cancers, HCC is the most common histological subtype, accounting for 70%-85% of all liver cancers worldwide[2]. Unlike most solid cancers, HCC incidence rates are increasing in many parts of the world including the United States and Europe, possibly due to hepatitis C virus (HCV) associated cirrhosis acquired *via* intravenous drug injection in the sixties and the obesity epidemic leading to non-alcoholic steatohepatitis (NASH) and NASH cirrhosis[2] Approximately 90% of HCCs are associated with cirrhosis or a known underlying risk factor for chronic liver disease. Worldwide, approximately 54% of cases can be attributed to hepatitis B virus infection, 31% to Hepatitis C virus infection leaving approximately 15% associated with other causes such as chronic alcohol intake, non-alcoholic steato-hepatitis and environmental factors[3]. Patients are usually asymptomatic until they present with decompensation of their cirrhosis due to venous extension or replacement of functional liver tissue by tumor tissue. Extra-hepatic spread is present at the time of diagnosis in up to 15% of cases. The most common sites for metastases are the lungs, abdominal lymph nodes, bone, and adrenal glands[4].

Early diagnosis of HCC can be achieved by surveillance of populations at high risk[3,5]. Ultrasonography (US) is the imaging modality most widely used with sensitivity ranging from 58% to 89% and specificity greater than 90%[6]. Multiphase computerized tomography (CT) (sensitivity 68% and specificity 93%) or dynamic magnetic resonance imaging (MRI) (sensitivity 81% and specificity 85%)[7], can be used in specific cases[3]. Alpha-fetoprotein (AFP) is the most widely tested biomarker in HCC. It is generally accepted that serum levels greater than 500 μ/L in high-risk patients are diagnostic for HCC. However, negative values do not rule out tumor presence[8]. Due to its low sensitivity and specificity the use of AFP as a surveillance tool is not recommended by the European association for the study of the liver (EASL) and American association for the study of liver disease (AASLD)[3,5].

According to the EASL and AASLD guidelines, one dynamic imaging technique (CT or MRI) showing defined radiological features suffices for diagnosing tumors > 1-2 cm in diameter. An approach utilizing two techniques is recommended when imaging is suboptimal or in centers not routinely treating patients with HCC. The role of contrast-enhanced ultrasound (CEUS) with Sonazoid for detecting HCC is gaining popularity (sensitivity of 95% and specificity of 93%)[9], while angiography and positron emission tomography (PET)-scan are not routinely used for early diagnosis[3,5], Liver biopsy is the gold standard for diagnosis in cases where imaging results are equivocal.

HCC STAGING

Several staging systems have been proposed to provide a clinical classification of HCC.

Currently, EASL and AASLD guidelines support the Barcelona-Clinic Liver Cancer (BCLC) classification for prognostic prediction and treatment allocation in HCC[10]. BCLC staging classification is comprised of 4 stages that are based on the extent of the primary lesion, performance status, presence of constitutional symptoms, vascular invasion, extrahepatic spread, and Okuda stage. The Okuda classification takes into account tumor size (imaging or surgery) and liver function status (ascites, jaundice and serum albumin)[11].

BCLC early stage (A) includes patients with asymptomatic small tumors suitable for resection, transplantation or per-cutaneous treatments. Intermediate stage (B) comprises patients with asymptomatic multinodular HCC. Advanced stage (C) includes patients with symptomatic tumors and/or an invasive tumoral pattern (vascular invasion/extrahepatic spread). End-stage disease (D) contains patients with advanced tumor and liver disease (Okuda stage Ⅲ or Eastern Cooperative Oncology Group performance status of 3 or 4) that should receive best supportive care.

TREATMENT STRATEGIES AND ALLOCATION

The potentially curative treatments for HCC are resection and liver transplantation. However, most patients with HCC present with advanced disease and underlying liver dysfunction and are not suitable candidates for these treatments. Thus, they generally have a poor prognosis with median survival time of less than 1 year[12]. Other treatments are loco-ablative and systemic therapies. Treatment allocation is preferably performed through a multi-disciplinary team according to the BCLC allocation system.

RESECTION

Hepatic resection is the treatment of choice for HCC in non-cirrhotic patients. In well-selected candidates the expected 5-year survival is 60%-80%. In cirrhotic patients, the expected 5-year survival rate post-resection is 60%, with a peri-operative mortality of 2%-3% and blood transfusion requirements of less than 10%[3,13,14]. Patient selection was traditionally based on the Child-Pugh classification, but this may significantly underestimate the severity of the liver disease. Portal hypertension is a major risk factor for post-operative hepatic decompensation[5]. Patients with hepatic venous pressure gradient (HVPG) value

< 10 mmHg have a small chance of developing clinical decompensation (< 10%). For each 1 mmHg increase in HVPG there is an 11% higher risk of clinical decompensation, thus HVPG of 15 mmHg has 55% higher chance of developing decompensation compared with a HVPG of 10 mmHg at equivalent MELD and albumin values[15]. Platelet count has also been confirmed as an independent predictor of survival in resected HCCs[3,5].

In patients properly selected for resection, the main predictors of survival are tumor size, number of tumor nodules and the presence of microsatellites and vascular invasion[16]. Microscopic vascular invasion involves 20% of tumors of up to 2 cm in diameter, 30%-60% of cases in nodules 2-5 cm and up to 60%-90% in nodules above 5 cm in size[16]. As for the number of tumors, multivariate analyses revealed that the presence of multiple tumors is an independent risk factor for postoperative recurrence[13].

Tumor recurrence represents the major complication of liver resection. Post-resection tumor recurrence rate exceeds 70% at 5 years[13,17]. For macroscopically solitary HCC, anatomic resection aiming at 2 cm margins provides better survival outcome and reduces recurrence rate as compared to narrow resection margins.

Another factor that may influence recurrence rate is the primary tumor location. HCC recurrence rates were found to be significantly higher in left-sided resection (41% at 1 year and up to 90% at 5 years), compared with right-sided resection (18% at 1 year and up to 72% at 5 years)[18]. Long-term survival was also significantly lower in patients with left-sided resection[18]. The hypothesized reason is the larger size of liver remnants harboring a risk for local recurrence, as opposed to right hepatectomy which harbors a higher risk for hepatic decompensation[5].

Several adjuvant treatments to prevent post operative recurrence have been assessed. Interferon was the most frequently evaluated adjuvant with conflicting results. Other strategies such as systemic chemotherapy, chemoembolization, internal radiation, immune therapies and retinoids were also tested with disappointing results. According to EASL and AASLD guidelines, pre or post-resection adjuvant therapy is currently not recommended[3,5].

Recently, laparoscopic liver resection (LLR) for HCC has been assessed. The best indications for LLR are solitary lesions, less than 5 cm in diameter, located in the anterior segments, at a distance from the line of transection, the hepatic hilum, and the vena cava[19]. However, surgical indications have continued to evolve and tumor size and posterior location are no longer considered contraindication to laparoscopic surgery[14]. According to a recent meta-analysis[20], LLR had a significantly lower hazard ratio of mortality (HR = 0.64; P = 0.04) with similar rates of recurrence (HR = 0.79; P = 0.37) as compared to open liver resection (OLR). Furthermore, the LLR group had a lower operative blood loss and lower relative risk of total postoperative complications, lower duration of hospital stay and fewer days of intravenous narcotic use. A literature review of western and Middle Eastern LLR experience concluded that comparative studies did not demonstrate any significant difference in terms of overall survival and recurrence rate between LLR and OLR. No seeding was reported[21]. Moreover, the main clinical advantage of laparoscopy for cirrhotic patients is a significantly lower rate of postoperative decompensation and lower blood transfusion requirement[14].

LIVER TRANSPLANTATION

Liver transplantation combines tumor removal with treatment of the underlying liver disease and cirrhosis. Initial enthusiasm for this modality in HCC was hampered by high rates of tumor recurrence. In 1996, a prospective cohort study defined restrictive selection criteria that led to superior survival for transplant patients in comparison to other treatment options for HCC[22]. Since then, these selection criteria have become universally known as the Milan criteria.

An international consensus conference held in 2010 in Zurich, Switzerland, re-affirmed the Milan criteria as the reference benchmark for selection of HCC patients for liver transplantation, and the basis for comparison with other suggested criteria. In addition, they recommended that liver transplantation should be reserved for HCC patients who have a predicted 5-year survival comparable to non-HCC patients[23].

Currently, liver transplantation achieves excellent results in patients with limited tumor load. Patients fulfilling the Milan criteria (HCC nodule less than 5 cm or up to three nodules of less than 3 cm) have a 1-year survival exceeding 85% and a 5-year survival of 75% after liver transplantation, with tumor recurrence in less than 10%. This survival matches post-transplant survival of most other transplantation indications[24,25]. Survival is significantly reduced in patients undergoing liver transplantation for HCV-associated HCC as compared to HCC associated with other liver disease. The evidence of a beneficial effect of post-transplantation antiviral treatment and viral clearance on liver fibrosis, tumor recurrence, and survival is encouraging but has to be confirmed[26].

The model for end stage liver disease (MELD) score is the most clinically used tool for organ allocation to patients on the liver transplantation waiting list[27]. In order to give patients with HCC equal opportunity for transplantation, those patients meeting the Milan criteria are given 22 calculated MELD points and a 10% point increase for every 3 mo on the waiting list.

Patients on the liver transplantation waiting list have a high cumulative probability to drop-out from the list due to intra or extrahepatic tumor progression. This probability has been reported to be 7%-11% at 6 mo and 38% at 12 mo following enrollment[17].

Adjuvant therapies for patients within the Milan criteria while on the waiting list are used in most centers to prevent tumor progression. However, the impact of these treatments on drop-out rate, recurrence and survival is only estimated from non-randomized studies. Considering the strength of evidence available, the EASL and AASLD recommendation is to treat patients waiting for transplantation with percutaneous local ablation, and as a second choice with chemoembolization when the waiting period is estimated to exceed 6 mo[3].

Down staging is a term used to describe treating tumors and decreasing their size to within the Milan criteria to allow transplantation. Two prospective studies showed that in patients with large tumors that were successfully down-staged, the post transplantation survival was similar to patients who initially met the criteria for transplantation[28,29]. Patients with progressive disease, in whom loco-regional therapy intervention is not considered appropriate or is ineffective, should be removed from the waiting list.

In some countries, mainly in Asia, living-donor liver transplantation (LDLT) using the right, or less often, the left liver lobe is the only option for liver transplantation. Currently, LDLT comprises less than 5% of adult liver transplants[30]. Some studies have suggested a higher risk of tumor recurrence in LDLT as compared to deceased donors, but no difference in outcome could be identified according to type of graft. A higher risk of recurrence was noted in patients with a short delay between diagnosis and liver transplantation, not allowing enough time for the biological behavior of the tumor to manifest[23].

Salvage liver transplantation (SLT) for patients with HCC recurrence after initial liver resection is becoming more widely accepted in centers around the world. In a Korean multicenter study, the prognosis of patients following SLT was affected not only by the Milan criteria at the time of SLT, but also by the biological behavior of the recurrent HCC after initial liver resection[31]. The interval between initial resection and HCC recurrence, AFP level at the time of SLT and the Milan criteria at SLT were independent risk factors for low overall and recurrence-free survival of the salvage LT recipient.

LOCO-ABLATIVE THERAPIES

Local ablation is considered the first line treatment option for patients at early stages (BCLC 0-A) not suitable for surgical therapies. Destruction of the tumor is achieved by injection of chemical substances (ethanol, acetic acid, or boiling saline) or by modifying the temperature (radiofrequency, microwave, laser, cryotherapy).

Percutaneous local injection

Percutaneous ethanol injection (PEI) is the most studied method of local treatment. PEI can achieve necrosis of 90-100% of HCC smaller than 2 cm, 70% in tumors of 2-3 cm and to 50% in HCC of 3-5 cm[32,33]. Patients with Child-Pugh A and successful tumor necrosis may achieve a 5-years survival of 50%, comparable with the outcome of resection in those candidates[33]. PEI requires repeated injections on separate days and is less effective in tumors larger than 3 cm, because the injected ethanol cannot access the entire tumor volume. This may be due to the presence of intra-tumoral septa, resulting in 43% local recurrence rates in lesions exceeding 3 cm[34].

Radiofrequency ablation

Radiofrequency ablation (RFA) induces thermal injury to the tissue through electromagnetic energy deposition. RFA requires fewer treatment sessions as compared to PEI in order to achieve comparable anti-tumoral effects. In randomized controlled trials comparing RFA to PEI for the treatment of early-stage HCC, RFA had a higher anti-cancer effect, leading to a lower recurrence rate (2 year local recurrence rate: 2%-18% *vs* 11%-45%, respectively)[35-37]. The best RFA outcomes have been reported in Child-Pugh A patients with early-stage HCC. Five-year survival rates as high as 51%-64%, may be reached in selected patients[33]. The main drawback of radiofrequency is its higher cost and the higher rate (up to 10%) of adverse events (mainly pleural effusion and peritoneal bleeding)[5,33,37]. Procedure-related mortality is 0%-0.3%. Sub-capsular location and poor tumor differentiation have been associated with increased risk of peritoneal seeding. According to EASL practice guidelines, RFA is recommended in most instances as the main ablative therapy in tumors less than 5 cm while PEI is recommended in cases where RFA is not technically feasible (around 10%-15%)[3]. For the RFA procedure to be considered technically successful, the tumor and at least a 5 mm safety margin must be included in the ablation zone[38]. Kudo *et al*[39] reported that the local recurrence rate at 2 years after RFA was 2.6% in HCC patients with a $\geqslant$ 5 mm safety margin, as opposed to 20.8% in HCC patients without such a safety margin (P = 0.01).

Transarterial chemoembolization

Arterial obstruction of branches of the hepatic artery by transarterial chemoembolization (TACE) induces ischemic tumor necrosis with a high rate of objective responses. The procedure combines trans-catheter delivery of chemotherapy emulsion with lipiodol followed by vascular occlusion with embolic agents. TACE achieves partial responses in 15%-55% of patients, and significantly delays tumor progression and macro-vascular invasion[3]. Overall, the median survival after TACE for intermediate HCC is about 20 mo, an improvement over conservative therapy. TACE is the standard of care for patients with non-surgical HCC that are ineligible for per-cutaneous ablation, provided there is no extra-hepatic tumor spread and no portal

vein thrombosis. Patients with advanced liver disease (Child-Pugh class C) and/or intermediate-BCLC staging should not be considered for this treatment due to increased risk of liver failure and death[3,5].

Possible side effects of TACE are nausea, vomiting, bone marrow suppression, alopecia and potentially renal failure. "Post-embolization syndrome" appears in more than 50% of the patients and consists of fever, abdominal pain and a moderate degree of ileus. The syndrome is usually self-limited and lasts less than 48 h. A minority of patients may develop severe infectious complications such as hepatic abscess or cholecystitis.

Chemoembolization with Drug-Eluting Beads (TACE-DEB) improves anti-tumoral activity and reduces systemic exposure, *via* well-controlled embolization with accurate size beads. Embolic microspheres release chemotherapeutic agents in a controlled mode over a 1-wk period. A randomized phase II study comparing TACE and TACE-DEB reported a significant reduction in liver toxicity and drug-related adverse events for the latter arm, associated with a non-significant trend of better anti-tumoral effect[40].

Treatment response is assessed by the decrease in the concentration of tumor markers and by specific imaging characteristics on CT or MRI one month after therapy. Persistence of contrast uptake at the tumor edge indicates treatment failure.

Adverse outcome of TACE can be predicted by the response to the first TACE utilizing the Assessment for Retreatment with TACE score. An increase of aspartate aminotransferase and Child-Pugh score and the absence of radiologic tumor response after the first TACE, all predict poor response to another TACE[41].

Radio-embolization

Radio-embolization also known as selective internal radio-embolization (SIRT) is defined as the trans-arterial delivery of radioactive substances in the form of microspheres containing yttrium-90 (^{90}Y), iodine-131 (^{131}I) iodized oil, or similar agents. Currently, the most popular radio-embolization technique uses microspheres coated with ^{90}Y, a beta-emitting isotope[33]. ^{90}Y microspheres have minimally embolic effect, thus, treatment can be safely used in patients with portal vein thrombosis[42]. Contraindications for the use of ^{90}Y microspheres include significant hepato-pulmonary shunting and the risk of deposition in the gastrointestinal tract. Therefore, ^{99m}Tc macro-aggregated albumin scan prior to treatment is mandatory. Post-radio-embolization syndrome (PRS) can range from mild flu-like symptoms, abdominal discomfort, and cachexia, to hepatic dysfunction, development of portal hypertension, radiation pneumonitis, pancreatitis and vascular injury. The incidence of PRS ranges from 20% to 55%[33,43].

There are currently two commercially available ^{90}Y microspheres: TheraSphere (MDS Nordion, Ottawa, Canada) is made of glass and SIR-Spheres (Sirtex Medical, Sydney, Australia) are made of resin. Glass microspheres are minimally embolic with higher specific activity and lower number of spheres as compared to resin microspheres[44].

In a cohort study reporting long-term outcomes, the median survival time following radio-embolization was 17.2 mo for patients with Child-Pugh A disease and 14.8 mo in patients with Child-Pugh B without portal vein thrombosis (PVT) or extra-hepatic disease. Evidence of PVT decreased the median survival time to 10.4 mo in Child-Pugh A patients and to 5.6 mo in Child–Pugh B patients[45]. Similar results were observed in a phase Ⅱ study where the median survival was 18 mo in non-PVT Child-Pugh A patients and 6 mo in patients with Child-Pugh B disease and PVT[46].

To date, there was only one comparative study that assessed the relative safety and efficacy of TACE *vs* SIRT in patients with un-resectable HCC. In the SIRTACE open-label study, single-session SIRT appeared to be as safe and effective as multiple sessions of TACE[47].

STEREOTACTIC BODY RADIOTHERAPY

The use of radiation therapy for the treatment of HCC has been limited by the poor tolerance of the whole liver to radiation, allowing no more than 30-35 Gy to be delivered and a subsequent high risk of developing radiation induced liver disease; a clinical syndrome characterized by an anicteric hepatomegaly, ascites and elevated liver enzymes, 2 wk to 4 mo after hepatic irradiation[48]. However, technological developments in radiotherapy planning and treatment delivery have offered a significant benefit for patients with advanced HCC. The characteristics of stereotactic body radiotherapy (SBRT) include highly conformal radiation, iso-dose distribution around the planned treatment volume with minimal collateral damage to critical structures and organs by very tight margins and rapid fall-off of the radiation dose in the normal liver parenchyma outside of the treatment field[49]. Proper patient selection for this therapy includes unresectable HCC patients with Child-Pugh class A-B8 how are able to remain immobile in the radiation suite for a potentially extended period of time, tumors of up to 6 cm in diameter and a distance of at least 5 mm from neighboring organs such as the stomach wall or the small intestine[50]. To date, only small phase I/II and retrospective studies have been performed, but with encouraging results[48,51]. SBRT can be a complementary treatment to TACE, since the ischemic effects of TACE are less potent in the surrounding well-oxygenated periphery of the HCC tumor where radiation is the most effective. A retrospective study suggests a survival advantage in large tumors for the combination of SBRT and TACE as compared to TACE alone[52]. Due to the limited data regarding SBRT, it is considered under investigation, and not yet recommended by the EASL guidelines as part of the

HCC therapy regimen.

SYSTEMIC THERAPIES

Sorafenib, is an oral multi-tyrosine kinase inhibitor (TKI), the first and only drug that demonstrated a survival benefit in patients with advanced HCC. The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol study was a large double-blinded placebo controlled phase Ⅲ study that demonstrated improved survival for treated patients. Sorafenib increased the median overall survival from 7.9 mo in the placebo group to 10.7 mo in the treatment group, a 31% decrease in the relative risk of death[53]. A similar survival benefit was demonstrated in a parallel phase Ⅲ trial conducted in the Asian-Pacific population[54]. Median overall survival was 6.5 mo in the sorafenib group *vs* 4.2 mo in the placebo group. The most common grade 3 drug-related adverse events observed in these studies were diarrhea and hand-foot skin reaction, which occurred in 8%-9%, and 8%-16% of patients, respectively. Drug discontinuation due to adverse events was 15% in the sorafenib arm and 7% in the placebo arm.

Sorafenib is indicated for patients with well-preserved liver function (Child-Pugh A class) and with advanced tumors (BCLC C) or tumors progressing despite loco-regional therapies[3,5,55].

Two randomized controlled trials and four cohort studies assessed the combined treatment of TACE and sorafenib[55]. Despite encouraging initial results, this treatment combination is not recommended by any of the HCC treatment guidelines.

TARGETED MOLECULES UNDER CLINICAL DEVELOPMENT

Angiogenesis is currently the most extensively studied therapeutic target of HCC. The efficacy of novel anti-angiogenic TKIs for advanced HCC has been investigated in several phase Ⅲ RCTs. However, to date, none of these drugs has shown superior efficacy to sorafenib[56].

Sunitinib is an oral multi-TKI approved for the treatment of renal cell carcinoma, gastrointestinal stromal tumors and pancreatic neuroendocrine tumors. Although phase Ⅱ studies showed potential efficacy, overall objective response rate was < 3%. Grade 3/4 adverse events were observed in 5%-10% of patients and hematologic adverse events in approximately 20%. Treatment-related deaths due to severe liver dysfunction were recorded in 5.8%-10.8%[57,58]. A phase Ⅲ, multicenter, randomized open-label study of sunitinib *vs* sorafenib was prematurely discontinued for safety issues and futility reasons. Overall survival with sunitinib was significantly inferior to sorafenib[59]. This drug is presently not recommended for treatment of HCC.

Linifanib is an oral TKI targeting vascular endothelial growth factor (VEGF) and platelet-derived growth factor. One open-label, phase Ⅲ trial, compared Linifanib with sorafenib as first-line therapy in advanced Child-Pugh A HCC patients. Overall survival was similar among the two groups and predefined superiority and non-inferiority overall survival targets were not met for Linifanib. Secondary endpoints such as time to progression favored Linifanib while safety results favored Sorafenib[60]. This drug is presently not recommended for treatment of HCC.

Brivanib, an oral VEGF receptor and fibroblast growth factor receptors (FGFR) TKI was evaluated in a multinational, randomized, double-blind, phase Ⅲ trial as compared to sorafenib for first-line treatment of HCC. Overall survival for brivanib in the per-protocol population did not meet non-inferiority targets. However, both agents had similar antitumor activity, based on secondary efficacy end points. Grade 3/4 adverse events were more frequent in Brivanib treated patients[61].

Everolimus (RAD001), an mTOR Inhibitor, has been extensively studied for the treatment of HCC. The EVOLVE-1 trial was a phase Ⅲ study that evaluated everolimus *vs* placebo for advanced HCC following sorafenib failure. Everolimus did not improve overall survival as compared to placebo[62].

CONCLUSION

In conclusion, HCC is a growing cause of mortality in cirrhotic patients and is one of the only solid tumors whose incidence is rising. Currently the best chance for prolonged survival is early diagnosis. This can be achieved by strict adherence to surveillance protocols. Unfortunately, only a small percentage of eligible patients adhere to such programs[63], although preliminary data imply increased adherence in recent years. Patients with active alcohol consumption and those with previous injection-drug abuse are the groups with highest risk for poor adherence to surveillance and as a result may present with more advanced tumors at diagnosis[64].

The only curative therapies currently available are hepatic resection or liver transplantation which are only suitable for a small number of patients. RFA in small tumors may also be curative. The large majority of patients are not candidates for these therapies and for them treatment is only palliative. Successful treatmet of HCC relies on adherence to protocols and on advancing knowledge through enrolling patients into clinical trials. In our institution treatment decisions are made by a multi-disciplinary team based on EASL guidelines following the BCLC criteria for treatment allocation. We recommend enrollment into clinical trials with new systemic medications to any patient who has failed conventional loco-ablative therapy and sorafenib. Significant research is being conducted to develop other agents and combinations that may help improve

outcomes for these patients, but so far results have been disappointing. In the battle against this deadly disease, we are on the right path, but there is still a long way to go.

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MINIREVIEWS

Role of diet on non-alcoholic fatty liver disease: An updated narrative review

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Abstract

The purpose of this article review is to update what is known about the role of diet on non-alcoholic fatty liver disease (NAFLD). NAFLD is the most common cause of chronic liver disease in the developed world and is considered to be a spectrum, ranging from fatty infiltration of the liver alone (steatosis), which may lead to fatty infiltration with inflammation known as non alcoholic steatohepatitis While the majority of individuals with risk factors like obesity and insulin resistance have steatosis, only few people may develop steatohepatitis. Current treatment relies on weight loss and exercise, although various insulin-sensitizing medications appear promising. Weight loss alone by dietary changes has been shown to lead to histological improvement in fatty liver making nutrition therapy to become a cornerstone of treatment for NAFLD. Supplementation of vitamin E, C and omega 3 fatty acids are under consideration with some conflicting data. Moreover, research has been showed that saturated fat, trans-fatty acid, carbohydrate, and simple sugars (fructose and sucrose) may play significant role in the intrahepatic fat accumulation. However, true associations with specific nutrients yet to be clarified.

Key words: Diet; Non-alcoholic fatty acids; Fatty acids; Obesity; Insulin resistance

Core tip: The beneficial effects of weight loss and exercise have been well documented by many authors in reducing the steatosis, inflammation and fibrosis. Vitamin E can also be used with safety in adults only with biopsy proven non alcoholic steatohepatitis. Consumption of high fructose syrup to the development of non-alcoholic fatty liver disease is still under debate. The data for vitamin C shows no clear effect while the supplementation of n-3 fatty acids and probiotics is still conflicting but shows promise.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the spectrum of hepatic disorders embracing uncomplicated fatty liver and nonalcoholic steatohepatitis (NASH) is associated with features of metabolic syndrome (MS) and several hepatic and extra-hepatic complications[1]. Medications such as tamoxifen, methotrexate and corticosteroids may be considered as a secondary causes of MAFLD, rarely though. Additionally, rapid weight loss, total parental nutrition and lipodystrophy may also aggravate fatty liver disease. NAFLD may also progressed to non-alcoholic steatohepatitis, a term that includes symptoms of hepatocellular damage plus inflammation and/or fibrosis[2]. The diagnosis of NAFLD remains under-recognized, as most patients are asymptomatic until late stages of disease. Liver biopsy is the gold standard in diagnosing NAFLD and the most accurate tool for grading fibrosis however is invasive and carries the risk of complications[1]. Although literature information is emerging, it is not clear what type of diet is more likely to cause fatty liver. Since it is very difficult to reduce and maintain weight loss, it looks more feasible for someone to change the dietary composition of a particular diet as a more realistic method to treat NAFLD without the need of decreasing in Kcal intake. Therefore, it is more important to look for associations between NAFLD and specific nutrients.

EPIDEMIOLOGY

Excessive fat deposition in the liver is seen in about thirty percent (30%) of the adult general population. NAFLD is very common in the general population and may effects form children to elderly[3]. This increase in the prevalence of NAFLD is possibly due to the fact that the obesity rates have been also increased the last 2 decades[3].

The prevalence of non-alcoholic fatty liver disease ranges from 9% to 36.9% of the population in different parts of the world[4-6].

Approximately 20% of the United States population suffers from non-alcoholic fatty liver, and the prevalence of this condition is increasing[7]. NAFLD is also high in elderly people. A chinese case-control study examined 4226 adults above 60 years of age from a previous cohort investigated and compared them to 3145 randomly selected younger controls (< 60 years) from the same cohort. NAFDL was higher in the elderly (26.7%) than in the non-elderly (22.8%) and similar in the elderly between men and women (26.6% *vs* 27.0%, $P > 0.05$)[8]. Similar results presented by a cross-sectional study of 6905 nonobese (BMI < 25) subjects. Risk factors for the development of NAFLD were assessed in a subsequent prospective study in NAFLD-free individuals at baseline, 494 of who had developed NAFLD during the 5-year follow-up. Prevalence of NAFL was found to be 7.27%[9].

PATHOGENESIS

Even though the pathogenesis of nonalcoholic fatty liver disease is not clear yet, the most important factor of the development of NAFLD is insulin resistance. Insulin résistance increase fat breakdown from adipose tissue, which in turn, increases circulating free fatty acids having as a final result the retention of lipids within the liver, called steatosis[10]. De novo synthesis of fatty acids is also regulated by hyperinsulinemia and hyperglycemia. This is a result of transcription factors such as sterol regulatory binding protein-1c and carbohydrate response element binding protein[11]. Then, the mitochondrial-oxidation system is overloaded by the extra amount of fatty acids leading to the accumulation of free fatty acid within the hepatocytes. Finally, production of free oxygen radicals is generated by the cytochrome P450 4A and 2E1 isoenzymes-lypoxygenases[12].

Age- related data even though still undefined might reveal some connection of alterations in cholesterol synthesis in patients with NAFLD[13]. Finally this lipid peroxidation leads to the release of malondial-dehyde and 4-hydroxynonenal, which causes cell death and protein cross-linkage, resulting in the formation of Mallory's hyaline in the hepatocyte[14]. They also activate stellate cells, which lead to collagen synthesis and fibrosis[15]. Altered distribution of inflammatory cytokines in the different body compartments may further contribute to worsening NAFLD course in the elderly[16].

HISTOLOGY

Recently, information from consensus conference defined NASH as steatosis that includes hepatocellular ballooning plus lobular inflammation. However, in the absence of inflammation, subjects with steatosis in conjunction with peri-cellular fibrosis may also considered to present NASH[2]. This histological distinction between NASH and simple steatosis, yet to be clarified. Histologically, a minimum of 5% steatosis is required to confirm NAFLD. The histologic features of steatohepatitis, which include steatosis, inflammation, ballooning hepatocyte necrosis, are similar to those of alcoholic liver disease. A new development system for grading and staging was recently developed by Alkhouri *et al*[17]. The diagnosis of NASH was based on Brunt's criteria. Histological features were scored: steatosis (0-3), lobular inflammation (0-3), ballooning (0-2), and PI (0-2). The new score was called the Pediatric NAFLD Histological Score or PNHS and was found to have excellent correlation with NASH.

DIAGNOSIS

Even though significant liver disease can exist with normal levels of transaminases, increased levels of

the hepatic enzymes aspartate aminotransferase and alanine aminotransferase (ALT) are usually very good predictors of the presence of NAFLD and NASH. Serum ALT levels can be found up to 10 times higher than normal in general population with fatty liver disease[18-21]. The last few years, different non-invasive tests have been developed to estimate liver fibrosis (FibroTest)[22] and simple steatosis (SteatoTest)[23]. However, both of them have not been widely adopted[24].

Histological examination of biopsy samples can assess the presence of necro-inflammation and fibrosis[25,26], and can differentiate between macro- and micro-vesicular steatosis, thus it remains the reference standard for the grading and staging of NAFLD[27]. However, it is subject to sampling error due to histological heterogeneity[28,29] scoring is semi-quantitative, limiting its ability to detect modest changes, and scoring systems vary between reports precluding direct comparisons. Ultrasound provides semi-quantitative estimates of hepatic steatosis based on diffuse increases in echogenicity[30]. Reported sensitivity and specificity vary between 60%-94% and 66%-95%, respectively[30]. Even though it is important to diagnose NAFLD, special attention must be give when it comes for the diagnosis of NASH. Symptoms and physical examination may not be enough while presence of MS most of the times will reveal the presence of NASH. Increased liver enzymes have been found to highly related with NASH, however, may not be reliable[31]. Lately, it has come to the literature a new model namely the fragment of keratin 18 (CK18), which is for now, the best marker for detecting NASH, however showed lower accuracy with sensitivity (60%)[32].

TREATMENT

Drug management

Improvement of insulin sensitivity remains the main strategic treatment for NAFLD as well as the modification of all others underlying metabolic risk factors. One of the most common drugs, metformin, it has been used to reduce hyperinsulinemia and to improve insulin resistance. Data for mice studies has been shown to reverse fatty liver in obese, leptin deficient mice[33]. Moreover, a trial using adults who underwent a therapy of 4 mo with metformin demonstrated significant reduction in serum ALT[34]. Nobili *et al*[35] found that metformin was no more effective than lifestyle interventions improving liver enzymes or histology. Additionally, other studies have also failed to prove benefits of using metformin to improve liver histology[36]. Another agent, pioglitazone was found in a metanalysis[37] of reducing liver enzymes and inflammation and benefit of metabolism of glucose, however, the review failed to reveal an improvement of liver fibrosis. Statins are also a promising drug agent; two large studies examine the effect of statins in cardiovascular disease. The authors showed that NAFLD patients with high liver enzymes had lower cardiovascular events compared to patients with normal liver enzymes[38,39]. These results are very encouraging of using statins as a treatment for NAFLD patients with high liver enzymes. Losartan, which is also used in NAFLD patients as a anti-hypertensive drug, has been found to decrease liver fibrosis[40]. Finally, Telmisartan *et al*[41] has been found to reduce insulin resistance and fat deposition in the liver and seems to be looks even more promising in the near future.

Dietary modifications and exercise

One of the most effective method of treating NAFLD is weight loss and exercise together. In a recent review by Schwenger *et al*[42], the authors summarized the effects of weight loss and exercise intervention studies in obese patients with NAFLD. A randomized controlled trial conducted by Promrat *et al*[43] used a combination of diet, physical activity and behavior modification to trigger 7%-10% weight loss in obese NASH patients. Those who achieved a minimum of 7% weight loss had improvements in their liver histology. A similar study used NAFLD patients with elevated liver enzymes and central obesity to assess the effectiveness of lifestyle interventions. Patients were randomly assigned to either low (3 sessions/4 wk) or moderate (6 sessions/10 wk) physical activity intensity groups and were compared to a control group. The lifestyle interventions included physical activity and dietary guidance as well as behavior modification. The authors found that there was a decrease in aminotransferases, which was greater in the group with the moderate-intensity lifestyle compared to the control one[44].

Exercise alone has been also found to have positive results. Hallsworth *et al*[45] found that after 8 wk (3 times per week lasting 45-60 min) of resistance based exercise resulted in a reduction of liver lipids, and improvements of lipid oxidation, glucose control and insulin resistance.

Additionally a recent review conducted by Thoma *et al*[46] analyzed 23 studies using diet modification, physical activity, or a combination of both. He concluded that lifestyle modifications that led to weight reduction and/or increased physical activity greatly reduced liver fat and improved insulin sensitivity. More recently a study led by Montesi *et al*[47] found that intensive psychological counseling for physical activity improves physical fitness and liver fat independent of weight loss. Similar effects have been also verified by a recent meta-analysis study[48].

Dietary changes for 1-3 mo have shown to reduce liver enzymes and even normalize them (Table 1)[49-55].

Many study in adults[50,55,56] and children[56] have shown improvement in the histological profile that underwent a weight loss program. The type of weight loss with a traditional low fat diet or calorie restriction is still debatable. All of these studies though, have

Table 1 Nutritional recommendations for non-alcoholic fatty liver disease

Diet trials in NAFLD
Decrease of about 600-800 kcal per day[49]
Based on IBW, a reduction of caloric intake to < 25 kcals/kg per day[50]
Reduction of total fat intake of < 30% of energy intake with no more of 10% of saturated fatty acids[51-53]
Reduction of total Kcal per day of < 30 kcal/kg[54]
A diet low in calories and carbohydrates of 40%-45%[55]

NAFLD: Non-alcoholic fatty liver disease.

failed to examine any decrease of NAFLD as the final result[57-59]. However, the main outcome from these studies was that a reduction of total body weight between 5%-10% would have the most benefits to these patients. This probably verify the theory where the amount of fat that is delivered to the liver may play very important role in the lipid metabolism as well to the total real weight loss itself[55]. Therefore, it is important for dietitians and other health professionals to direct the patients with NAFLD to lose weight, as this interventions therapy seems to offer the most advantages. Rapid weight loss of more than 1.6 kg/wk has been also found in studies[60] to cause deterioration of the inflammation in people with NAFLD and may increase the progression pace of the disease by promoting increase of fatty breakdown from fatty tissue and increasing transport to the liver. Patel *et al*[61] observed that a reduction in BMI of at least 5% is associated with a significant decrease in liver fat and volume in patients with biopsy-proven NASH. Even for the normal weight people, losing weight has an effect on the improvement of non-alcoholic fatty liver disease. A Korean study of 180 subjects, compared with the stable group, the loss group showed an almost 19-fold increase in the odds of disappearance of non-alcoholic fatty liver disease[62].

Even though factors that determine the severity of NAFLD are still unclear in some studies[63], the exercise component is a recommended treatment. Physical activity intensity and histological severity of NAFLD were evaluated in 813 adults (males = 302, females = 511). Moderate-intensity exercise and total exercise per week was associated with decreased levels of NASH or stage of fibrosis. In the same study vigorous exercise was relate with beneficial results in subjects with NAFLD. The authors concluded that intensity of exercise may be more important than duration or total volume. Resistance training (RT) has been also found to be beneficial recently. Three months of RT improves hepatic fat content accompanied by favorable changes in body composition and ferritin and may serve as a complement to treatment of NAFLD[64]. In addition, intensive psychological counseling for PA produces hepatic effects the same as standard cognitive behavior counseling, improving physical fitness and liver fat independent of weight loss. Strategies promoting exercise are effective in motivated patients, particularly in lean NAFLD patients where large weight loss cannot be systematically pursued[47,65].

OTHER NUTRIENTS

Vitamin C

Vitamin C and E together with weight loss or without has been also examined in children with fatty liver disease. The authors concluded that significant histologic improvements (degree of steatosis, inflammation and ballooning degeneration) were produced by a weight loss of around 5 kg. However, the study failed to prove and beneficial effects of Vitamin C and E on weight loss[56]. Data for Vitamin C and its effects on NAFLD shows no clear beneficial effects. The statement of the most recent consensus was that vitamin C is not recommended for patients with NAFLD outside the context of research protocols[66].

Vitamin E

Oxidative stress and depletion of endogenous antioxidants are important in the pathogenesis of disease progression in NASH. Many drugs with antioxidant features were tried in studies for the treatment of NASH with variable conclusions. Vitamin E (a-tocopherol) is a well-known antioxidant and this feature is the best studied of its many other biological functions. The largest randomized controlled study on vitamin E, the PIVENS trial, demonstrated a greater histological improvement in inflammation in non-diabetic patients with biopsy-proven NASH compared with the placebo and pioglitazone groups. However, only 42% of patients receiving high dose vitamin E (800 IU/d) for 96 wk achieved an improvement in histological parameters compared with 19% in placebo-treated patients[67]. Recently, the Nonalcoholic Steatohepatitis Clinical Research Network conducted a multicenter study comparing metformin and vitamin E in 173 pediatric patients with NAFLD, who were followed up for 96 wk and underwent a post-treatment biopsy (the TONIC study). This study did not show significant benefits of vitamin E for aminotransferase levels; however, it did show differences in the histological characteristics (ballooning and NAFLD activity score) of the liver biopsy performed at 96 wk[68]. Several concerns have been raised regarding an increase in all-cause mortality with the long-term use of vitamin E[69]. Thus, the statement of the consensus was that the use of vitamin E is well supported for nondiabetic adults with biopsy-proven NASH[66].

n-3 fatty acids

A decrease rate in the development of NASH has been demonstrated by a diet high in n-3 PUFA in animal studies[70]. This is possibly due to the fact that n-3 PUFAs have the ability to regulate lipid processing to the liver by reducing oxidative stress and liver inflammation[70].

Capanni *et al*[71] examined the effects of n-3 PUFA

in non-alcoholic fatty liver disease in 42 patients who received 1 gm n-3 PUFA per day for 1 year. Both liver enzymes as well as ultrasound results were improved. A 53% reduction in NAFLD was also observed in 134 patients who received 2 gm of n-3 PUFA three times per day compared with a 35% reduction of NAFLD group who follow a diet low in kcal, respectively[72]. Other similar studies using n-3 PUFA to treat NAFLD have shown pararell results when used aminotransferases and ultrasound to asses fatty liver[73,74]. A recent systematic review and meta-analysis[75] found significant heterogeneity between these studies and concluded that although omega-3 PUFA supplementation may decrease liver fat (with no effects on aminotransferase levels), the optimal dose has not been established. Additional trials are needed to support the routine use of omega-3 PUFA in patients with NAFLD. To date, there is insufficient evidence to support the routine use of omega-3 PUFA supplementation in patients with NAFLD[66].

Fructose

The most common sugar found in fruit and soft drinks is high fructose corn syrup (HFCS). Sucrose is 50% fructose and 50% glucose. In a recent study that included health people the authors demonstrated an increase of liver enzymes of those subjects consuming ¼ of total calories per day in the form of sucrose[76]. In another similar study patients with fatty liver found to have twice the consumption of high fructose syrup compare with those without fatty liver disease (365 kcal *vs* 170 kcal)[77].

In another study, patients that consumed a diet high in calories and fructose were found to have an increase in hepatic fat deposition compared to the normal group[78]. Most recently, Sullivan *et al*[79] showed that children with NAFLD absorbed and metabolized fructose more effectively than lean subjects. Fructose ingestion was associated with an exacerbated metabolic profile[79]. In a 4-wk randomized, controlled, double-blinded beverage intervention study, Jin *et al*[80] demonstrated that reduction of dietary fructose in Hispanic-American adolescents with NAFLD improved several important factors related to cardiovascular disease risk, including adipose insulin sensitivity, high sensitivity C-reactive protein and low-density lipoprotein oxidation. On the other side a recent study by Kanerva *et al*[81] found that high fructose intake was inversely associated with risk of NAFLD in older Finnish adult. A latest meta-analysis of 21 intervention studies concluded that there was insufficient evidence to draw a conclusion for effects of HFCS or sucrose on NAFLD[82].

Prebiotics and probiotics

Dietary prebiotic consumption, which modulates gut microbiota[83] although associated with subjective satiety, reduced postprandial glucose and insulin concentrations and exhibits inconsistent results regarding total energy intake, body weight, gut peptides, insulin sensitivity, serum lipids, inflammatory markers and immune function[84]. Despite the positive results in animals[85], data of probiotics on metabolic effect in humans is still conflicting. Further studies are needed to identify strategies to target gut microbiota composition as an innovative NAFLD treatment in humans.

CONCLUSION

NAFLD is one of the major causes of liver diseases in the world. As the disease progresses from simple steatosis to steatohepatitis, and finally, cirrhosis should alarm health professionals to look over in order to avoid high mortality rates that have been found to related with the disease. The treatment should lie on the management of the "insulin resistance-metabolic syndrome" and not in fatty liver disease itself. The significant recognition of the disease will involve a challenge in educating people as well in the initiation of the appropriate interventions. Weight loss and exercise has been proven in reducing the steatosis inflammation and reversion of fibrosis in some cases. Vitamin E can also be used with safety in adults only with biopsy proven NASH. Consumption of high fructose syrup to the development of NAFLD is still under debate. The data for vitamin C shows no clear effect while the supplementation of n-3 FA and probiotics is still conflicting but shows promise.

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MINIREVIEWS

Variations and mutations in the hepatitis B virus genome and their associations with clinical characteristics

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Abstract

Hepatitis B virus (HBV) infection is major global issue, because chronic HBV infection is strongly associated with liver cancer. HBV spread worldwide with various mutations and variations. This variability, called quasispecies, is derived from no proof-reading capacity of viral reverse transcriptase. So far, thousands of studies reported that the variety of genome is closely related to the geographic distribution and clinical characteristics. Recent technological advances including capillary sequencer and next generation sequencer have made in easier to analyze mutations. The variety of HBV genome is related to not only antigenicity of HBs-antigen but also resistance to antiviral therapies. Understanding of these variations is important for the development of diagnostic tools and the appropriate therapy for chronic hepatitis B. In this review, recent publications in relation to HBV mutations and variations are updated and summarized.

Key words: Hepatitis B virus; Mutation; Quasispecies

Core tip: Hepatitis B virus infection is major global issue. HBV spread worldwide with various mutations and variations. So far, thousands of studies reported that the variety of genome is closely related to the geographic distribution and clinical characteristics. Recent technological advances have made in easier to analyze mutations. Understanding of these variations is important for the development of diagnostic tools and the appropriate therapy for chronic hepatitis B.

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INTRODUCTION

Hepatitis B virus (HBV) was first discovered by Blumberg *et al*[1] in 1965, and the relationship between

Table 1 Position of hepatitis B virus regions and transcripts

ORF	Protein	Position	Amino acids	Protein
Pre-S/S	Pre-S1	2854-3211	119	LHBs
	Pre-S2	3211-155	55	MHBs/LHBs
	S	155-835	226	SHBs/MHBs/LHBs
P	P	2357-1623	843	Polymerase
X	X	1374-1838	154	HBxAg
Pre-C/C	Pre-C	1814-1901	29	HBeAg, HBcAg
	C	1901-2458	183	

ORF: Open reading frame; S: Surface protein; LHBs: Hepatitis B large surface protein; MHBs: Hepatitis B medium surface protein; SHBs: Hepatitis B small surface protein; P: Polymerase; HBxAg: Hepatitis B X antigen; C: Core; HBeAg: Hepatitis B e antigen; HBcAg: Hepatitis B c antigen.

HBV and acute hepatitis after blood transfusion was reported by Okochi in 1968[2]. At that time, most studies were based on immunological and serological methods. Molecular-based analyses progressed rapidly after the HBV particle was discovered[3] and the HBV genome cloned[4].

HBV infection is major global issue, and is a particular concern in Asia and Africa. Although HBV itself is not directly cytotoxic, the immune response to HBV infection causes liver damage and eventually leads to liver cirrhosis and hepatocellular carcinoma (HCC)[5]. More than 350 million people worldwide are thought to be chronically infected with HBV and 1-2 million people die every year from HBV-related cirrhosis and HCC[6]. The long-term outcomes of chronic hepatitis B (CHB) vary among different countries. The annual incidence of cirrhosis is estimated to range from 2% to 6% in hepatitis B e antigen (HBeAg)-positive patients and from 8% to 10% in HBeAg-negative patients. The annual incidence of HCC ranges from 2% to 3% in cirrhotic patients[7]. The goal of treating CHB is to suppress HBV replication before significant and irreversible liver damage occurs, such as end-stage decompensated cirrhosis and HCC. There are currently two main treatment options for chronic HBV infection, interferon (IFN) and nucleos(t)ide analogues.

HBV GENOME AND REPLICATION

HBV is approximately 42 nm in size. It is an incomplete double-stranded DNA virus from the genus *Orthohepadnavirus* and family *Hepadnaviridae*. Its genome consists of full-length coding minus strand DNA and incomplete noncoding plus strand DNA. The viral particle, a Dane particle, comprises an envelope and a core particle (Figure 1). The envelope is composed of a double lipid layer and three envelope proteins: L (large), M (medium), and S (small). The core particle (27 nm in size) consists of the core protein (HBc antigen) and the incomplete double-stranded DNA genome.

The HBV genome is contained within the capsid. It is approximately 3200 bp long, with four overlapping open reading frames (ORFs), which encode the polymerase (P), core (C), surface antigen (S), and X protein (Figure 1B)[8]. Seven viral proteins (HBeAg, HBcAg, LHBs, MHBs, SHBs, polymerase, and HBx) are produced from transcripts (Table 1).

The entry of HBV into human hepatocytes is the initial step of viral infection. It has been reported that the pre-S1 sequence at amino acids 2-48 mediates the attachment of the virus to its target cell[9]. After invading the target cells, HBV is transported to the nucleus where covalently closed circular DNA is constructed as the replication template of HBV.

Following infection, the HBV DNA is integrated into the host's cellular DNA. HBV integration induces various kinds of secondary genetic alterations within the host's genome, including deletions, translocations, and genomic instability[10]. It has been reported that the loss of chromosomal integrity in HCC is attributable to deletions in some chromosomes. In particular, losses in chromosomes 1p, 4q, 5q, 6q, 8p, 9p, 13q, 16p, 16q and 17p have been detected in 25%-45% of patients, whereas gains occur in chromosomes 1p, 6p, 8q, and 17q in 30%-55% of patients[11].

Unlike other DNA viruses, reverse transcriptase is necessary for HBV replication. Because reverse transcriptase has no proof-reading capacity, DNA mutations frequently occur during replication. In general, the mutation rate of the hepadonaviruses is estimated to be 2×10^{4} base substitutions/site/year. This mutation rate is approximately 100 times higher than that of other DNA viruses, but 100-1000 times lower than that of RNA viruses[12]. The mutation rate of HBV is reported to be in the range of $1.4\text{-}3.2 \times 10^{-5}$ base substitutions/site/year[13].

Mutations and variations that occur naturally or during antiviral therapy play important roles in viral latency, the pathogenesis of liver disease, immune escape, and resistance to antiviral therapies.

HBV GENOTYPES/SUBTYPES AND MUTATIONS

The major hepatitis B s antigen (HBsAg) protein carries a pair of mutually exclusive determinants, d or y and w or r, which are associated with variations in single amino acids at positions 122 and 160, respectively. Differences in the epitope result in four major serotypes (adr, adw, ayr and ayw) and ten subtypes (Figure 2)[14]. The serotypes and subtypes show differing geographic distributions and affect the antigenic characteristics of HBV[15,16].

HBV has been classified into at least 10 genotypes (A-J) according to the divergence of their viral DNA sequences, with distinct geographic distributions (Table 2)[17-19]. In addition, many studies have revealed that the HBV genotype is strongly associated with disease progression and responses to antiviral therapies[20,21].

HBV/A is mainly distributed in Africa (HBV/A1),

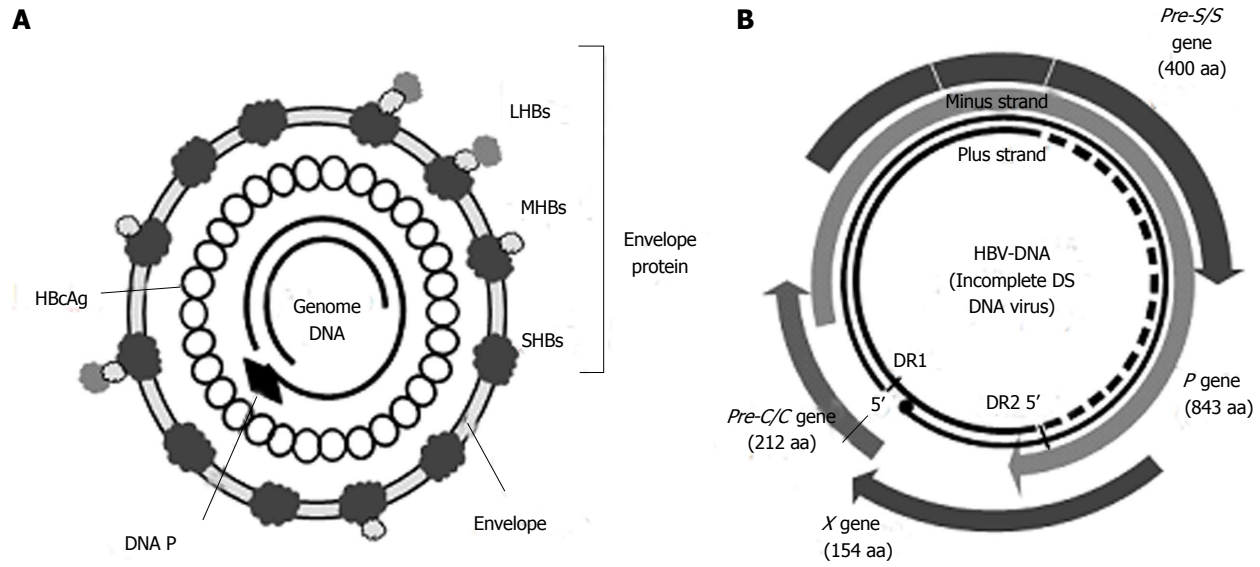

Figure 1 Structures of the hepatitis B virus (Dane particle) (A) and genome (B). aa: Amino acids; C: Core; DS: Double-stranded; LHBs: Hepatitis B large surface protein; HBV: Hepatitis B virus; MHBs: Hepatitis B medium surface protein; P: Polymerase; SHBs: Hepatitis B small surface protein; HBcAg: Hepatitis B c antigen.

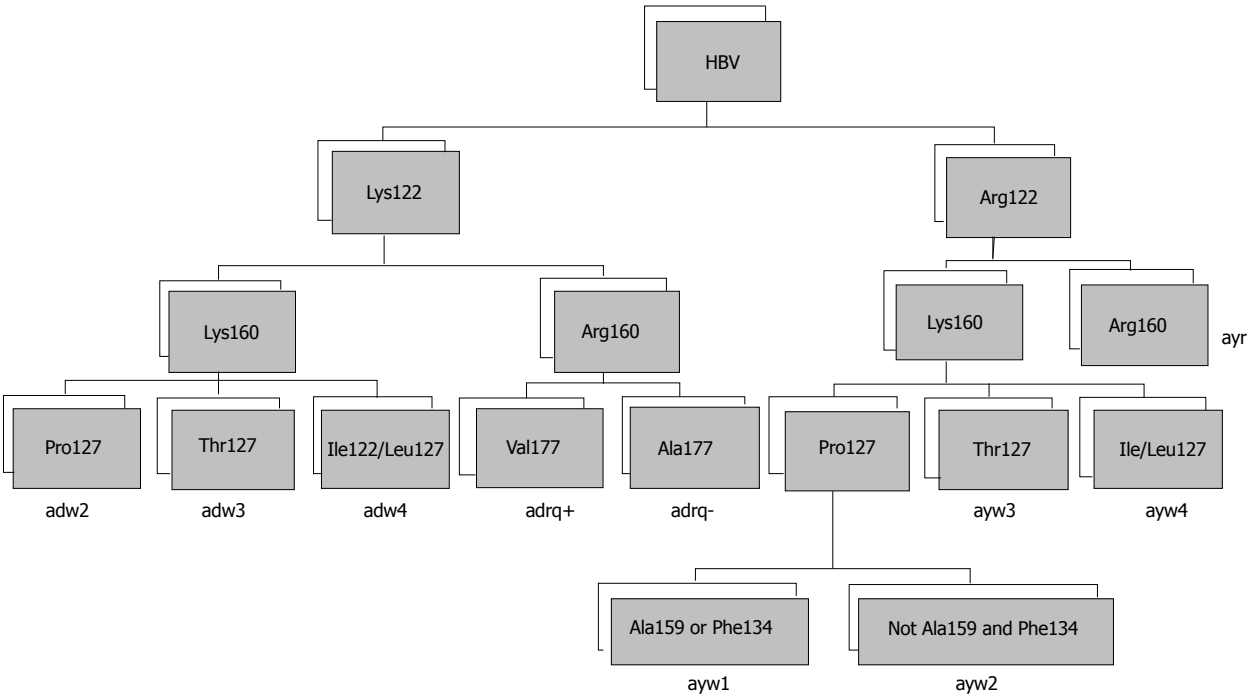

Figure 2 Algorithms for determining hepatitis B virus subtype from the primary structure of the *S* gene. HBV: Hepatitis B virus.

the United States of America, and Europe (HBV/A2). Several reports from Africa, India, and Brazil have shown that HBV/A1 is associated with a high incidence of HCC in younger patients without cirrhosis[22]. By contrast, HBV/A2 is reported to be associated with a lower incidence of HCC than HBV/D and HBV/F[23,24]. HBV/A2 readily progresses to chronic infection after an acute infection, and is a major genotype in cases of vertical transmission[25]. HBV/B is mainly distributed in Asia, and is subclassified into HBV/B1/Bj and HBV/B2-5/Ba. HBV/B1 is found in Japan and is the most asymptomatic genotype. HBV/B2-5 is mainly detected in South-East Asia and has similar clinical characteristics to HBV/C. HBV/B and HBV/C are prevalent in the Far East and in South-East Asia. Several studies from Taiwan, Thailand, China, and Japan have shown that HBV/C is more aggressive and is associated with a greater risk of HCC than HBV/B[26-29]. HBV/D is detected worldwide, with HBV/D1 in Central Asia, HBV/D2 in Russia, HBV/D3 in Inner Mongolia, and HBV/D4 in Africa. HBV/D is reportedly associated with worse clinical outcomes than HBV/A[23,30].

The therapeutic efficacy of antiviral drugs is also

Table 2 Hepatitis B virus subtypes and genotypes and their geographic distributions

Genotype/ subgenotype	Geographic location
A	
A1	Saharan Africa, India
A2	Northern Europe
A3	Western Africa
B	
B1 (Bj)	Japan
B2-5	East and Southeast Asia
B6	Alaska, Northern Canada
C	
C1-3	Taiwan, China, South Korea, Southeast Asia
C4	Australia
C5	Philippines, Vietnam
D	
D1-5	Africa, Europe, Mediterranean countries and India
E	West Africa
F	Central and South America
G	France, Germany, United States
H	Central America
I	Vietnam and Laos
J	Ryukyu, Japan (Kalimantan)

related to the HBV genotype. HBV/A and HBV/B had better persistent response to IFN than HBV/C and HBV/D. A meta-analysis revealed that the response to IFN, including HBeAg seroconversion, loss of HBeAg and loss of HBV DNA, is better in HBV/A compared with HBV/D, and the response of HBV/B is better than that of HBV/C[31]. Whereas the HBeAg seroconversion rate by one-year treatment were respectively HBV/A 47%, HBV/B 44%, HBV/C 28%, and HBV/D 25%, HBsAg seroclearance rates were respectively HBV/A 14%, HBV/B 9%, HBV/C 3%, and HBV/D 2%[32]. It has also been reported that the HBs and HBe seroconversion rates are higher for HBV/A than for other genotypes[33]. A recent study reported that the response to IFN in HBV/E was worse than that in other genotypes[34]. However, the therapeutic responses to nucleotide analogue have been shown as mostly similar. Several meta-analyses revealed that HBV/B had no different response to lamivudine as HBV/C[35,36]. Though few studies were available, the therapeutic efficacy to other nucleos(t)ide analogues except lamivudine was same among genotypes[37]. It would be because the genomic variety in relation to antiviral resistance within polymerase region were mostly same among genotypes (Table 3).

METHODS AVAILABLE FOR DETECTING MUTATIONS

Recent technological advances have made it easier to detect mutations in HBV DNA. Several approaches can be used to detect HBV genomic mutations. Polymerase chain reaction (PCR) amplification with direct Sanger sequencing is perhaps the most commonly used method, but it cannot detect variations in < 20% of viral quasispecies. By contrast, line probe assays can detect specific variants occurring in > 5% of viral quasispecies[38,39]. Other highly sensitive methods include restriction fragment length polymorphism analysis[40], clone-based sequencing[41], and real-time PCR. More recently, several next-generation sequencing methods have been developed, including ultra-deep pyrosequencing, which can detect thousands of clonally amplified regions[42-45]. However, this method also has some limitations and it is unclear whether the variants found in different positions are actually located in the same clones because the next-generation sequencing read is shorter than the Sanger sequence read. Furthermore, it is still difficult to analyze insertion and deletion variants.

CHARACTERISTICS OF THE *PRE-S/S* GENE

The S ORF (nt 2854-835) encodes three different translated genes: pre-S1, pre-S2, and S domain. The pre-S domain is essential for viral binding to hepatocyte receptors and contains several epitopes that are targeted by T and B cells. The S domain is also important in the production of HBsAg[9]. There are three forms of HBV surface proteins (S, M, and L), of different sizes[46]. The 24-kDa S protein, which contains 226 amino acids, is the major component of the envelope protein and is involved in particle budding. The 33-kDa M protein contains the S protein and an additional 55 amino acids encoded by the *pre-S2* gene. Finally, the 39-kDa L protein contains the M protein and an additional 108 or 119 amino acids, the sequence of which depends on the genotype[47]. The three HBsAg types share a common region, consisting of the main antigenic loop (amino acids 124-147), which is called the "a" determinant region. The "a" determinant region is the main epitope to induce a protective immune response. It is located in the major hydrophilic region (MHR) of the S protein, which is between amino acids 103 and 173. The MHR forms a two-loop structure. Mutations and variations in the *S* gene have been reported in many countries[48]. Although these mutations occur naturally, they are also generated during immunoglobulin therapy or vaccine-induced immunity. Variations in the "a" determinant region cause changes in HBsAg antigenicity and may prevent the detection of HBsAg in HBsAg screening assays (Figure 3)[49,50]. These mutations also occur naturally in developing countries where nucleos(t)ide analogues are less frequently used than in developed countries[51,52].

HBsAg is an important diagnostic marker and its expression level is related to the efficacy of an antiviral treatment. Recent studies have revealed that the HBsAg titer correlates with the level of HBV DNA

Table 3 Nucleotide alignment of polymerase region among different genotypes

Position	169	173	180		184		202		204			207		213						221					231		236		238		248		250
Consensus	I[1]	V[1]	L[1]	A[1]	T	-	S[1]	Y	M[1]	D	D	V	-	S	V	Q	H	L	-	F	T	A	V	-	L	-	N[1]	P	N	-	N	F	M[1]
A1_AY233288_SouthAfrica	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-
A2_AJ309370_France	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	R	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-
B1_D23679_Japan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	A	-	-	-	-	-	-	-	H	-	-	-	-
B2_AB073639_Taiwan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	A	-	-	-	-	-	-	-	H	-	-	-	-
B3_M54923_Indonesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	A	-	-	-	-	-	-	-	Q	-	-	-	-
C1_AB112471_Thailand	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	I	-	-	-	-	-	-	-	-	-	-
C2_AB014376_Japan	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	S	I	-	-	-	-	-	-	-	-	-	-
D1_FJ386590_China	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-
D2_JF754597_Turkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-
D3_EU594434_Estonia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E_X75664_Senegal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	Y	-	S	-	-	-	-	-	-	-	-	-	-	-
F_AF223963_Argentina	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	Y	-	-	-	-	V	-	-	T	S	-	-	-	-
G_AB064312_United States	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	D	-	-	-	-
I_AB241408_Vietnam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]Indicated the known antiviral resistant against nucleos(t)ide analogue.

and hepatocarcinogenesis. However, mutations in the *pre-S/S* region are strongly related to HBs antigenicity, and it is reported that the presence of *pre-S/S* variants correlates negatively with the HBsAg titer[53]. Mutations in the S region result in antigenic variations and may allow HBV to escape vaccination. Such mutations are known as "vaccine escape mutations". A study of HBsAg-negative patients from Hong Kong revealed that a variety of mutations, including deletions in the promoter region, abolition of the pre-S2/S start codon, disruption of the pre-S2/S mRNA splice site, nucleotide duplications, and missense mutations in the "a" determinant region, contribute to defects in HBsAg production (Table 4)[54].

The HBs antigen was discovered pathologically as ground glass hepatocytes (GGH) in 1973[55]. Different types of GGHs are associated with the expression patterns of surface/core antigens and the stage of virus replication. Type Ⅰ GGHs express an inclusion-like pattern of HBsAg and carry mutants with deletions in the pre-S1 region. By contrast, type Ⅱ GGHs are distributed in clusters, emerge in the late replicative phase, and contain mutants with deletions in the pre-S2 region. Because the pre-S2 region includes an epitope targeted by cytotoxic T lymphocytes, type Ⅱ GGHs may represent an immune escape mutant[56]. It has also been reported that pre-S mutants could induce endoplasmic reticulum stress, followed by oxidative DNA damage and genomic instability[57,58]. Recent studies have also shown that the *pre-S2* region upregulates human telomerase reverse transcriptase expression and transactivates forkhead box P3 expression, which may promote the development of HCC[59]. Clinical studies have revealed that pre-S deletions, pre-S2 start codon mutations, and the *T53C* mutation in the pre-S2 region are related to the development of HCC[60].

CHARACTERISTICS OF THE *P* GENE

The *P* gene encodes the 843-amino-acid virus-specific DNA polymerase and partially overlaps the other three genes. The DNA polymerase is located in the core of the virus. It acts as the DNA primer and exhibits reverse transcriptase, RNaseH, and DNA-dependent DNA polymerase activities.

A tyrosine (Y)-methionine (M)-aspartic acid (D)-aspartic acid (D) motif (YMDD) starting at codon 203 forms the enzyme activity center of the polymerase There are several well-known hot spots in the HBV DNA where mutations lead to the emergence of antiviral drug resistance. In particular, long-term treatment with lamivudine sometimes leads to the emergence of YMDD variants and breakthrough hepatitis[61]. Nucleos(t)ide analogues approved for the treatment of CHB include lamivudine,

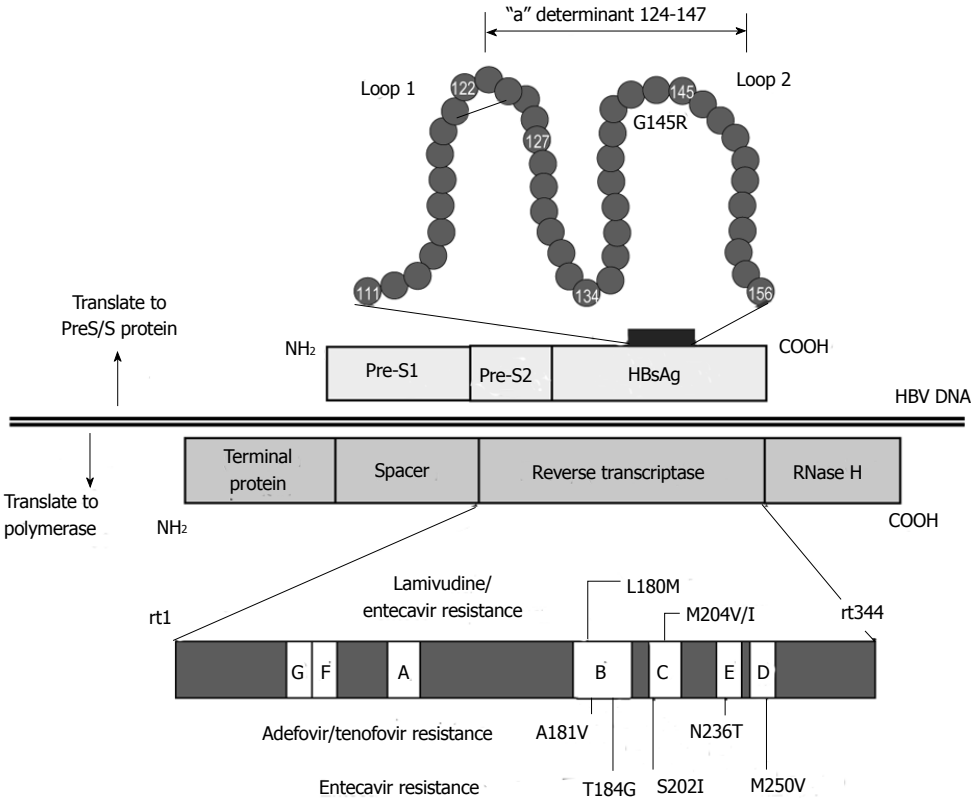

Figure 3 Structures of the overlapping *P* and *S* genes. HBsAg: Hepatitis B s antigen; P: Polymerase; S: Surface.

adefovir, entecavir, telbivudine, and tenofovir. Nucleos(t)ide analogues have a similar structure to natural nucleotides and compete with natural nucleotides for binding sites on the polymerase during DNA synthesis. Incorporation of nucleos(t)ide analogues instead of natural nucleotides disrupts DNA synthesis and suppresses viral replication.

M204M/I is a well-known mutation that confers resistance to L-nucleosides, including lamivudine and telbivudine. The M204V/I mutation is also associated with compensatory mutations, such as L80V/I, I169T, V173L, L180M, T184S/G, S202I, and Q215S[62]. Mutations A181T and N236T, which are located outside the YMDD motif, are major mutations that confer resistance to alkyl-phosphonates, such as adefovir and tenofovir[63]. The mutations T184G/S, S202I/G, and M250V in combination with L180M and M204V confer resistance to D-cyclopentanes, including entecavir (Tables 4 and 5)[64].

The overlapping region of the *P* and *S* genes is important for drug resistance and HBs antigenicity (Table 3). A triple mutation in the P protein (V173L + L180M + M204V) is accompanied by a double mutation in the S protein (E164D + I195M). This mutant may confer antiviral resistance and promotes vaccine escape[65,66]. Furthermore, the introduction of an rtA181T (A181T in reverse transcriptase) surface nonsense mutation (rtA181T/sW172*) reduced viral replication and increased drug resistance compared with the introduction of an rtA181T surface missense mutation (rtA181T/sW172S)[67].

CHARACTERISTICS OF THE *X* GENE

The X ORF (nt 1374-1838) encodes HBx, a 154-amino-acid 16.5 kDa protein. HBx is a multifunctional protein that modulates transcription, signal transduction, cell-cycle progression, protein degradation pathways, apoptosis, and genetic stability by interacting with a variety of host factors[68,69].

HBx protein is strongly associated with the development of HCC. HBx activates cAMP and several transcription factors, including nuclear factor κB and activating transcription factor 2. It also stimulates RAS, SRC, and c-JUN, resulting in activation of the RAS–RAF oncogenic pathways[70].

Deletion of the basal core promoter (BCP) causes a frame shift in the *X* gene, leading to the production of a truncated X protein. The truncated X protein is frequently detected in HCC, and is thought to contribute to hepatocarcinogenesis by upregulating *RAS* and *MYC*. Despite the deletions of nt 1637-1667, which regulate p53-dependent transcription, and nt 1733-1754, corresponding to the SP1-binding region in the CP domain, truncated X protein is still capable of regulating various transcription factors and competes with protein p53. Moreover, because amino-acid mutations at positions 130 and 131 of the X protein overlap the core promoter region, these mutations are associated with the progression of CHB and

Table 4 Representative mutations and clinical characteristics

Region	Mutation	Clinical characteristics
Pre-S/S	P120S/E, K122R, T126A, P127T, Q129H/R, L134S, K141E, P142S, D144A/E/V, G145R/A	OBI/HBsAg decrease
	Pre-S deletion	HCC
Pre-C/C	A1896T, G1899A	HBe seroconversion HCC
X	C1653T, T1753C, A1762T, G1764A	HCC
P	L180M, A181V, T184G, S202I, M204V/I N236T, M250V	NA resistance

S: Surface; OBI: Occult hepatitis B virus infection; HCC: Hepatocellular carcinoma; HBe: Hepatitis B e; HBsAg: Hepatitis B s antigen.

hepatocarcinogenesis.

Several mutations in the *X* gene are reported to be are associated with hepatocarcinogenesis. Liao *et al*[71] reviewed 85 case–control studies and reported that G1896A (OR = 1.46), G1899A (OR = 3.02), the pre-S1 deletion (OR = 2.94), and pre-S2 deletion (OR = 3.02) were significantly associated with the development of HCC. The A1762T/G1764A double mutant, T1753V and C1653T in the BCP were also associated with HCC. Similar results have reported in another meta-analysis of case–control studies[72], and several other mutations in the *X* gene are associated with hepatocarcinogenesis[73,74].

CHARACTERISTICS OF THE PRE-CORE/CORE GENOME

The pre-C/core region contains two regions; pre-C (nt 1814-1901) and core (nt 1901-2452). The core ORF encodes an 183-amino-acid core protein (HBcAg) and the pre-C ORF encodes the 29-amino-acid protein that connects to the N-terminal tail of the core protein. Although the *pre-C/C* gene produces HBcAg and HBeAg, only the cleaved form of HBeAg is released from infected cells into the blood, together with the HBV particle. Although the function of HBeAg is not completely understood, it may act as an immune "tolerogen", contributing to the establishment of chronic infection[75].

The BCP and the adjacent pre-C region are crucial for the replication of HBV. The BCP binds to various liver factors and pre-C forms a pregenomic RNA structure that acts as the encapsidation signal[8]. Changes in viral replication may influence the progression of liver diseases[76]. In particular, nucleotide mutation G1896A, which replaces tryptophan with a stop codon at codon 28, is the most common and important factor responsible for the inhibition of HBeAg production[77,78].

A relatively common double mutation (A1762T and G1764A) in the BCP is responsible for reduced pre-C mRNA synthesis[79].

Table 5 Mutations associated with resistance to nucleos(t)ide analogues

Nucleos(t)ide analogue	Resistance-conferring mutations
Lamivudine	I169T, V173L, L180M, T184G, S202G, M204V/I, M250V
Adefovir	A181V/T, N236T
Entecavir	I169T, V173L, L180M, T184G, S202G, M204V/I, M250V
Telbivudine	I169T, V173L, L180M, T184G, S202G, M204V/I, M250V
Tenofovir	N236T

CONCLUSION

The treatment of CHB has changed dramatically in recent years. However, there is increasing evidence that viral variations and mutations that allow the virus to escape antiviral therapies are clinically important. HBV mutations are also closely related to the serological status of the patients. Understanding the viral mutations and their associations with the clinical characteristics of HBV infection should contribute to improvements in diagnostic procedures and therapeutic guidelines. Recent technological advances have made it easier to assess the HBV genome and detect possible variations or mutations in it. We believe it is important to discuss and implement generalized methods that are suitable for use worldwide.

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MINIREVIEWS

Prevention of hepatocellular carcinoma: Focusing on antioxidant therapy

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Abstract

Oxidative stress has been investigated in the context of alcoholic liver injury for many years and shown to be a causal factor of chronic hepatitis C (CHC), nonalcoholic steatohepatitis (NASH), drug-induced liver injury, Wilson's disease, and hemochromatosis. In CHC, it has been demonstrated that oxidative stress plays an important role in hepatocarcinogenesis. In cases with persistent hepatitis due to failure of hepatitis C virus eradication, or chronic liver disease, such as NASH, the treatment of which remains unestablished, it is important to reduce serum alanine aminotransferase levels and prevent liver fibrosis and development of hepatocellular carcinoma. This also suggests the importance of antioxidant therapy. Among treatment options where it would be expected that anti-inflammatory activity plays a role in their confirmed efficacy for chronic hepatitis, iron depletion therapy, glycyrrhizin, ursodeoxycholic acid, Sho-Saiko-To, and vitamin E can all be considered antioxidant therapies. To date, however, the ability of these treatments to prevent cancer has been confirmed only in CHC. Nevertheless, anti-inflammatory and anti-fibrotic effects have been demonstrated in other liver diseases and these therapies may potentially be effective for cancer prevention.

Key words: Chronic hepatitis; Antioxidant therapy; Hepatocellular carcinoma; Prevention; Iron depletion therapy

Core tip: Among treatment options where it would be expected that anti-inflammatory activity plays a role in their confirmed efficacy for chronic hepatitis, iron depletion therapy, glycyrrhizin, ursodeoxycholic acid, Sho-Saiko-To, and vitamin E can all be considered antioxidant therapies. In chronic liver diseases, it has been demonstrated that antioxidant therapy may potentially be effective for suppressing inflammation and liver fibrosis and expected to prevent carcinogenesis.

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INTRODUCTION

Oxidative stress has been investigated for many years as a possible cause of alcoholic liver injury. Recently, it has attracted attention as one of the causal factors for a variety of liver diseases, such as chronic hepatitis C (CHC), nonalcoholic steatohepatitis (NASH), drug-induced liver injury, Wilson's disease, and hemochromatosis. Furthermore, it has been demonstrated that oxidative stress plays an important role in hepatocarcinogenesis in CHC.

Recent studies have shown that excess hepatic iron accumulation in CHC patients contributes to liver injury[1-3]. It is believed that free iron in the liver facilitates the formation of reactive oxygen species (ROS), including hydroxyl radicals (•OH), which cause oxidative damage to numerous cellular components, including lipids, proteins and nucleic acids, and also cause an up-regulation of collagen synthesis[4]. Further, •OH is known to generate promutagenic bases such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), which has been implicated in spontaneous DNA mutagenesis and carcinogenesis[5,6]. Although the mechanism of hepatocarcinogenesis due to hepatitis C virus (HCV) infection remains unclear, long-term follow-up studies indicate that most patients with progressive liver disease who develop cirrhosis and/or hepatocellular carcinoma (HCC) have persistently elevated or fluctuating serum alanine aminotransferase (ALT) levels, suggesting that they have a background of chronic active inflammation and regeneration of the liver[7]. Further, we have demonstrated in a 6-year follow-up study of CHC patients that iron depletion therapy, consisting of intermittent phlebotomies and a low-iron diet, significantly reduced serum ALT levels, the histological hepatic fibrosis grade, and hepatic 8-OHdG levels[3].

In cases with persistent hepatitis due to failure of HCV eradication and chronic liver disease, such as NASH, the treatment of which remains unestablished, it is important to reduce serum ALT levels and prevent liver fibrosis and development of HCC. This also suggests the importance of antioxidant therapy. To date, reported effective treatment options expected to exert anti-inflammatory activity for chronic hepatitis include iron depletion therapy, glycyrrhizin, ursodeoxycholic acid, and Sho-Saiko-To, which can be considered as antioxidant therapies. Cancer prevention by antioxidants such as vitamin E has also been investigated (Table 1). Here, we review iron depletion as an antioxidant therapy for the treatment of the inflammatory effects of chronic hepatitis to reduce fibrosis and prevent cancer, as illustrated in this paper by an analysis of own cases.

IRON DEPLETION THERAPY

The liver is the major iron storage organ in the body; thus, it is not surprising that disorders of iron metabolism are involved in chronic liver diseases. We have shown previously that in Long-Evans cinnamon rats, an abrupt accumulation of iron in the liver causes spontaneous hepatitis and subsequent development of HCC[8]. Free iron in the liver is believed to catalyze the formation of ROS[4]. In particular, the Fenton reaction, in which Fe^{3+}, •OH and OH^- are produced in the presence of Fe^{2+} and H_2O_2, generates large amounts of highly toxic promutagenic ROS hydroxyl radicals (•OH)[9,10].

Iron overload in the setting of hereditary hemochromatosis has long been known to be associated with an increased risk for HCC[11]. Standard of care is phlebotomy to reduce total body iron levels and achieve normal ferritin levels. Although for ethical reasons the beneficial effect of phlebotomy has never been formally demonstrated in controlled trials, Bomford *et al*[12] reported that the percentage survival 5 years after diagnosis was 66% in 85 patients treated by phlebotomy, and 18% in 26 untreated patients who died before phlebotomy had become widely accepted. Hepatic iron accumulation has also been reported in patients with CHC[13] but the mechanisms responsible for this have not been fully elucidated. Possibly, inflammatory cytokines stimulate iron uptake *via* up-regulation of transferrin receptor expression in hepatocytes, as described previously[14]. Nishina *et al*[15] demonstrated in mice that HCV-induced reactive oxygen species may down-regulate hepcidin transcription which leads to increased duodenal iron transport and macrophage iron release, causing hepatic iron accumulation. We have reported previously that iron depletion improves serum ALT levels as well as hepatic oxidative DNA damage in patients with CHC, and that long-term phlebotomy together with a low-iron diet lowers the risk of developing HCC[3,16]. In this cohort study, we undertook weekly phlebotomy (200 g) until the patients achieved a state of mild iron deficiency, and we followed this by monthly maintenance phlebotomy for 107 mo (median). Patients were advised to consume a low-iron diet (5-7 mg iron/d). We have continuously followed these patients, with the result shown in Figure 1. If dietary iron intake is not restricted, phlebotomy may lead to enhanced iron absorption; therefore, a low-iron diet is essential for a successful outcome of this treatment.

It was recently reported that a high frequency of patients with NASH develop HCC. NASH is a severe form of nonalcoholic fatty liver disease (NAFLD)[17] suggested by Day *et al*[18] to require two hits for its development, (1) excess accumulation of triglyceride in the hepatocyte; and (2) factors such as free radicals capable of inducing oxidative stress. Slight increases of hepatic iron concentration have been reported in NAFLD/NASH patients[19]. Although the exact mechanisms involved in iron overload remain to be clarified, it can be hypothesized that insulin plays a role by stimulating cellular iron uptake through

Table 1 Clinical trials of chemoprevention effects in hepatocarcinogenesis

Therapy	Ref.	Year	Study design	Treated patients/control	Disease	Combined medication	Hepatocarcinogenesis rate
Phlebotomy	Kato *et al*[16]	2007	Open labeled	35/40	Chronic hepatitis C	None	Hepatocarcinogenesis rates in iron depletion and control were 5.7% and 17.5% at the end of the fifth year, and 8.6% and 39% in the tenth year, respectively (P = 0.018)
Glycyrrhizin	Ikeda[29]	2007	Case-control	244/102	Chronic hepatitis C	None	Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the fifth year, and 21.5% and 35.5% at the 10th year, respectively (P = 0.021)
Glycyrrhizin	Arase *et al*[32]	1997	Case-control	84/109	Chronic hepatitis C	None	The 10th-year rates of cumulative HCC incidence for the treated and untreated group were 7% and 12%, and the 15th-yr rates were 12% and 25%, respectively (P = 0.032)
Ursodeoxycholic Acid	Tarao *et al*[44]	2005	Case-control	56/46	Hepatitis C virus -associated liver cirrhosis	Sho-saiko-to, Ursodeo-xycholic acid	The cumulative 5-yr incidence of HCC in the patients treated with UDCA was 17.9% and was significantly lower than that in patients not treated with UDCA (39.1%; P = 0.025)
Vitamin E	Kakizaki *et al*[48]	2001	Randomized controlled	44/39	Chronic hepatitis C	None	Cumulative tumor-free survival tended to be higher in the Vit E group than in controls, albeit statistically insignificant
Sho-saiko-to	Oka *et al*[64]	1995	Randomized open controlled	130/130	Cirrhosis from chronic liver disease	None	The cumulative incidence curve for 5 yr of the trial group was lower than that of the control group (P = 0.071), albeit statistically insignificant

HCC: Hepatocellular carcinoma; UDCA: Ursodeoxycholic acid.

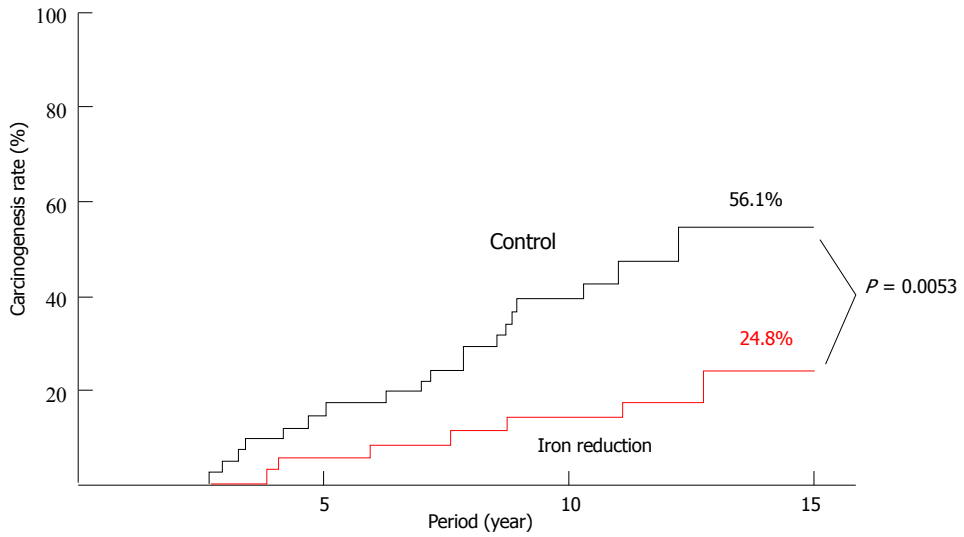

Figure 1 Crude hepatocarcinogenesis rate in iron reduction and control groups.

increased transferrin receptor expression[20]. Facchini *et al*[21] reported an improvement in ALT levels and plasma insulin concentrations following phlebotomy in 17 NAFLD patients with impaired glucose tolerance. Riquelme *et al*[22] reported histological resolution of NASH after iron depletion therapy in a case report. According to Fargion *et al*[23], HOMA-IR and ALT were significantly reduced after phlebotomy in 42 patients with NAFLD. Sumida *et al*[24] also reported that aspartate aminotransferase (AST) and ALT were reduced by phlebotomy in 9 Japanese patients with NASH. Valenti *et al*[25] reported that 64 NAFLD patients treated by phlebotomy achieved significant reduction in insulin resistance compared with 64 NAFLD patients who underwent lifestyle modifications only. Fujita *et al*[26] showed that iron reduction resulting from a-combination of phlebotomy and a low iron diet resulted not only in improvement of ALT levels but also normalization of hepatic levels of 8-OHdG in 11 NASH patients. In a phase Ⅱ trial on 31 patients with NAFLD, phlebotomy resulted in a significant improvement in the NAFLD activity score (NAS), AST and ALT[27]. In a phase Ⅲ trial, Valenti *et al*[28] studied 38 NAFLD patients randomized to phlebotomy (n = 21) or

lifestyle changes alone (n = 17). It was concluded that phlebotomy was associated with improvement in NAS, AST, ALT and γGT without adverse events.

Because it has been reported that iron depletion therapy has anti-inflammatory effects in NASH, it may also contribute to the prevention of hepatocarcinogenesis in these patients. However, it has been reported that it is not effective in all cases[25]. It would therefore be valuable to establish a method for selecting those NASH patients most likely to benefit clinically from iron depletion therapy.

GLYCYRRHIZIN (GLYCYRRHIZIC ACID), STRONGER NEO-MINOPHAGEN C

Glycyrrhizin is a triterpene glycoside from licorice root (Glycyrrhiza glabra) and consists of one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. Glycyrrhizin is widely used in patients with chronic viral hepatitis because of its anti-inflammatory action and beneficial effects on ALT levels and histology[29]. The anti-inflammatory action of glycyrrhizin is believed to be due to its protective effect on the hepatic cellular membrane, which may explain its ability to lower the serum transaminase level in patients with chronic hepatitis. Kiso *et al*[30] demonstrated that GA inhibited free radical generation and lipid peroxidation *in vitro*. Stronger neo-minophagen C (SNMC, Minophagen Pharmaceutical, Tokyo, Japan), was first reported by Yamamoto *et al* in 1958, and has now been used in the treatment of chronic liver disease for more than 50 years. SNMC is a compound GA tablet that includes GA (2 mg) together with glycine acid (20 mg) and L-cysteine hydrochloride (1 mg)[31]. In 1977, Arase *et al*[32] confirmed its ability to reduced aminotransferase levels in patients with histologically-documented chronic hepatitis in a double-blind randomized controlled trial using a dose of 40 mL daily for a month. According to a retrospective study of 84 patients (Group A) who had been treated with SNMC at a dose of 100 mL daily and 109 patients (Group B) who could not be treated with SNMC or interferon, 36% of Group A achieved ALT normalization. The 10-year HCC rates in Groups A and B were 7% and 12%, respectively, and the 15-year rates 12% and 25%[32]. van Rossum *et al*[33] performed a double-blind randomized placebo-controlled trial in which glycyrrhizin was administered three times per week for 4 wk, but reported that only 10% of the European patients so treated normalized their ALT levels. These investigators also performed an open study in which SNMC was administered six times per week at a dose of 100 mg for 4 wk. At the end of treatment, 20% (3 of 15) of the patients achieved normal ALT levels[34]. Shiota *et al*[35] demonstrated in mice treated with diethylnitrosamine as a model of hepatocarcinogenesis due to viral hepatitis that AST and albumin values were significantly improved and the occurrence of HCC decreased in the glycyrrhizin group. A long-term prospective randomized trial in humans is actually difficult from both ethical and medical viewpoints. Therefore, Ikeda[29] retrospectively analyzed 346 patients with chronic hepatitis with high alanine transaminase, 244 of whom had received glycyrrhizin injections. Carcinogenesis rates in the treated and untreated groups were 13.3% and 26.0% at the fifth year, and 21.5% and 35.5% at the 10th year, respectively.

URSODEOXYCHOLIC ACID

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid which has cytoprotective effects not only in chronic cholestatic liver disease, but also in various other liver diseases. The therapeutic properties of UDCA include hypercholeresis, protection of cell membranes by replacing hydrophobic bile acids, and immunomodulation[36-38]. Ljubuncic *et al*[39] reported that UDCA can act as an antioxidant blocking hydrophobic bile acids which otherwise oxidatively activate Kupffer cells to generate reactive oxygen species *in vitro*. Mitsuyoshi *et al*[40] also proposed the antioxidant effect of UDCA in cultured rat hepatocytes. They demonstrated that UDCA increased thiol-containing proteins such as metallothionein, and activated γ-glutamylcysteine synthetase, which regulates glutathione.

A decrease of serum transaminase levels in patients with chronic hepatitis after UDCA administration was first reported from a pilot study by Leuschner *et al*[41]. The effect was confirmed in a double-blind study by Crosignani *et al*[42] and Bellentani *et al*[43], who also established efficacy in long-term treatment. A retrospective study by Tarao *et al*[44] implied an association of UDCA use with lower incidence of hepatocellular carcinoma in hepatitis C virus-associated liver cirrhosis.

Crosignani *et al*[42] reported that 250 mg/d of UDCA was effective in improving biochemical markers of liver function, but no further improvement could be attained with higher doses in patients with chronic hepatitis. By contrast, according to a large multicenter randomized controlled dose study of UDCA for chronic hepatitis C, Takano *et al*[45] suggested that UDCA at a dose of 600 mg/d was optimal.

VITAMIN E (α-TOCOPHEROL)

Vitamin E, an essential lipid-soluble nutrient, is a potent peroxyl radical scavenger that prevents the propagation of free radicals in membranes and in plasma lipoproteins[46]. Vitamin E has been shown to protect against liver damage induced by oxidative stress in animal experiments[47,48]. In 1997, von Herbay *et al*[49] treated 23 chronic hepatitis C patients refractory to interferon therapy with high doses of vitamin E (2 x 400 IU α-tocopherol/d) for 12 wk. In 11 of these patients, ALT and AST levels improved during treatment. Mahmood *et al*[50] also suggested that Vitamin E can act as a supportive therapy to

protect the liver from damage caused by oxidative stress. In their study, 17 CHC patients, receiving anti-inflammatory drug therapy at least 6 mo prior to Vitamin E administration, were given α-tocopherol 500 mg/d, orally, for a period of 3 mo. The ALT level was lowered in those patients initially with high levels (ALT > 70 IU/L). The thioredoxin (TRX) level was reduced in all patients. Houglum *et al*[51] showed that treatment of 6 interferon-refractory patients with α-tocopherol (1200 IU/d for 8 wk) decreased the level of carbonyl modification of plasma proteins, a sensitive index of oxidative stress, but it did not significantly affect serum ALT levels, hepatitis C virus titers, or the histologically-determined degree of hepatocellular inflammation or fibrosis. These results suggest that the treatment may need to be prolonged. In 2000, the effect of vitamin E against NASH/NAFLD was first reported in a cohort[52]. Eleven children < 16 years old with NAFLD were prescribed oral vitamin E (400-1200 IU/d for 4-10 mo) with the result that serum ALT, AST and alkaline phosphatase decreased significantly during treatment. However, liver histology was not assessed. In a small, uncontrolled pilot trial, Hasegawa *et al*[53] demonstrated improvement in fibrosis in 66% of NASH patients who took vitamin E in doses of 300 mg/d for 1 year. In 2003, according to a prospective, double-blind, randomized, placebo-controlled trial with 45 NASH patients, combination vitamin E and C (1000 IU and 1000 mg per day, respectively) was well tolerated and effective in improving fibrosis scores[54]. Nonetheless, no improvement in inflammatory activity or ALT was seen with this combination. Two subsequent studies by Kugelmas *et al*[55] in 16 NASH patients and Vajro *et al*[56] in 28 NAFLD children also showed no improvement in ALT levels[55,56]. Dufour *et al*[57] showed that two years of treatment with UDCA (12-15 mg/kg per day) and vitamin E (400 IU twice a day) improved laboratory values (AST, ALT) and hepatic steatosis of NASH patients compared with UDCA alone or placebo. Sanyal *et al*[58] concluded that vitamin E therapy was associated with a significantly higher rate of improvement in hepatic steatosis, lobular inflammation, ALT and AST.

SHO-SAIKO-TO (TJ-9, XIAO-CHAI-HU-TANG)

Sho-saiko-to is a traditional Chinese herbal medicine derived from seven species of medicinal plants[59]. Although the mechanism by which Sho-saiko-to protects hepatocytes against liver disease is not fully elucidated, clinical trials have shown its efficacy in patients with chronic hepatitis and liver cirrhosis in Japan, Korea and China. Some plausible actions as an antioxidant have been reported, for example, scavenging free radicals[60,61] and reducing the hepatic level of malondialdehyde, a product of lipid peroxidation[62]. Shiota *et al*[61] demonstrated that TJ-9 lowered diethylnitrosamine-induced ROS, resulting in reduction of 8-OHdG formation and hepatocarcinogenesis in rats. A double-blind multicenter trial reported an improvement in AST and ALT values in 116 chronic hepatitis patients treated with TJ-9 for 12 wk[63]. Oka *et al*[64] showed a weak not statistically significant benefit of TJ-9 treatment at a daily dose of 7.5 g and decreased hepatic carcinogenesis rate in a randomized study of patients with cirrhotic chronic liver disease.

CONCLUSION

In CHC cases with persistent inflammation where HCV eradication is difficult, it has been reported that a combination of antioxidant therapies is an effective method to prevent the onset of liver cirrhosis and HCC. In other chronic liver diseases, in particular NASH, it has been demonstrated that antioxidant therapy may potentially be effective for suppressing inflammation and liver fibrosis and expected to prevent carcinogenesis.

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MINIREVIEWS

Occult hepatitis B virus infection and blood transfusion

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Abstract

Transfusion-transmitted infections including hepatitis B virus (HBV) have been a major concern in transfusion medicine. Implementation of HBV nucleic acid testing (NAT) has revealed occult HBV infection (OBI) in blood donors. In the mid-1980s, hepatitis B core antibody (HBc) testing was introduced to screen blood donors in HBV non-endemic countries to prevent transmission of non-A and non-B hepatitis. That test remains in use for preventing of potential transmission of HBV from hepatitis B surface antigen (HBsAg)-negative blood donors, even though anti-hepatitis C virus tests have been introduced. Studies of anti-HBc-positive donors have revealed an HBV DNA positivity rate of 0%-15%. As of 2012, 30 countries have implemented HBV NAT. The prevalence of OBI in blood donors was estimated to be 8.55 per 1 million donations, according to a 2008 international survey. OBI is transmissible by blood transfusion. The clinical outcome of occult HBV transmission primarily depends on recipient immune status and the number of HBV DNA copies present in the blood products. The presence of donor anti-HBs reduces the risk of HBV infection by approximately five-fold. The risk of HBV transmission may be lower in endemic areas than in non-endemic areas, because most recipients have already been exposed to HBV. Blood safety for HBV, including OBI, has substantially improved, but the possibility for OBI transmission remains.

Key words: Occult hepatitis B infection; Transfusion; Anti-hepatitis B core antibody; Nucleic acid testing; Blood service

Core tip: Hepatitis B surface antigen negative but hepatitis B virus (HBV) DNA positive blood products can evoke hepatitis in blood recipients. Anti-hepatitis B core and HBV nucleic acid testing screening tests are necessary to prevent occult HBV infection transmission by transfusion. Anti-HBs antibody in donors and recipients can protect against hepatitis B infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection *via* blood transfusion

is a major concern in transfusion medicine[1-4]. Screening tests for hepatitis B surface antigens (HBsAg) and anti-hepatitis B core (HBc) antibodies detect HBV transmissible blood and prevent recipient HBV infection. After the introduction of HBV nucleic acid tests (NAT) in blood donor screening, the residual risk of HBV infection by transfusion decreased[5,6]. Implementation of this test revealed occult hepatitis B virus infection (OBI) in blood donors. OBI is defined as the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals who tested negative for HBsAg[7]. The amount of viral DNA in the serum is typically very low in cases of true OBI. Because testing liver tissue is not always practical or possible, OBI is often diagnosed through serum HBV DNA and viral marker tests[8,9].

A positive OBI test may be found in blood donors as a result of various clinical conditions, including: (1) the incubation period of acute infections; (2) the tail-end stage of chronic hepatitis B; (3) low-level viral replication after recovery from hepatitis; and (4) escape mutants not detected by current HBsAg tests[10,11]. HBV transmission by blood transfusion from an OBI donor was first reported in 1978[12]. An increasing number of studies on OBI infectivity of blood products have recently been published[13,14]. In this review, we summarize the role of blood screening tests for HBV infections and update the known risks of OBI transfusion transmission.

ANTI-HBC ANTIBODY

Hepatitis B core antigen (HBcAg) appears in hepatocytes within 2 wk after HBV infection; infectious viremia including HBsAg and polymerase are present in the blood after 3 wk. Anti-HBc IgG forms during the recovery phase of infection and is persistent for life, thus, the presence of this antibody in blood indicates past HBV infection[15]. The analytic sensitivity of HBsAg tests in the 1980s was lower than that of current assays. In 1983, Nath *et al*[16] found that 1 of 16 samples with anti-HBc in the absence of anti-HBs was found to have HBsAg when tested with a more sensitive test. Therefore, additional screening for HBV and surrogate tests for non-A, non-B hepatitis were necessary in the 1980s until the anti-hepatitis C virus (HCV) antibody test became available[17]. The anti-HBc test was introduced in the mid-1980s for screening of blood donors in HBV non-endemic countries, such as the United States. Even after the introduction of the anti-HCV test in the early 1990s, the anti-HBc test continues to be used for donor screening in many countries to prevent potential transmission of HBV from HBsAg-negative donors[18,19]. Several studies have reported effective screening of blood for anti-HBc[20,21]. However, HBV endemic countries were unable to implement anti-HBc screening because many blood products would be discarded due to positive screening tests even though most of the blood would be safe for transfusion. Cases of posttransfusion hepatitis B from positive carrier blood and posttransfusion fulminant hepatitis B from blood containing precore-defective HBV mutants have been reported in Norway and Japan, respectively, countries that did not screen donors for anti-HBc[22,23]. In 1989, Japan introduced anti-HBc testing with a modified algorithm in which anti-HBc-reactive blood with titers < 1:32 or ⩾ 1:32 with anti-HBs ⩾ 200 mIU/mL were used for transfusion[24].

Anti-HBc prevalence is related to regional hepatitis B prevalence, and both are typically proportional to one another. The prevalence rates of anti-HBc in blood donors in the United States are 0.23%[25]; United Kingdom, 0.56%[21]; Denmark, 0.70%[26]; Japan, 1.1%[27]; Germany, 1.88%[28]; Italy, 4.85%[29]; India, 10.82%[30]; South Korea, 13.5%[31]; Egypt, 14.2%[32]; Greece, 14.9%[33]; and Pakistan, 17.28%[34] (Figure 1). O'Brien *et al*[35] reported 5585 (1.13%) anti-HBc repeat-reactive blood donors among 493344 blood donors in Canada, of which 29 (0.52%) were HBsAg-negative but HBV DNA-positive[35]. The anti-HBc test lacks specificity and reactivity of the test reagents varies by manufacturer. Therefore, comparison of anti-HBc positivity should be conducted with caution; it is better to describe general features rather than directly comparing studies. Efforts to improve test specificity have included the addition of reductants such as dithiothreitol and cysteine[36]. High donor HBsAg antibody (anti-HBs) levels (> 100 mIU/mL) are assumed to be putatively protective against the transfusion transmission of HBV[37]. Anti-HBc only or isolated anti-HBc are defined as anti-HBc positive without HBsAg and anti-HBs. An HBV DNA positivity of 0%-15% among those donors positive for anti-HBc only was reported in studies performed in Greece, China, Japan, and Germany[10]. Therefore, the presence of anti-HBc only does not necessarily indicate active viral replication and transmission potential.

NAT IN BLOOD SERVICE

Improved molecular methods for the detection of viral nucleic acids made it possible to introduce NAT for donor screening in the late 1990s[38]. By 1997, several countries in Europe had initiated voluntary screening of pooled plasma donations using NAT, and a directive was issued by the EU requiring HCV RNA testing for all plasma intended for fractionation in Europe by July 1, 1999. Routine NAT for HBV was first introduced in German blood transfusion services in January of 1997[39]. The United States began screening source plasma pools for HCV and human immunodeficiency virus (HIV)-1 RNA in early 1998 under the Food and Drug Administration's Investigational New Drug (IND) program. Currently, more than 90% of whole blood and nearly all source plasma are screened for HCV and HIV by NAT. NAT for blood screening is typically conducted using a multiplex polymerase chain reaction

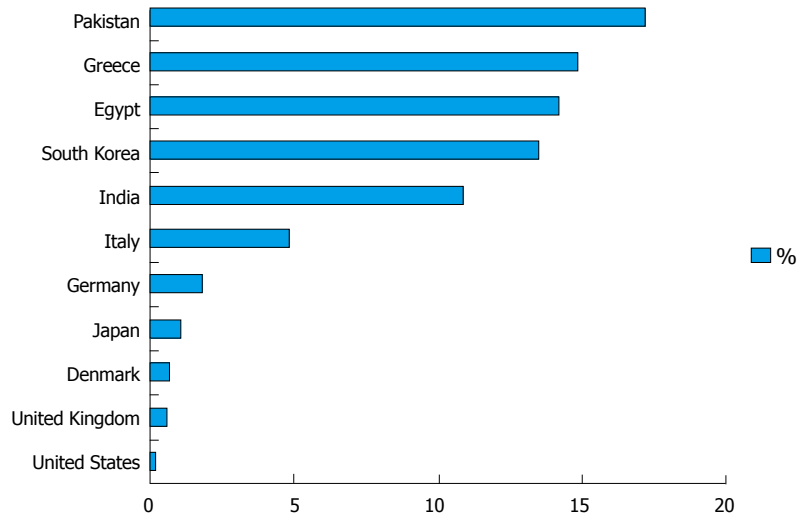

Figure 1 Prevalence of anti-hepatitis B core in blood donors.

method that simultaneously detects the presence of HIV, HCV and HBV[40]. And a multiplex reaction positive case requires discrimination testing[41]. NAT can be performed on pooled samples to reduce running costs[42]. In pooling test system, primary NAT positive cases require a secondary confirmation procedure for identification of positivity of individual donation samples. The American Red Cross implemented automated triplex NAT for HIV, HCV, and HBV in June of 2009[25]. Japan implemented NAT in November 1997 and all donations have been screened since October 1999. Japanese Red Cross Blood Centers serologically screened 500 samples that were pooled and tested for HBV, HCV and HIV-1. To increase HBV detection sensitivity in Japan, the sample pooling size was reduced to 50 in 2000, and 20 in 2004[43].

As of 2012, 30 countries have implemented HBV NAT[44,45], as shown in Table 1. According to international survey results, there were a total of 170 HBV NAT-only positive donors among 19887649 blood donations in 2008, and the prevalence of OBI among blood donors was an estimated 8.55 per 1 million donations[44]. In Taiwan, 8 (0.13%) HBV NAT-only positive donors were identified among 5,973 random donor samples[46]. In China, 22 among 165371 HBsAg-negative plasma samples were identified as OBI-positive; their alanine aminotransferase levels were normal and their viral loads low, with a median of 14 IU/mL[47]. In Iran, 4% of 1000 healthy blood donors were anti-HBc- and OBI-positive, and 8.23% of 11,240 volunteer donors were anti-HBc positive and OBI-positive in Mexico[48,49]. To detect OBI, the HBV DNA test is substantial, and minipool NAT can be used to reduce the cost of testing in developing countries. However, in HBV endemic areas, minipool NAT can result in many primary positive cases, which means that many blood products must be retained until the positivity of individual sample is resolved through a secondary confirmation test. Therefore, it is better to implement NAT for individual–donations rather than minipools for NAT in HBV endemic areas[50-53]. HBV replicates more slowly than HIV or HCV, and its doubling time during the ramp-up phase was estimated to be 2.56 d by Biswas *et al*[54]. Therefore, minipool NAT for HBV detection is less effective than for HIV or HCV detection. Each country should develop its own blood screening strategy based on HBV prevalence, yields of infectious units by different screening methods and cost-effectiveness of testing methods.

OBI TRANSMISSION BY BLOOD TRANSFUSION

Occult HBV is transmissible by blood transfusion, although the transmission rate is considered to be very low. The clinical outcome of OBI transmission mainly depends on the immune status and copies of HBV DNA in blood products of the recipient. A look-back program by the Japanese Red Cross showed that window period-derived blood components evoked 50% (11/22) seroconversion in recipients, but tail-end chronic hepatitis B infections caused only 3% (1/33) seroconversion in recipients[55]. Satake *et al*[55] concluded that the blood infectivity rate during the window period was 10-fold higher than the transmission rates from occult carriers with low-titer anti-HBc. In Canada, a look-back study identified 9.7% anti-HBc-positive recipients and 4 HBV DNA-positive, HBsAg-negative, anti-HBc-positive donors[35]. However, hepatitis cases were reported to have occurred as the result of transfusion of anti-HBc-positive, anti-HBs-positive (12 IU/L), HBV DNA-positive (180 IU/mL) blood product[56].

A study on blood component infectivity by Allain *et al*[14] reported that the presence of anti-HBs in donors reduces the risk of HBV infection by approximately five-fold, while therapeutic fresh frozen plasma over platelet concentrate increases the risk by approximately three-fold by logistic regression analysis[14]. A case of OBI transmission by plasma has been reported, but not by red blood cells[57,58]. Because the amount of plasma containing viral particles is

Table 1 Introduction of hepatitis B virus nucleic acid testing in donor screening

Year	Country
1997	Germany
1999	Austria, Japan
2004	Singapore, Spain
2005	Poland, France (OT + army), South Africa
2006	Greece, Italy, Portugal, Thailand
2007	Hong Kong, Kuwait, Malaysia, New Zealand, Slovenia, Switzerland
2008	Finland, Israel, Latvia, Netherlands, Taiwan
2009	United States, Denmark, Ireland, United Kingdom (England and Wales)
2010	Australia, United Kingdom (Scotland), Canada
2012	South Korea

very small in RBCs, they are considered to be less infective than plasma products. In a look-back study in Taiwan, Su *et al*[59] identified 12 (0.11%) OBI-positive donors among 10824 repository samples; they also identified no post-transfusion hepatitis cases among the recipients. They suggested that the risk of HBV transmission is lower in hyperendemic areas such as Taiwan than in non-endemic areas, because most recipients have already experienced an HBV infection. In a look-back study in Hyogo-prefecture, one of 12 recipients was diagnosed with post-transfusion hepatitis B. Of the remaining 11 recipients, 7 were lost to follow-up, and 4 were negative for HBV[60].

CONCLUSION

The life-saving role of blood transfusion makes it an essential component of modern medical practice. However, blood used for transfusion is not always free of transmissible diseases. According to the 2008 World Health Organization Global Database on Blood Safety, approximately 92 million blood donations are collected worldwide each year. Of these donations, 48% are collected in high-income countries. Blood products from these countries are screened for HBV, HCV, and HIV. However, 39 countries do not routinely test blood donations for transfusion-transmissible infections[61]. HBsAg-positive blood products, including OBI, could be transfused to patients in these counties more frequently than in the countries that screen blood. Although the prevalence of OBI in blood donors differs by country, a 2008 international survey estimated it to be 8.55 per 1 million donations. To reduce the risk of HBV transmission, HBsAg testing was introduced, followed by anti-HBc and HBV NAT in countries where additional testing was feasible. These approaches were also effective for the prevention of transfusion-transmission of OBI. A modified algorithm for anti-HBc screening of blood donors was implemented in intermediate HBV endemic areas, such as Japan, where the test alone could not be introduced because of the resultant high blood discard rates. Unlike anti-HBc screening, HBV NAT can detect infections during window periods. Therefore, despite higher costs, HBV NAT is more suitable for screening in areas with endemic HBV.

Although OBI infectivity depends on recipient immunity and blood product type, it is transmissible by transfusion. One study observed higher genetic diversity in occult HBV genotype B and C strains from South East Asian blood donors[62]. Epigenetic factors have also been identified in HBV cccDNA molecules, and studies to understand OBI immunopathogenesis are currently underway[63-68]. Even with HBV NAT screening, there is a risk of false-negative results. NAT using sample pooling systems in particular cannot detect low-level viremia. Pathogen inactivation technologies that destroy viral DNA or RNA in blood products using chemical agents and ultraviolet illumination have been introduced as alternatives in blood services[69,70]. HBV vaccination of potential recipients is also an important preventive measure. In conclusion, following the implementation of anti-HBc and HBV NAT screening, blood safety for HBV including OBI has improved substantially, but the potential for OBI transmission remains.

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MINIREVIEWS

Staging of liver fibrosis or cirrhosis: The role of hepatic venous pressure gradient measurement

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Abstract

Liver fibrosis is a common histological change of chronic liver injury and it is closely related with portal hypertension which is hemodynamic complication of chronic liver disease. Currently, liver fibrosis has been known as a reversible dynamic process in previous literatures. Although liver biopsy is a gold standard for assessing the stage of liver fibrosis, it may not completely represent the stage of liver fibrosis because of sampling error or semi-quantative measurement. Recent evidences suggested that histologic, clinical, hemodynamic, and biologic features are closely associated in patients with chronic liver disease. Hepatic venous pressure gradient (HVPG) measurement has been known as a modality to evaluate the portal pressure. The HVPG measurement has been used clinically for fibrosis diagnosis, risk stratification, preoperative screening for liver resection, monitoring the efficacy of medical treatments, and assessing the prognosis of liver fibrosis. Therefore, the HVPG measurement can be used to monitor areas the chronic liver disease but also other important areas of chronic liver disease.

Key words: Fibrosis; Liver; Venous pressure

Core tip: Hepatic venous pressure gradient (HVPG) measurement has been used in the clinical fields such as diagnosis of fibrosis, risk stratification, preoperative screening for liver resection, monitoring of the efficacy of medical treatment, and assessing the prognosis of liver fibrosis. HVPG measurement, along with monitoring stage the liver fibrosis, will play important roles in the field of chronic liver disease.

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INTRODUCTION

Liver fibrosis is one of the leading causes of mortality because it changes the architecture of certain organs and disrupts normal function[1,2]. Liver fibrosis is a histological consequence of the wound-healing process resulting from chronic liver diseases such as viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease, and other liver disorders. Deposition of excess extracellular matrix (ECM) that is rich in fibril-forming collagens is a typical finding of liver fibrosis[3]. The excess deposition of the ECM changes the normal architecture of the liver resulting in pathophysiologic damage to the organ.

Liver cirrhosis (LC) is defined as an advanced stage of liver fibrosis with distortion of the hepatic vasculature and architecture. Histologically, regenerative nodules with fibrous tissues form in response to chronic injury and lead to LC[4,5]. Consequently, disruption of the liver architecture due to liver fibrosis and/or cirrhosis causes hemodynamic instability and portal hypertension. The development of portal hypertension is a hallmark of LC.

Portal hypertension is a clinical syndrome defined by an increase in the hepatic venous pressure gradient (HVPG) above 5 mmHg due to increased hepatic resistance[6]. Portal hypertension occurs in patients with fibrosis in the sinusoid of the liver, and portal hypertension is one of the causes of several severe complications of LC (variceal bleeding, ascites, peritonitis, or hepatic encephalopathy) that are associated with its mortality[7,8].

The concept of wedged hepatic venous pressure (WHVP) was described by Myers and Taylor. WHVP can be measured by occlusion of hepatic vein using catheterization[9]. For many years, a safe, simple, reproducible, and less invasive method has been used to measure the HVPG. HVPG means the difference between the portal vein pressure and the hepatic vein pressure. Measuring both the free hepatic venous pressure (FHVP) and the WHVP has been the standard method for estimating HVPG[10-12].

It has been proposed that serial HVPG measurements can estimate the stage of fibrosis or cirrhosis regardless of the etiology[13,14]. In addition, the HVPG measurement has been used clinically for fibrosis diagnosis, risk stratification, preoperative screening for liver resection, monitoring the efficacy of medical treatments, and assessing the prognosis of liver fibrosis. Therefore, the HVPG measurement can be used to monitor areas the chronic liver disease but also other important areas of chronic liver disease[15]. This review presents the role of the HVPG measurement in staging liver fibrosis and LC.

LIVER FIBROSIS AND CIRRHOSIS

Because liver fibrosis is positively related to prognosis, accurate staging of liver fibrosis gives important clinical implications in the management of chronic liver disease[16]. As such, there is a strong demand for reliable liver fibrosis biomarkers that provide insight into the disease etiology, diagnosis, therapy, and prognosis[17]. Currently, diagnostic modalities range from blood biomarkers to genomics as well as to even more advanced techniques such as elastography, or magnetic resonance imaging[17].

The mechanism of liver fibrosis is thought to be associated with the hepatic damage of various etiologic factors followed by the activation of hepatic stellate cells (HSC) within the liver that develop into myofibroblasts[18]. HSCs are resident peri-sinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells[19]. The main cells affected by liver fibrosis are the HSCs and fibroblasts, which are activated by soluble mediators produced by activated Kupffer cells or inflammatory cells in the course of chronic liver disease[20,21].

HSC activation

HSCs activation consists of 3 phases (initiation, perpetuation, and final resolution phase when liver injury resolves)[19]. Initiation is the first phase occurred during HSC activation resulting from paracrine activation by all other cells such as sinusoid endothelium, hepatocytes, cholangiocyte, and platelets. Hepatocyte apoptosis caused by injury also promotes HSCs initiation through a process mediated in part by Fas, tumor necrosis factor-related apoptosis-inducing ligand, endothelial cells, platelets, and Kupffer cells[19,22,23].

The perpetuation phase of HSCs results from the chronic stimulation that signals for cell maintenance of the activated form and induces liver fibrosis[24]. The perpetuation of HSC activation causes changes in cell behavior, including proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, and retinoid loss. The perpetuation phase involves autocrine and paracrine loops. The effect of these changes leads to the accumulation of the ECM in the liver. Activated HSCs become directly fibrogenic by increasing the synthesis and deposition of ECM proteins[24]. The contractility of HSCs may be one of the main causes of elevated portal resistance during liver fibrosis. The collagen bands in LC contain a large numbers of activated HSCs[25]. Liver fibrosis is caused by an unbalance between matrix production and degradation. A main component of ECM remodeling is the family of matrix-metalloproteinases[26]. In addition, the tissue inhibitors of metalloproteinases and the uroplasminogen activator receptor or its inhibitor as well as other components of the plasmin system are closely related to ECM degradation[26-28]. In the case of liver fibrosis resolution, HSCs are either driven to apoptosis or prompted to revert to a quiescent HSC[5,24].

LC

The change from initial liver fibrosis to LC involves the

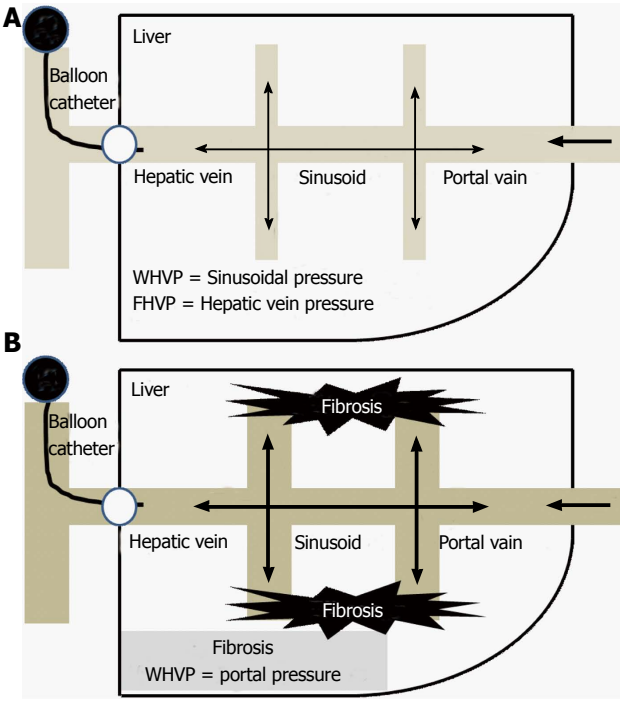

Figure 1 Increase of hepatic venous pressure gradient in liver fibrosis. A: In normal liver, wedge pressure is equivalent to hepatic sinusoidal pressure; B: In cirrhosis, pressure is equivalent to that in the portal vein, and then wedge pressure can be considered equivalent to the portal vein pressure. WHVP: Wedged hepatic venous pressure; FHVP: Free hepatic venous pressure.

inflammation and activation of HSCs with subsequent fibrogenesis, angiogenesis, and parenchymal disruption caused by vascular occlusion[29]. Histologically, LC is characterized by a vascularized fibrosis septum that allows for communication between portal tracts and to the central veins, resulting in liver nodules that are devoid of a central vein and surrounded by a fibrotic band[30]. This vascular distortion leads to shunting of the blood supply between the portal vein and artery and disrupts the exchange between hepatic sinusoids and the liver parenchyma. The hepatic sinusoids are lined by endothelium that is located on a sheet of permeable connective tissue in the space of Disse, in which HSCs and other cells also rest[31]. Hepatocytes, perform most of the known liver functions and line the other side of the space of Disse. In LC, the space of Disse becomes occupied with fibrous tissue, and the endothelium loses its functions, a process known as sinusoidal capillarization[32].

Increased resistance to portal blood flow is the main cause of increased portal pressure in LC. Portal hypertension results from the structural distortion that is associated with advanced fibrosis and LC (Figure 1). LC and the resultant vascular distortion were previously regarded as irreversible. However, recent reliable data suggest that LC regression or even reversal is possible[33].

LIVER BIOPSY

Liver biopsy is currently the gold standard for assessing liver fibrosis and has been used for the diagnosis of fibrosis, risk stratification, prognosis evaluation, and differential diagnoses. In the 1960s, the introduction of the liver biopsy brought about substantial change in the field of liver disease[34]. Currently, several semi-quantitative scoring systems are available for the diagnosis of liver fibrosis (METAVIR, Knodell, and the Ishak score) (Table 1)[35]. Typically, liver fibrosis is scored in stages and necro-inflammation is evaluated by grade. Liver fibrosis is histologically staged by assessing the amount of fibrosis and level of architectural disorganization. Until now, these semi-quantitative scoring systems have been used in many clinical trials and for the evaluation of chronic liver disease. Though the current scoring systems apply a common principle to assess the status of chronic liver disease, none of them specifically describes the relation between these scoring systems and the level of liver fibrosis[36]. Because LC is defined as a diffuse process in which the normal lobules are replaced by architecturally abnormal nodules separated by fibrous tissue, the semi-quantitative nature of these histologic scoring systems do not fully represent the actual state of the liver nor do they include the histologic features of LC have been traditionally linked to clinical outcomes[37]. In addition, tissue obtained by liver biopsy is only a small portion of the entire liver (1/50000); therefore, sampling error may be inevitable[38].

Taken together, liver histology may be the best-standardized method for evaluating the status of chronic liver disease. However, a comprehensive study of chronic liver disease requires data that combine all histological, hemodynamic, and clinical features as well as clinical endpoints, such as the onset of complications of cirrhosis related to LC and the incidence of death[33,39,40].

HEPATIC VENOUS PRESSURE GRADIENT

Methods

Hemodynamically, the sinusoidal connection dissipates the pressure backup from the wedged catheter. Consequently, WHVP is slightly lower than the directly measured portal pressure. In LC, the inter-sinusoidal communication becomes disrupted by the fibrosis septum and tissue, thus the reduction of WHVP becomes blocked. Therefore, the WHVP accurately represents the portal pressure (Figure 1)[41]. The use of a balloon catheter allows for the occlusion of a branch of the large hepatic vein at the lobar and sub-lobar levels. As a result, the hemodynamic stage of a large portion of the liver can be measured *via* the HVPG.

Three veins (antecubital, femoral, or right jugular vein) have been commonly used as route for catheter insertion for the HVPG measurement. A 6 or 7 French balloon catheter is placed in the hepatic vein through a guide track made in the vein to measure the FHVP and FHVP. The WHVP is measured by inflating the balloon. And then, the HVPG is calculated by subtracting the

Table 1 Scoring system of liver fibrosis in liver biopsy

	Score						
METAVIR score	0 No fibrosis	1 Portal fibrosis without septa	1 Portal fibrosis without septa	2 Septal fibrosis (portal-portal)	3 Septal fibrosis (portal-central)	4 Cirrhosis	4 Cirrhosis
Ishak score	1 No fibrosis	1 Some portal tract fibrotic ± short fibrous septa	2 Most portal tract fibrotic ± short fibrous septa	3 Portal tract fibrotic with occasional portal to portal bridging	4 Portal tract fibrotic with marked portal to portal and portal to central bridging	5 Marked portal to portal and/or portal to central with occasional nodules	6 Cirrhosis

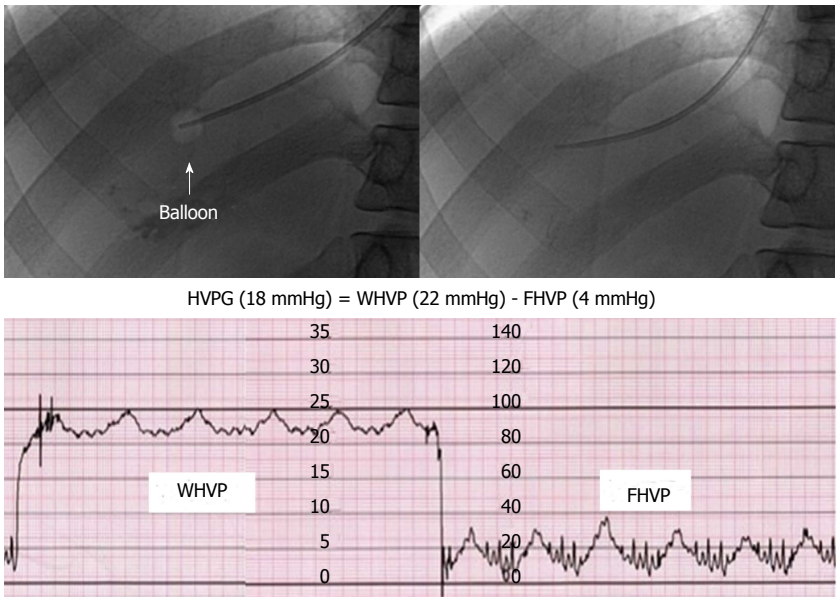

Figure 2 Method for hepatic venous pressure gradient measurement. HVPG: Hepatic venous pressure gradient; WHVP: Wedged hepatic venous pressure; FHVP: Free hepatic venous pressure.

FHVP from the WHVP (Figure 2)[11,42].

Clinical implications

The HVPG measurement has been a useful tool for the diagnosis, evaluation and assessment of the severity and prognosis of chronic liver disease and cirrhosis, including the risk assessment of the LC related complications[43]. Compensated LC is defined according to the presence of varices[44]. Patients with an HVPG $\leqslant$ 10 mmHg had a 90% possibility of maintaining compensated LC during a median follow-up of four years[45]. Ripoll *et al*[46] demonstrated that an HVPG > 10 mmHg increases the risk of clinical decompensation such as bleeding, ascites, hyperbilirubinemia, or encephalopathy. In other reports, patients with LC and an HVPG > 16 mmHg or > 20 mmHg showed a poor prognosis[47-49].

Liver fibrosis

In patients with stage 1 compensated LC, the sensitivity and specificity of the HVPG in predicting stage 1 compensated LC were 78% and 81% at an HVPG of 6 mmHg, respectively[50]. Other reports have also suggested a significant correlation between the HVPG and fibrosis stage[51]. The area under receiver operating characteristic (AUROC) curve of HVPG for the diagnosis of advanced fibrosis was 0.906. The HVPG > 13 mmHg revealed a sensitivity of 79% and specificity of 89% in the prediction of advanced fibrosis[51]. In another study, the HVPG showed a good AUROC of 0.85 for the prediction of advanced fibrosis among patients with chronic viral hepatitis and a sensitivity and specificity of 80% and 77%, respectively, which were superior to that of other serologic biomarkers[52]. In addition, it has been demonstrated that the HVPG is associated with critical complications such as portal hypertension, HCC, and survival[53,54]. Repeated HVPG measurements might assess the progression of fibrosis to cirrhosis despite the lack of other etiologic factors[13,55].

Currently, liver stiffness measurements by transient elastography have been a promising and safe method used to monitor fibrosis progression and to predict portal hypertension in patients with chronic liver disease[56-58]. In patients who had undergone liver transplantation, the HVPG score was correlated with the liver stiffness measurement in the overall population[56]. The positive relation between liver stiffness and the HVPG score has been found in patients with LC, especially those with an HVPG < 10 mmHg[56]. The AUROC for predicting HVPG values of 10 mmHg and 12 mmHg is reported as 0.76 and 0.99 (cutoff value 13.6 kPa and 34.9 kPa), respectively[56,59]. In another study, HVPG scores of > 6 mmHg and > 10 mmHg were

predicted by a cutoff value of 8.7 kPa and 21 kPa, respectively[60].

Variceal bleeding

In patients with LC, the annual incidence rate of variceal bleeding is estimated to be 4%. However, this bleeding risk might be as high as 15% according to the size of the varices[61], and an HVPG > 10 mmHg is considered a good predictor of the development of varices[45]. In one study, an HVPG score of 11 mmHg had sensitivity and specificity for variceal hemorrhage of 92.4% and 27.7%, respectively[62].

In patients with LC, the probability of incidenct bleeding at 3 years after being diagnosed with LC was significantly higher in poor responders than in good responders to therapy with beta-blockers alone or beta-blockers with isosorbide mononitrate[63]. Regarding the primary prophylaxis, few studies have investigated the hemodynamic response to pharmacological therapy because of the difficulties in creating this kind of clinical trials.

In cases of acute variceal bleeding, the HVPG measurement was a good predictor of the prognosis and therapeutic efficacy of medication. Previous studies have suggested that an HVPG of > 12 mmHg is a good indicator of variceal bleeding in patients with LC[64,65]. In patients with acute variceal bleeding, the early prognosis in patients with alcoholic LC was closely related to the HVPG score measured within two days of hospital admission[66,67]. In addition, an HVPG of > 20 mmHg was significantly related to a long hospital stay, numerous blood transfusion, and a lower one-year mortality of 64%[49]. Albrades *et al*[66] demonstrated that an HVPG > 20 mmHg is independently related to the early prognosis of patients with acute variceal bleeding and should be treated with a vasoactive, antibiotic, or endoscopic therapy.

In patients with acute variceal bleeding, emergent endoscopic treatment [endoscopic injection sclerotherapy (EIS) or endoscopic band ligation (EBL)] has been commonly used. In one study, the HVPG was estimated to have significantly increased after endoscopic therapy compared to the pre-treatment HVPG score in the EBL (pre-treatment, 18 mmHg; post-treatment, 21 mmHg) and EIS groups (pre-treatment, 18 mmHg; post-treatment, 22 mmHg)[68]. However, in the EBL group, the HVPG recovered to the pretreatment values within two days after the endoscopic therapy, while in the EIS group, the HVPG score remained high during the five days of follow-up. As a result, the EIS was associated with a continued increase in HVPG in patients with acute variceal bleeding. It is well known that a reduction of 12 mmHg or more or at least than 20% from baseline HVPG score leads to decreased the risk of rebleeding and mortality[69,70]. Another report suggested that an HVPG reduction of > 10% from the baseline value is the best target to induce the greatest response to primary prophylaxis[71].

If patients with LC do not receive pharmacologic treatment, the risk of rebleeding increases to 55%-67%. The use of endoscopic therapy (EIS or EBL), a transjugular intrahepatic portosystemic shunt, or other types of shunts also reduce the risk of rebleeding[72,73]. However, the likelihood that a patient will fail to hemodynamically respond to treatment varies between from 45% to 63%[74]. Another study has suggested that HVPG monitoring was more effective when used with EBL and secondary prophylaxis therapy for variceal rebleeding than it was with EBL alone[75]. The HVPG has been shown to predict bleeding outcome such as variceal rebleeding and mortality[48,49,76].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the main cause of death in patients with LC[77]. A recent report proposed that non-selective beta-blockers decrease the incidence and progression of HCC *via* a reduction of the inflammatory materials from the gut to the liver and by inhibiting translocation[78]. Ripoll *et al*[79] suggested that portal hypertension is a significant predictor of HCC and an HVPG > 10 mmHg increase the risk of HCC by a six fold. In patients with decompensated alcoholic LC, the HVPG may be a predictor for the development of HCC[80]. Bruix *et al*[81] suggested that a high HVPG score is significantly related with decompensation after HCC operation. Another study demonstrated that a high HVPG score was related with mortality after liver resection for HCC[82]. However, in the field of HCC, few data are available about on role of HVPG. Further studies are needed.

Prognosis

Until now, the HVPG measurement has been used for the evaluation of LC prognosis in many studies[10]. The HVPG is a useful tool for the evaluation of viral recurrence after transplantation[83]. In viral LC, lamivudine monotherapy for chronic viral hepatitis has been found to reduce the HVPG effectively in patients with virologic suppression and biochemical remission[84]. One study demonstrated that the HVPG was positively associated with mortality and liver dysfunction after liver resection in patients with HCC[85]. They also concluded that preoperative HVPG measurements should be taken routinely for the evaluation of the prognosis. Moreover, Suk *et al*[86] demonstrated that a repeated HVPG measurement was necessary for the prediction of mortality in patients with decompensated LC. Measurement of the HVPG and albumin have been considered as significant predictors for the development of clinical decompensation in patients with compensated LC[87].

Suk *et al*[86] also reported that the HVPG measurement was better at predicting mortality than the model for end-stage liver disease (MELD) or MELD including serum sodium (MELD-Na) were. In patients with portal hypertension, monitoring the HVPG

Table 2 Pathologic, hemodynamic, and clinical stage of liver fibrosis

Classification	Stages				
METAVIR score	F0-F3	F4	F4	F4	F4
HVPG (mmHg)		> 6	> 10	> 12	> 16 > 20
Clinical class		Stage 1 Compensated	Stage 2 Compensated Varices	Stage 3 Decompensated Varices Ascites	Stage 4-5 Decompensated Variceal bleeding Ascites Other complications
1-yr mortality (%)		1	3	10-30	60-100

This table was modified from ref.[54]. HVPG: Hepatic venous pressure gradient.

after the treatment provides substantial prognostic information[70]. However, other studies have suggested that the MELD-Na is the most predictive of one year mortality in patients with decompensated cirrhosis[88]. However, the combined use of the HVPG and the MELD/MELD-Na score does not improve the prognostic accuracy. To properly evaluate the prognostic accuracy of the MELD, MELD-Na, and HVPG, future studies are needed[86].

A NEW CLASSIFICATION SYSTEM FOR LIVER FIBROSIS

Recently, a new classification system for LC that combines histologic, clinical, hemodynamic and biologic features has been suggested (Table 2)[14,43,54]. This system classifies liver fibrosis according to the presence of compensation or decompensation which is mainly defined by clinical findings[44,89].

At the METAVIR F1-F3 stages (the non-cirrhosis stage of chronic liver disease) without histologic or clinical evidence of LC, the HVPG is expected to be within the normal range (1-5 mmHg). The cirrhotic stage of METAVIR F4 is sub-classified into two stages: compensation and decompensation. Clinical decompensation is defined as the development of ascites, a variceal hemorrhage, encephalopathy, and/or jaundice. The compensated stage can be further classified into stage 1 without varices or stage 2 with varices[54]. Portal hypertension is considered moderate or subclinical when 6 mmHg < HVPG $\leqslant$ 10 mmHg (stage 1 compensated LC)[54]. A clinically significant case is defined when the HVPG is > 10 mmHg (stage 2 compensated LC). A severe case is defined when the HVPG is > 12 mmHg (stage 3 or 4 decompensated LC) (Table 2).

Does new classification system correctly represent liver fibrosis?

In a previous study, the one year outcome probabilities were calculated according to the clinical stage of LC[44]. Recently, D'Amico *et al*[90] proposed that the development of varices and decompensating events in cirrhosis should be divided into five prognostic stages with significantly increasing mortality risks. However, no definite staging system is yet widely accepted for clinical practice in chronic liver disease and there is little evidence available regarding the correlation between hemodynamic (exact score according to stages), pathologic (Laennec fibrosis scoring system according to stages), and clinical staging (complications of LC according to stages) of chronic liver disease[91-94]. Therefore, further studies on the recent classification system that combines histologic, clinical, hemodynamic and biologic findings are needed in the future.

CONCLUSION

The HVPG measurement is a safe, simple, and reproducible method of quantifying liver fibrosis. The recent concept considers LC as a dynamic and potentially reversible disease. There are many stages in liver fibrosis of chronic liver disease. Of these stages, HVPG measurement is a method of evaluating the presence and severity of liver fibrosis. The HVPG measurement has been used clinically for fibrosis diagnosis, risk stratification, preoperative screening for liver resection, monitoring the efficacy of medical treatments, and assessing the prognosis of liver fibrosis. HVPG measurement, along with monitoring stage the liver fibrosis, will play important roles in the field of chronic liver disease. Therefore, measuring HVPG in addition to monitoring hemodynamic effects or staging liver fibrosis will play an important role in the management of chronic liver disease.

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MINIREVIEWS

Evidence-based consensus on the diagnosis, prevention and management of hepatitis C virus disease

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Abstract

Hepatitis C virus (HCV) is a potent human pathogen and is one of the main causes of chronic hepatitis round the world. The present review describes the evidence-based consensus on the diagnosis, prevention and management of HCV disease. Various techniques, for the detection of anti-HCV immunoglobulin G immunoassays, detection of HCV RNA by identifying virus-specific molecules nucleic acid testings, recognition of core antigen for diagnosis of HCV, quantitative antigen assay, have been used to detect HCV RNA and core antigen. Advanced technologies such as nanoparticle-based diagnostic assays, loop-mediated isothermal amplification and aptamers and Ortho trak-C assay have also come to the front that provides best detection results with greater ease and specificity for detection of HCV. It is of immense importance to prevent this infection especially among the sexual partners, injecting drug users, mother-to-infant transmission of HCV, household contact, healthcare workers and people who get tattoos and piercing on their skin. Management of this infection is intended to eradicate it out of the body of patients. Management includes examining the treatment (efficacy and protection), assessment of hepatic condition before commencing therapy, controlling the parameters upon which dual and triple therapies work, monitoring the body after treatment and adjusting the co-factors. Examining the treatment in some special groups of people (HIV/HCV co-infected, hemodialysis patients, renal transplanted patients).

Key words: Hepatitis C virus; Enzyme immunoassay; Nucleic acid testing; Loop-mediated isothermal amplification; Sustained viral response; Telaprevir; Boceprevir; Liver transplant

Core tip: The present review describes the evidence-based consensus on the diagnosis, prevention and management of hepatitis C virus (HCV) disease. Besides the conventional techniques more advanced technologies have come to the front that provides best detection with greater ease and specificity. It is of immense importance to prevent this infection among the sexual partners, injecting drug users, mother-to-infant transmission of HCV, household contact, healthcare workers and people who get tattoos and piercing on their skin. Management includes examining the treatment, assessment of hepatic condition before

commencing therapy, controlling the parameters upon which dual and triple therapies work, monitoring after treatment and adjusting the co-factors.

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INTRODUCTION

Hepatitis is a viral infection of the liver that ultimately causes liver to become swollen thus inflammation occurs. Hepatitis C is caused mostly due to viral agent, smoke, pollution and unhygienic condition of the surrounding[1]. Hepatitis C virus (HCV) is an infectious particle that causes cirrhosis and carcinoma of hepato-cellular components round the globe[2]. HCV prevalence is 4.95% in general and 57% in injecting drug use (IDU) population of Pakistan[3]. HCV is small in size measuring 55-65 nm. It is a positive stranded RNA virus and belongs to the family of *Flaviviridae*. Its RNA genome constitutes a single open reading frame made up of 9600 nucleotide bases long. In addition to envelope proteins E1 and E2, core protein comprises structural proteins and non-structural proteins include NS1, NS2, NS3, NS4a, NS4b, NS5a and NS5b[4].

Mainly, six genotypes exist for HCV isolates having a difference of 30%-35% in their nucleotide sequences and multiple subtypes differ up to 20% to 25%[5]. 1a and 1b genotypes are the most prevalent ones in the Western Europe and United States then 2 and 3 genotypes come next in the order. While genotype 4 is widespread in Egypt, genotype 5 is common in South Africa, and genotype 6 is in Southeast Asia[6]. In patients from Canada and Belgium, another seventh genotype has also been identified[7].

Three common assay procedures have been used to diagnose the infection. These comprise some anti-HCV antibody assay, detection of HCV-RNA and very recently Hepatitis C Virus core antigen assay[8].

This review will focus on the diagnosis, prevention and management of HCV infection.

DIAGNOSIS

Enzyme immunoassay (EIA) and HCV RNA assay are performed to detect the presence of anti-HCV antibodies in suspected patients of acute hepatitis C. HCV RNA assay is a sensitive technique with a lower detection limit of 50 IU/mL or it can be less than this value[9]. In the absence of anti-HCV antibodies, the presence of HCV RNA is a pinpoint of acute HCV infection. It is further confirmed after a few days or weeks through seroconversion (*i.e.,* the emergence of anti-HCV antibodies). At the time of diagnosis, both of HCV RNA and anti-HCV antibodies can be found in patients with acute hepatitis. In this case, it is not easy to distinguish acute hepatitis C with chronic hepatitis C exacerbating acute infection. If both anti-HCV antibodies and HCV RNA are absent then acute infection is improbable. When HCV RNA is absent but anti-HCV antibodies are present acute infection of HCV is unlikely. As HCV RNA can be undetectable temporarily so retest should be taken of these patients after a few weeks. It is mainly because immune response partially takes control of replication of virus before chronic hepatitis C infection occurs[10]. This case is observed also in the patients who have recovered from long-ago HCV infection.

In case of chronic hepatitis C, both HCV RNA and anti-HCV antibodies (detecting 50 IU/mL or less with a sensitive technique) must be present[11,12]. Replication of HCV is detectable only by the third-generation EIAs that has been profoundly observed in hemodialysis and immunodepressed patients[13,14]. In a research study, B-cell epitopes and antigenic regions were recognized after cloning the genome of the HCV[6]. The development of screening tests for anti-HCV IgG, requires synthetic peptides including the epitopes that were immunodominant and recombinant proteins[15,16]. Recently, Food and Drug Administration approved an anti-HCV IgG assay for clinical use in United States[17]. However, these tests are unable to identify active HCV infection in IgG positive patient. As this antibody may be detected in patients who have evaded viral infection. In order to diagnose active HCV infections, nucleic acid testing (NAT) can be carried out for the detection of HCV RNA. But it cannot be used in laboratories frequently as it requires specialized technical staff and expensive tools. However, it can be used to detect HCV RNA. For that matter, serological assays independent of NAT are mostly used for easy identification of HCV infection[18].

Detection of IgG against HCV

Several immunoassays have been developed for the detection of anti-HCV IgG in plasma or serum samples. A recombinant protein expressed in yeast containing an epitope from NS4 section of HCV genome was used to carry out first-generation assays. These were successful in identifying anti-HCV IgG in posttransfusion HCV patients (80%) and caused a significant decrease in the infections. But due to their lack in specificity and sensitivity[19], there was a need to establish second- and third-generation assays. These assays involved use of multiantigens and antigens of core, NS3 and NS4. These modifications proved good for enhanced sensitivity and specificity[16].

Recognition of specific molecules of virus through NATS

The detection of HCV RNA is the most reliable marker

for diagnosis HCV infection. HCV RNA is detectable within 1 wk after its exposure in serum or plasma. As described earlier, NATs recently in use for detecting its RNA work on the principle of polymerase chain reaction (PCR), transcription mediated amplification, and branched DNA signal amplification. Another set of assays named as Qualitative and quantitative PCR assays have been accepted for use in laboratory by regular authorities in Europe and United States for diagnosing HCV RNA. The qualitative assay is not used now but ultrasensitive quantitative NATs have a broad range and can even detect RNA as little as 5 IU/mL. Moreover, HCV genotyping is also applicable in this regard. It is done through restriction fragment length polymorphism analysis, direct sequencing and reverse hybridization to genotype-specific oligonucleotide probes[20].

Role of core protein in HCV diagnosis

This is another very prominent technique for the diagnosis of HCV infection. Core protein is an RNA-binding protein that makes the viral nucleocapsid. A host signal peptidase cleaves it from polyprotein at the C-terminus, generating an immature form of the core protein[21], and further processing of signal peptide present at the C-terminus of the core is done by a host signal peptide peptidase, yielding its mature form[22]. It is highly antigenic in its nature as it can evoke immune responses. HCV nucleocapsid (core) may participate valuably in the vaccine development as it is the most conserved viral antigen. Various studies showed the presence of core protein in nearly 80%-92% of HCV positive patients with anti-HCV antibody[8]. Therefore it can be used as a diagnostic marker. In few studies, HCV core assay is less sensitive than anti-HCV antibody assay or HCV-RNA assay. Quantification of total core antigen of HCV is a precise indirect marker of its replication in the infected people. The sensitivity of HCV-RNA test was found to be 99% that of HCV core assay was noted as 98%[23]. In fact, more studies are required to further validate the beneficial aspect of HCV core estimation over HCV-RNA detection for the identification of the infection. The role of the core protein can be determined more extensively relating to HCV entry, replication and virion production. The culture system may be proved to help in developing antiviral agents targeting different stages of virus cycle, and these may be potential therapeutic agents.

Quantitative antigen assay

In Europe, the Architect HCV Ag assay (Abbott) is commercially available. It employs an automated platform and is an immunoassay based on a chemiluminescence in which monoclonal antibody specific to the HCV core antigen are coated on microparticles[24]. It has been found from many studies that HCV core antigen can be detected within the first 2 wk of acute infection. It has shown to be 96% to 100% specific and 80% to 99% sensitive. This assay has an advantage that it is an immunoassay and works like molecular assays in that it does not require processing of samples and active infection is confirmed through positive result. It has one disadvantage of being lower sensitive than NAT; approximately 1000 IU/mL of HCV RNA is its lower limit of detection. This assay is not available in United States yet.

Up-coming technologies for HCV detection

A research study showed that biomarkers can be detected by the recently developed prototype nanoparticle-based diagnostic assays in diseases like HCV. Gold nanoparticles and quantum dots (QDs) are the most commonly used nanoparticles[25]. QDs are composed of semiconductor substances that upon excitation give off light at different spectra[26]. Using biochip-based assay 1 ng/mL was obtained. An oligonucleotide probe containing RNA conjugated with quantum dots targeting NS5B protein of hepatitis C virus[27]. Moreover, gold nanoparticles varying from 2 to 50 nmol/L in size have been used to detect HCV RNA and anti-HCV[28]. Antibodies to HIV, HBV, HCV, have been detected through a multiplex platform that uses a micro-fluidic chip and quantum dots coated with antigen are embedded in beads made of polystyrene having a sensitivity of pM concentration[29,30]. Another important method working on the principle of amplification, named loop-mediated isothermal amplification (LAMP) has been used for detection of HCV RNA[31,32]. Several other techniques have been estimated for the diagnosis of HCV. Currently used technology is aptamers which are used as capture molecules. These are small, single-stranded oligonucleotides that can be folded into three-dimensional structures and able to identify target molecules including proteins, cells, and chemicals[33]. They have the potential to bind with their targets with high specificity and attraction[34].

Ortho trak-C assay

According to a research conducted by Fabrizi *et al*[35], an immunoassay that quantitatively measures the total HCV core Ag irrespective of the presence or absence of anti-HCV antibodies in human serum or plasma was used. It is a manual method that uses several monoclonal antibodies specific to various regions of the core antigen of HCV. It employed a microwell plate and onto which monoclonal antibodies were coated that bound with antigen. Horseradish peroxidase was conjugated with Fab segments of monoclonal antibodies and bound with captured antigen. The method comprised four steps: In the first step, incubation of the control material or patient sample (100 μL) in an uncoated microwell was done with a pretreatment reagent (50 μL) at 56 ℃ for 30 min. So as to facilitate the liberation of the core antigen of HCV to be detected, this pretreatment step was undergone

for the dissociation of any immune complexes. In the second step, the dilution and incubation of pretreated sample (100 μL) was done at 25 ℃ for 60 min in a monoclonal antibodies coated microwell that captured the immunoreactive core antigen. In order to remove any unbound material the wells were washed. In the third step, the conjugate (200 μL), containing Fab fragments of monoclonal antibody conjugated to horseradish peroxidase, was added into the microwell. The mixture was incubated at 25 ℃ for 30 min. The conjugate got bound with the core antigen that was bound to the capture antibody coating the surface of microwell. The core antigen connected the capture and conjugate antibody reagents. Again washing was done at the end of the step to remove any unbound antigen. The fourth step was based on an enzyme detection system consisted of hydrogen peroxide and *o*-phenylenediamine (OPD) that was added to the microwell. The *o*-phenylenediamine was oxidized, in the presence of bound conjugate that produced a colored end product. In this reaction, hydrogen peroxide divalently oxidized the peroxidase producing an intermediate compound that was reduced to initial state after interacting with hydrogen-ion-donating OPD. As a result, orange coloration of oxidized form of OPD was produced. In order to stop the reaction, sulfuric acid was added. The intensity of the color is proportional to the amount of bound conjugate and thus it can be said that it is a function of the amount of core antigen of HCV in the sample.

Through a microwell reader (photometer) designed to measure absorbance of light in a microwell (at 490 nm, using a 620 nm reference), the intensity of the color is measured. The samples and controls were tested in duplicate, and the mean optical density (OD) of the duplicate tests was used. Variation more than 25% between the optical densities of two samples pointed towards invalidity of those samples and they were allowed to be re-tested. If optical density more than recommended cut off corresponding sample of was obtained only then the sample was considered positive. The concentration of core antigen of HCV was measured through a standard curve[35].

PREVENTION OF HCV

Approximately 3% of the population of world is affected by HCV and according to estimation last stage of liver disease develops in 30% of patients[36]. Several research studies are being conducted for developing new therapeutic and prophylactic vaccines for hepatitis C virus. However, these efforts to produce effective vaccines have been damaged due to some of characteristics in common with many RNA viruses and the reasons are: First is RNA viruses display high antigenic and genetic diversity thus the mutation rate is very high inside the host. Second is the induction of strong immune responses that cannot eradicate the virus or avoid re-infection. Third is that there is no cell culture system developed so far or small animal model to develop vaccines. Thus so far, the vaccines developed have been tested on chimpanzees but they are unable to avoid HCV infection in them[37].

As there is no vaccine available for HCV so its prevention is even more difficult than HBV. It needs to have an integrated strategy including safe injection practices, and screening of blood donations[38]. The incidence of HCV infection is at its elevated level among the people who inject drugs (PWID)[39]. Nelson *et al*[41] estimated that in 2010, almost 10 million PWID were positive with HCV antibody and the global prevalence of HCV was 67% in that population[40]. Martin et al has used mathematical modeling to obtain the consequence of collaborative high-coverage needle and syringe programs (NSPs), opiate substitution treatment (OST) among PWID and treatment of HCV on its incidence and prevalence. For the prevention of HCV Page et al has emphasized upon the challenges of behavioral interference[39]. Many of the researchers have discerned about less effectiveness of harm-reduction programs in the prevention of human immunodeficiency virus (HIV) and HCV infection among the people who inject drugs. Many other strategies are still required. Results from their work have shown that from the last 10 years the prevalence of chronic HCV can be reduced up to more than 45% further needs antiviral treatment combined with the NSPs and OSTs that can reduce the rate of treatment to a large extent to get reduction in prevalence of HCV. In order to prevent HCV infection, its vaccine should become available. Two researchers named Cox and Thomas have stressed upon the development of HCV especially for people who inject drugs. Advanced research would be required for vaccine production[42].

Following are the modes of transmission of hepatitis C which cause people at risk of acquiring this infection:

Transmission of HCV infection through sexual contact

People at threat: Sexual transmission of HCV does not appear to be so common, as the studies has proved its spread in < 1% of couples yearly, among monogamous heterosexual partners. Meanwhile, researchers have currently discovered several cases of male sex partners (MSM) infected acutely with HCV and found it to be among men. Majorly these male sex partners had also HIV co-infection. However, the risk factor that is considered to be the strongest in the acquisition of HCV is injecting drug use. Many of the cohort and case studies have identified the prevalence and incidence of HCV among HIV-negative MSM, non-injecting drug users to be low apparently.

Factors related to elevated threat of sexually transmitted HCV: Acquisition of HCV has an enhanced risk associated with it in case of heterosexuals that have multiple sexual partners. Several risk factors have

Table 1 Approaches to prevent intra-venous drug users infection among hepatitis C virus

Reduce the risk of viral transmission among IDUs
Doctors should be educated properly
Lessen the use of injecting drug use
Use of sterilized syringes and other instrument even for diagnosis
Medical staff should be informed to inject safely
Hepatic disorder should be decreased in infected IDUs
Proper counseling should be undergone
Antiviral therapy for HCV
Combination of all kinds of services should be provided to infected patients

IDUs: Intra-venous drug users; HCV: Hepatitis C virus.

been identified by investigators that are related to HCV transmitted sexually among male sex partners: (1) HIV co-infection; (2) during sex, use of leisure drugs, *e.g.,* methamphetamine and gamma-hydroxybutyric acid (GHB); (3) unprotected intercourse through anus; (4) the sexual attempts that cause damage or bleeding from genital mucosa, group sex and fisting; and (5) ulcerative diseases that are transmitted sexually (syphilis, lymphogranuloma venereum proctitis) co-incidently[43-46].

Transmission of HCV via injection drug use

Infection of HCV among IDUs: In several parts of the world, injection drug use has become the most widespread threat factor for current cases especially in the United States. Acquisition of HCV is quite rapid when IDU is started meanwhile within short time interval a person becomes easy prone towards HCV infection. The occurrence of antibody of hepatitis C virus HCV antibody in IDUs enhances with many factors including age, intensity of injection, frequency of use and time from when injecting was initiated. As per a potential survey done form 2000-2007, HCV infection has an incidence of 27 out of 100 persons annually[47]. Re-infection has been observed in 26% patients who had previously eliminated the initial infection. Many co-researchers have demonstrated re-infection and super-infection[48]. Table 1 shows different approaches to prevent HCV infection among IDUs.

Factors Related to Transmission of HCV *via* Injection Drug Use: Injection Drug Use is considered to be a risk factor for the transmission of HCV. It spreads through sharing of syringes and needles. Moreover, many researchers have described that the equipment (*e.g.,* drug cookers, rinse water and filtration cottons) shared with the infected person is used in the making and injecting drugs. There is a dire need for counseling so to evade the use of contaminated syringes, needles and already prepared drug along with equipment. As an interesting fact, the persistency of HCV in syringes that are contaminated and having a large residual volume is very high[49]. According to a research study the survival of HCV is 16 h at room temperature outside the body but in the environment it cannot survive after 4 d[50]. Table 2 indicates different ways to prevent HCV infection among IDUs.

Table 2 Ways to prevent hepatitis C virus infection among intra-venous drug users

Already used needles and syringes should not be used
New and sterilized needle should be used for injecting drug
Illegal drugs should not be used
Disinfected equipment should be used to make drugs
Always wash hands before and after injecting
Carefully throw the syringes after using it
Safely cleanse the place of injecting drugs

Transmission of HCV from mother-to-child

Risk of mother-to-child transmission: Transmission of infection from mother-to-child is more common in cases of hepatitis B or HIV than that in hepatitis C, but it occurs in its case also. This type of transference is apparent mostly in HCV viremic women. These women have detectable amount of RNA in the peripheral blood. According to research, the transmission of HCV through mother-to-infant was found to be 4.3% from year 1992 to 2000 and a study conducted by Pembrey *et al*[51] depicted the rates of transmission from 3%-10%.

Factors associated with HCV transmission from mother-to-child: The most common risk factor that becomes the cause of HCV transference from mother-to-child is co-infection of HIV in mother with HCV viremia detectable during pregnancy[52-54]. Several other factors have also been observed and they are; injection drug use by mother, if infant is a female, intrapartum events leading to exposure of infant maternal blood infected with HCV, rupture of membrane for a long time while managing labor and obstetric procedures like vaginal/perineal lacerations. Contrary to it this transmission has not been found to be related to breast feeding or type of delivery whether Cesarean or vaginal[55]. If breast feeding is being done safely (if there is no damage or bleeding to the nipples) then it can reduce the risk of HCV transmission or breast feeding can be halted if there is any crackled nipple) until it gets healed.

Transmission of HCV through household

Risk of transmission: Some studies related to epidemiology have proved that HCV infection can also be caused by having family contacts of seropositive persons. Exposure to shared parenteral things is a critical factor that includes vertical transmission from mother to infant; dental or medical processes or injections; or if spouses and partners have any sexual exposure. HCV infection was found to increase among siblings and family members of patients that were infected with chronic liver disease. In case if children were infected with HCV there was no elevated threat

of this infection was found in parents or siblings. In several research studies the increased risk among partners and family members had a correspondence with the intensity of liver disease in the infected patient, any sexual contact with him, the period of exposure to him and the number of contacts infected with hepatitis c virus[56].

Transmission in healthcare units

Hepatitis C virus has been a serious problem in centers where hemodialysis is done[57,58]. Many steps have been taken by these centers in order to prevent hepatitis C virus infection, which are: HCV infected patients are grouped or isolated in separate rooms of dialysis center, adherence to infection-control rules has been increased for example the screening for HCV at regular intervals, not to reuse the shared vials or syringes and regularly vaccinating HCV infected patients with hepatitis A and B virus[57]. Resultantly, HCV infection has been reduced in dialysis centers. But the problem is that every healthcare setting has not taken these steps. Another factor which is challenging for most developing countries is that there is no viral hepatitis control program at the Ministry of Health level. Due to the absence, indirect and distorted trials for prevention are undergone for all types of hepatitis not just for HCV. Therefore, to counter transmission of HCV especially in healthcare units, infection control programs are required.

Transmission through tattooing and piercing

In United States, there is no outburst of HCV infection has been notified by CDC as per licensed tattooing or piercing commercially. Urbanus *et al*[59] observed it for several years and pointed out that these practices seemed to be safe[59,60]. However, like everywhere else, exceptions are also here. Miller *et al*[61] and Sameul *et al*[62] separately found that in United States and Australia, the prisoners who got handmade tattooing were infected with HCV. Tohme *et al*[60], thus, declared that noncommercial tattoos if received in places like homes, unsterile environment or friends might become the cause of transmitting this infection.

MANAGEMENT OF HCV INFECTION

Aim of treatment of hepatitis C virus

It is the aim of this therapy to wipe out HCV infection so to avoid various liver and extra-hepatic disorders *e.g.,* cirrhosis, fibrosis, hepato-cellular carcinoma, inflammation due to necrosis and mortality.

Endpoint of HCV treatment

Sustained virological response (SVR) is the endpoint of HCV treatment; it is characterized by 24th week non-detection of HCV RNA. It was assessed by a molecular method that had detection limit of < 15 IU/mL (SVR24). Approximately 99% of cases have been cured as explained by long-term follow up SVR studies[63].

Pre-therapeutic estimation

In order to establish a connection between liver disease and HCV infection, baseline virological characteristics should be determined so to assess the severity of liver disease. This assessment is suggested before commencing the treatment. It is important that cirrhosis must be identified in patients as the treatment and post-treatment response is related to the fibrosis phase. As proposing the type and duration of therapy is based on the absence of major fibrosis. If cirrhosis has been clinically evidenced then there is no need to do biopsy for assessment of fibrosis. If patients suffer from cirrhosis then they need to be screened for hepato-cellular carcinoma. The two tests can be applied to identify cirrhosis and they are; Liver stiffness measurement (LSM) and biomarkers. Both LSM and biomarkers can be used to measure the fibrosis of liver in patients suffering with chronic infection of HCV. This combination therapy can decrease the requirement to do biopsy of liver[64,65].

Quantification of HCV

Quantification of HCV is recommended to the patients who are willing to receive anti-viral therapy. The quantification is made with the help of a sensitive technique, and the concentration should be given in IU/mL. Genotyping should also be done before starting the treatment. The rate of response to treatment depends on specific genotypes and then subtypes[66]. Every genotype and subtype shows different response to therapy. Then genetics of host should be determined as IL28B genotyping helps in identifying infected persons who will achieve rapid virological response (RVR) and possess a considerable probability to get cured by dual therapy[67,68]. For any treatment to be applied, patient's preferences must be considered primarily. In case when patients infected with HCV genotype 1 become unable to remove virus when treated with dual therapy then should be treated with triple therapy. For other genotypes, Interferon-alpha based therapy should be used along with dual therapy.

Contra-indication to interferon-alpha and ribavirin therapy

Hezode *et al*[69] found that pegylated interferon-α/Ribavirin containing schedule of treating chronic HCV infection showed contra-indication in the following infected groups of patients; people suffering from epilepsy or psychosis, depression, couples who do not comply with the rules of contraception or pregnant women, concomitant severe diseases; disastrous liver disease.

Triple therapy based on telaprevir or boceprevir

Usually, both above mentioned dual therapy containing pegylated interferon-α/Ribavirin and triple

therapy based on TVR or BOC offer common contra-indications. In case of compensated cirrhosis, intense care is required for its treatment due to emergence of dangerous side effects after getting treated. As incidence of severe infections and hematological disorders is enhanced largely in case of triple versus dual therapy, particularly if serum albumin level is less than 3.5 g/dL or concentration of platelets becomes less than 100000 even sooner than commencing treatment[69].

Examining treatment

To monitor the treatment requires checking the efficiency, safety and side effects of treatment.

Efficiency of treatment

The efficiency of treatment can be monitored by repeating quantification of levels of HCV RNA. Monitoring of treatment efficacy is based on repeated measurements of HCV RNA levels. A precise and sensitive test should be used in order to get dynamic range in quantification. To ascertain the reliability of results, RNA can be measured in each patient using the same assay at various time points[70-72]. But to examine the efficiency of treatment and directions to follow for the duration of treatment, measurements of RNA levels should be taken at particular time points. If in case the result of quantification has some effect on the treatment only then measurements should be made. The treatment should be curtailed as response-guided therapy if it is to be discarded as it has uselessness and if it becomes successful then it is end of treatment and post- treatment SVR test. In order to measure SVR, Baseline assessment of HCV RNA levels should be done in dual therapy (pegylated interferon-α/Ribavirin), *i.e.,* 4th, 12th, 24th week, at the end of treatment or 12/24 wk after the completion of therapy. While in case of triple therapy with boceprevir, RNA should be quantified with the same schedule as that of dual therapy. It is referred in many Guidelines that the quantification of RNA with BOC therapy should be done after several weeks of dual therapy. TVR therapy acts on the same guidelines and schedule of treatment as BOC therapy uses.

On-treatment virological response

Depending on on-treatment virological response, kind of baseline, a low versus high, for HCV RNA level might be used for the guidance of decision-making for treatment during dual therapy is going on. Still there is no best discerning value of HCV RNA level has been agreed upon, while the range lies from 400000-800000 IU/mL[73-80]. If decrement in the value of HCV RNA is < log10^2 IU/mL with dual therapy, then the treatment should be stopped at 12th week and the rate of SVR is < 2% if treatment remains continued. At 24th week if the level of HCV RNA is still detectable then there is only 1%-3% chance of SVR so the therapy should be discontinued[73,74,79,81]. When detection tests were less sensitive than modern assays, the futility rule was defined at that time. By using modern assays, treatment should not be discontinued for people showing undetectable RNA. In BOC triple therapy, SPRINT-2 study has been analyzed order to derive futility rules. If level of RNA is greater than 100 IU/mL at 12th week of treatment then all medications should be stopped even also if that is detectable at 24th week and also when there is a viral breakthrough (reappearance of HCV RNA during the course of treatment post to virological response). For TVR triple therapy, ADVANCE database has been used to derive stopping rules. It its case the drugs should be discontinued at 4th week of treatment if RNA level is greater than 1000 IU/mL and also when there is breakthrough.

Dual therapy guided by virological response

The infected persons showing an undetectable HCV RNA and decrease of > log 10^2 in the 12th week can be grouped into three as per virological response: First is the RVR that defines an undetectable HCV RNA in the 4th week of therapy. Second is early virological response (EVR) or cEVR (complete EVR) that describes undetectable HCV RNA in the 12th week of therapy. Third that defines decrease of > log10^2 with HCV RNA detectable at week 12 and undetectable in the 24th week is thus referred to as delayed virological response (DVR) or pEVR (partial virological response).

Evaluation of safety post-treatment

It is necessary to monitor the patients after treatment so to avoid side effects and if they have encountered with them then they should be controlled. For instance, after peg-interferon-α injections sometimes flu-like symptoms start appearing. So paracetamol should be prescribed to control the symptoms. Other clinical side effects like irritability, allergic reactions, and severe fatigue should be checked with routine visit. Dermatological and hematological adverse reactions should be assessed both during and after treatment. Levels of thyroid stimulating hormone (TSH) should be quantified during treatment after every 12 wk[82]. Even the dosage of peg-IFN-α should be decreased when concentration of platelets and neutrophils drops lower than 50000/mm^3 and 750/mm^3 respectively. Even the medication can be stopped when there is a remarkable decrease in the amount of platelets and neutrophils, *i.e.,* less than 25000/mm^3 and 500/mm^3 respectively and severe depression. Treatment should be discontinued in case if any bacterial infection is detected during treatment[73,83,74].

Adjustment of cofactors

Cofactors such as body weight, lipids, alcohol and growth regulators should be adjusted. High value of body mass index (BMI) badly affects the response to

treatment (PegIFN/RBV)[84]. It should be suggested to reduce body weight before starting treatment. It can be co-related with the concentration of lipids as high body weight can be due to high level of lipids. The life cycle of hepatitis C virus is associated with the metabolism of lipids. Consumption of alcohol can have a severe impact on treatment actually it affects adherence to treatment[85]. Growth regulators limit the need for reducing drug dosage, *e.g.,* EPO (recombinant erythropoietin) improves the levels of hemoglobin by avoiding the decrease in the dose of ribavirin. Then a metabolic syndrome, type 2 diabetes and resistance to insulin, enhance the development of liver disease thus also can lead to hepato-cellular carcinoma. Furthermore, they may also be responsible for dampening the response to dual therapy[86].

Transplantation of liver

In case when liver is at the last stage of disease then only one treatment, liver transplantation (LT), is left. But there are still chances of recurrence of hepatitis C[87]. In order to achieve SVR, antiviral therapy should be initiated as quickly as possible in the patients who are suffering from acute liver disease but their treatment is probable before transplanting liver[88].

Treating the unusual groups

HIV-HCV co-infection: The rate of development of liver inflammation increases when HCV patients are co-infected with HIV. It happens especially in patients with weaken immune system and a low cell count of CD4-positive cells. These patients should be given antiretroviral treatment before starting anti-HCV treatment[89]. The use of these antiviral drugs, stavudine, zidovudine and didanosine should be evaded during PegIFN/ribavirin therapy[90]. Non-invasive techniques or biopsy should be used to measure severity of liver disorder.

Treatment of hemodialysis patients

As the risk of HCV infection development is enhanced in the patients undergoing hemodialysis. Actually this infection becomes the major cause of death in these patients. These patients require highly specialized antiviral therapy so ensure the survival of the person.

A new era of direct acting anti-HCV agents

Though the current standard therapy for chronic HCV infected patients is pegylated interferon-α in combination with ribavirin, however, a number of side effects of the current standard therapy usually make adherence to treatment difficult and reducing an SVR[91].

A new era of direct acting anti-viral (DAA) defined as agents/compounds that interfere with specific steps in the HCV life cycle through a direct interaction with the HCV encoded polyprotein and its cleavage products has emerged. To elevate SVR, DAAs are given in combination to the existing standard of care (pegylated interferon and ribavirin) for chronic hepatitis C infected patients with genotype 1 infection. Of these DAAs, 2 protease inhibitors, telaprevir and boceprevir, are in use for HCV treatment since 2011[92]. Other DAAs recently in use are TMC-435, vaniprevir, BI-201335, BMS-650032, Mericitabine (RG-7128), Tegobuvir (GS-9190), Daclatasvir (BMS-790052) and danoprevir. However, treatment outcome shows an overall efficacy rates between 70%-90% in no improvement in side effects.

Recently several other anti-viral agents are developed that directly target various stages of HCV replication and are proved very effective interferon-free therapy for HCV-infected patients[93]. Of these, the most important is oral nucleotide analogue "Sofosbuvir" (also known as GS-7977) that is an inhibitor of the HCV nonstructural protein 5B (NS5B) RNA-dependent RNA polymerase enzyme and is very effective against HCV. Sofosbuvir was recently approved for use in combination with ribavirin and/or pegylated interferon for chronic HCV infection, depending on the genotype. The recommended regimens and duration of therapy: (1) for genotype 1 or 4 chronic hepatitis C: Sofosbuvir, peginterferon alfa, and ribavirin for 12 wk; (2) for genotype 2: Sofosbuvir and ribavirin for 12 wk; (3) for genotype 3: Sofosbuvir and ribavirin for 24 wk; and (4) for hepatocellular carcinoma awaiting liver transplantation: Sofosbuvir and ribavirin for up to 48 wk or until liver transplantation (whichever occurs first). Sofosbuvir and ribavirin oral therapy is very effective against genotypes 2 and 3 HCV infections[94].

Renal transplant recipients infected with HCV

Recipients of kidney transplant when become infected with Hepatitis C might develop fibrosis of liver. If transplant is improperly carried out then the patients can become HCV positive and can be threatening for the life of the person[95]. The measurement of fibrosis should be ensured in these renal-transplant patients having HCV in serum.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Survival rates according to barcelona clinic liver cancer sub-staging system after transarterial embolization for intermediate hepatocellular carcinoma

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Abstract

AIM: To investigate the survival rates after transarterial embolization (TAE).

METHODS: One hundred third six hepatocellular carcinoma (HCC) patients [90 barcelona clinic liver cancer (BCLC) B] were submitted to TAE between August 2008 and December 2013 in a single center were retrospectively studied. TAE was performed *via* superselective catheterization followed by embolization with polyvinyl alcohol or microspheres. The date of the first embolization until death or the last follow-up date was used for the assessment of survival. The survival rates were calculated using the Kaplan-Meier method, and the groups were compared using the log-rank test.

RESULTS: The overall mean survival was 35.8 mo (95%CI: 25.1-52.0). The survival rates of the BCLC A patients (33.7%) were 98.9%, 79.0% and 58.0% at 12, 24 and 36 mo, respectively, and the mean survival was 38.1 mo (95%CI: 27.5-52.0). The survival rates of the BCLC B patients (66.2%) were 89.0%, 69.0% and 49.5% at 12, 24 and 36 mo, respectively, and the mean survival was 29.0 mo (95%CI: 17.2-34). The survival rates according to the BCLC B sub-staging showed significant differences between the groups, with mean survival rates in the B1, B2, B3 and B4 groups of 33.5 mo (95%CI: 32.8-34.3), 28.6 mo (95%CI: 27.5-29.8), 19.0 mo (95%CI: 17.2-20.9) and 13 mo, respectively (P = 0.013).

CONCLUSION: The BCLC sub-staging system could add additional prognosis information for post-embolization survival rates in HCC patients.

Key words: Hepatocellular carcinoma; Barcelona clinic liver cancer; Transarterial embolization; Subclassification

Core tip: This is the first study to apply the barcelona clinic liver cancer (BCLC) B subclassification in a survival analysis for hepatocellular carcinoma patients after transarterial embolization. Were observed significant differences in the mean survival rates among B1, B2, B3 and B4 patients. The BCLC B sub-staging system could be an additional tool for accessing prognosis in the post-embolization survival rates of hepatocellular carcinoma patients.

Scaffaro LA, Stella SF, Alvares-Da-Silva MR, Kruel CDP. Survival rates according to BCLC sub-staging system after transarterial embolization for intermediate hepatocellular carcinoma. *World J Hepatol* 2015; 7(3): 628-632 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i3/628.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i3.628

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related death[1,2]. Locoregional treatments, such as transarterial chemoembolization (TACE) and transarterial embolization (TAE), have been used for intermediate HCC patients, promoting an increase in overall survival[3-7]. An updated meta-analysis and a systematic review of randomized controlled trials comparing TACE with TAE did not proved significant difference in survival between this two techniques[6,7].

The intermediate stage of HCC affects a highly heterogeneous patient population and can present with varying tumor burdens and liver functionality that are usually staged in the same level as barcelona clinic liver cancer (BCLC) B[1,2,8].

Recently, some authors have proposed a sub-staging of BCLC B patients to facilitate therapeutic decisions, especially due to wide differences in response rates after transarterial treatments among intermediate HCC patients[8]. We analyzed the survival rates based on this sub-staging after TAE.

MATERIALS AND METHODS

We retrospectively analyzed a historical cohort of American Association for the Study of Liver Diseases diagnosis-based HCC patients[9] treated from June 2008 to December 2013 at the Gastroenterology Division of the Hepatology Unit of the Hospital de Clínicas de Porto Alegre.

TAE was indicated for patients with HCC BCLC A with nodules greater than 3 cm or without safe percutaneous access to ablative therapies and BCLC B with no signs of extra-hepatic disease[9].

BCLC D or BCLC C patients with evidence of extra-hepatic disease, portal vein thrombosis (or thrombosis of one of its branches), or hepatofugal portal flow were excluded. Patients with a definitive diagnosis of extra-hepatic metastasis, difficult to control ascites, other active malignant diseases or the following laboratorial anomalies were also excluded: serum creatinine above 1.5 mg/dL, total bilirubin above 3.0 mg/dL, platelets lower than 50000 mm^3 or a prothrombin time less than 50%.

Table 1 Barcelona clinic liver cancer B sub-stage categories

	BCLC sub staging			
	B1	B2	B3	B4
Child Pugh class	5-6-7	5-6	7	8-9
Beyond Milan and within up-to-7	In	Out	Out	Any
ECOG PS	0	0	0	0-1

BCLC: Barcelona clinic liver cancer; ECOG PS: The Eastern Cooperative Oncology Group performance status.

The BCLC B patients were sub-staged into four categories (Table 1). Group 1 comprised patients with Child-Pugh class A or B with a score of no more than 7, without current or previous decompensation, an Eastern Cooperative Oncology Group (ECOG) PS score of 0 and meeting the up-to-seven criteria. Group 2 comprised Child-Pugh A patients exceeding the up-to-seven criteria, without ascites or jaundice and an ECOG PS score of 0. Group 3 comprised patients with Child-Pugh class B exceeding the up-to-seven criteria and who had an ECOG PS score of 0. Group 4 comprised decompensated Child-Pugh B patients with severe ascites or jaundice, an ECOG PS score of 0 or 1 and who either did or did not exceed the up-to-seven criteria[8].

Procedure

TAE was performed by the same interventional radiologist through a common femoral access. Selective catheterization of the celiac trunk and the superior mesenteric artery were performed with a Cobra or Mikaelson 5F catheter to facilitate the liver blood flow study. The hepatic artery was selectively catheterized, followed by a superselective feeding branch tumor catheterization with a 2.8 F microcatheter (Progreat®, Terumo). In the PVA-TAE, a superselective injection of PVA (Cook, Bloomington, Indiana) was performed in the feeding artery as distal as possible. The ME-TAE injection was performed with ME embospheres (Biosphere medicals™, Rockland, MA, United States). The particle sizes were 100-300 microns for tumors up to 5 cm and 300-500 microns for tumors equal to or larger than 5 cm.

Statistical analysis

The categorical variables were described using frequency and percentages. The quantitative variables with symmetric distribution were expressed using their mean values and standard deviation; those with asymmetric distribution were described using the

Table 2 Baseline and tumoral characteristics

Characteristics	B1	B2	B3	B4	*P* value[1]
Age (yr)	62	61	60	60	0.77
Gender (male,%)	66	73	76	50	0.48
Caucasians (%)	95	99	96	100	0.28
HCV positive (%)	81	77	76	100	0.79
Alcohol (%)	33	33	34	0	1.00
PS (0) (%)	100	95	95	50	0.27
AST (U/L)	35	36	37	38	0.90
ALT (U/L)	64	67	69	70	0.80
GGT (U/L)	133	136	144	155	0.79
Platelets (× 1000/UL)	110	110	105	90	0.35
PT (%)	73	73	77	79	0.72
Albumin (U/L)	3.5	3.4	3.3	3.3	0.28
BT (mg/dL)	1.2	1.3	1.3	1.4	0.22
Creatinine	0.93	0.94	0.95	0.97	0.73
No. of TAE session	1.7	1.9	1.9	1.9	0.20

[1]*P* value analysis excluded B4 subgroup (only 2 patients). HCV: Hepatitis C virus; PS: Performance status; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transferase; PT: Prothrombine time; BT: Total bilirubin; TAE: Transarterial embolization.

median and inter-quartile interval (25th percentile - 75th percentile). The χ^2 test or Fisher's Exact Test was used to compare the categorical variables. Quantitative variables with symmetric distribution between groups were compared using Student's *t* test for the independent samples. Variables with asymmetric distribution were compared between the groups using the Mann-Whitney *U* test.

The date of the first embolization until death or the last follow-up date was used in the assessment of survival. The survival rates were calculated using the Kaplan-Meier method, and the groups were compared using the Log-Rank test.

Analyses were performed using SPSS software version 19.0 by a biomedical statician. The statistical level of significance was set at 0.05.

RESULTS

One hundred third six patients, 46 BCLC A (33.7%) and 90 BCLC B (66.25%), were treated with TAE. Among the BCLC B group, 48 (52.8%) patients were BCLC B1, 27 (30.2%) were BCLC B2, 13 (15.1%) were BCLC B3 and 2 (1.9%) was BCLC B4.

There were no significant differences at baseline in regard to demographic data, staging, laboratory or HCC characteristics according to the embolic agent. Table 2 summarizes the characteristics.

Throughout the analysis, the follow up time was 1 to 52 mo, with overall survival rates at 12, 24 and 36 mo of 98.5, 78.0 and 55.5%, respectively. The mean overall survival rate was 35.8 mo (95%CI: 25.1-52.0).

The survival rates of the BCLC A patients (33.7%) were 98.9%, 79.0% and 58.0% at 12, 24 and 36 mo, respectively, and the mean was 38.1 mo (95%CI: 25.0-52.0).

The survival rates of the BCLC B patients (66.2%) were 89%, 69% and 49.5% at 12, 24 and 36 mo, respectively, and the mean was 29.0 mo (95%CI: 17.2-34.3).

Table 3 Survival rates according to barcelona clinic liver cancer classification and subclassification

	n (%)	Mean survival rate (mo) (95%CI)
Overall	136 (100)	35.8 (25.0-52.0)
BCLC A	46 (33.7)	38.1 (25.0-52.0)
BCLC B	90 (66.2)	29.0 (17.2-34.3)
BCLC B1	48 (52.8)	33.6 (32.9-34.3)
BCLC B2	27 (30.2)	28.6 (27.5-29.8)
BCLC B3	13 (15.1)	19.0 (17.2-20.9)
BCLC B4	2 (1.9)	13.0 (25.0-52.0)

BCLC: Barcelona clinic liver cancer.

Table 4 Barcelona clinic liver cancer B substaging response rates according to mRECIST *n* (%)

	CR	PR	PD	SD
B1	12 (25)	29 (60.4)	3 (6.25)	3 (6.25)
B2	6 (22.2)	13 (48)	1 (3.7)	7 (26.1)
B3	1 (7.6)	4 (30.7)	1 (7.6)	7 (53.8)
B4	-	-	1 (50)	1 (50)

CR: Complete response; PR: Partial response; PD: Progressive disease; SD: Stable disease.

The survival rates according to BCLC B subclassification showed a significant difference between the groups, with mean survival rates for B1, B2, B3 and B4 of 33.6 mo (95%CI: 32.9-34.3), 28.6 mo (95%CI: 27.5-29.8), 19.0 mo (95%CI: 17.2-20.9) and 13 mo, respectively (P = 0.013). The median survival rates for B1, B2, B3 and B4 was 33, 28, 19 and 13 mo, respectively. Table 3 summarizes the survival rates, and Figure 1 shows the survival Kaplan-Meier curves according to BCLC B subclassification.

There was no differences in the response rates according to mRECIST among the BCLC B substaging, as demonstrated in Table 4.

DISCUSSION

The difficulty in finding an ideal treatment modality for BCLC B patients was been evaluated. Some authors showed a significant difference in survival between Child-Pugh B7 and B8 with survival means after TACE of 22 and 6 mo, respectively[10]. Survival differences between BCLC A and B patients after TACE were also demonstrated after drug-eluting beads chemoembolization (DEB-TACE), with survival rates after 12, 24 and 36 mo of 93.6%, 83.8% and 62%, respectively, in BCLC A, and 91.5%, 75% and 50.7% in BCLC B[11].

Therefore, Bolondi *et al*[8] proposed a refinement of intermediate HCC staging based on the Child-Pugh score, up-to-seven criteria, performance status and portal vein patency.

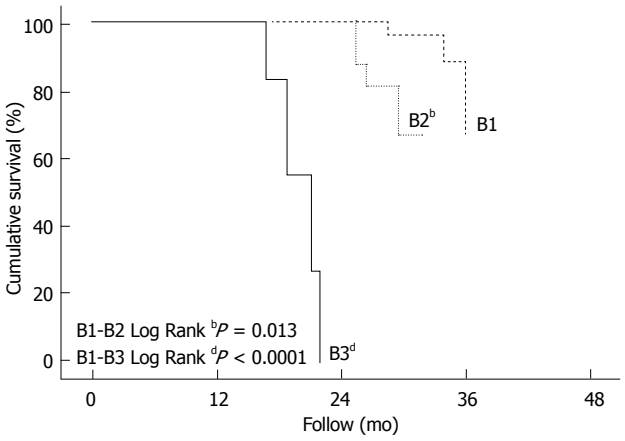

Figure 1 Kaplan-Meier curves.

Our study also applied the BCLC B sub-staging, and significant survival differences were observed in subgroups, with mean survival rates for B1, B2, B3 and B4 of 33.6 mo (95%CI: 32.9-34.3), 28.6 mo (95%CI: 27.5-29.8), 19.0 mo (95%CI: 17.220.9) and 13 mo respectively (P = 0.013).

Some authors also have been validated that proposal, depicting significant differences in median survival time between B1 and B2 and B2 and B3[12]. Another recently study also showed differences in 5-year survival rates using sub-stages, and had proposed a modification of this system based on alpha-fetoprotein (AFP) levels[13]. According to AFP levels (200 ng/mL), B1 was classified into B1a and B1b and B2 into B2a and B2b, without differences in survival among this subgroups, but a re-classification into modified mB1 (B1a), mB2 (B1b + B2a) and mB3 (B2b + B3) provided better prognostic prediction[13]. Since AFP levels were available in a small number of our sample, we did not performed this modified evaluation.

Even not considering the AFP levels, this differences in survival rates among the B sub-staging patients stressed distinct clinical conditions and tumor characteristics, even though it could represent a measure of interaction between underlying liver disease and tumor burden.

The present study has some limitations. It is single-centered, retrospective, and the choice of the embolizing agent was not standardized. Nevertheless, the survival rates we found suggest that this issue warrants further investigation.

In conclusion, the BCLC sub-staging system could be an additional tool for accessing prognosis in the post-embolization survival rates, having potential to better select the therapeutic approach for intermediate HCC patients.

COMMENTS

Background

The intermediate stage of hepatocellular carcinoma is composed of a group of heterogeneous characteristics related to tumor burden and clinical aspects. Therefore, the standard embolization treatment proposed for intermediate stage could have different responses in this group.

Research frontiers

Given the potential differences in patients in stage B, is not yet known the survival rates of patients divided into four subcategories of barcelona clinic liver cancer (BCLC) stage B, that underwent transarterial embolization (TAE).

Innovations and breakthroughs

The TAE is a minimal invasive technique that has been applied to patients in intermediate stage hepatocellular carcinoma (HCC). In the literature however there is debate about its role in patients in stage B of BCLC, given heterogeneity at this stage. This study aims to better characterize these patients by determining survival in the different sub stages.

Applications

This approach aim to predict prognosis in terms of survival in an attempt to optimize the therapeutic approach in this group of patients.

Terminology

BCLC is a classification used to guide therapeutic for patients with hepatocellular carcinoma. One of the available treatments, especially for patients with intermediate stage HCC, TAE is a non invasive trans catheter technique in which are injected embolizing agents aimed at restricting arterial tumor supply.

Peer-review

This is an interesting manuscript where the authors have analysed the difference in survival between different subgroups in BCLC Stage B HCC after TAE.

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CASE REPORT

Unusual presentation of severely disseminated and rapidly progressive hydatic cyst: Malignant hydatidosis

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Author contributions: Hammami A and Hellara O collected the data; Mnari W wrote the manuscript; Safer L and Saffar H critically revised the manuscript and contributed to the manuscript writing; Loussaief C, Bedioui F, Golli M and Chakroun M supervised the manuscript; all authors approved the final version of the manuscript.
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Informed consent: The authors declare that no patient data appears in this article.
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Abstract

The infection caused by the tapeworm *Echinococcus granulosus* leads to the development of hydatic disease. It is the most frequent mediterranean parasitic infection that commonly affects the liver and rarely involves multiple organs. Herein, we report an exceptional and confusing presentation of hepatopulmonary and splenic hydatidosis due to *Echinococcus granulosus* that caused diagnostic problems occuring in a 70-year-old man, treated with chemotherapy, with favorable outcome. This was a very unusual case of disseminated hydatid cyst highlighting the interest of keeping a high level of clinical suspicion of this diagnosis every time we have a cystic lesion of the liver.

Key words: Hydatid cyst; *Echinococcus granulosus*; Disseminated echinococcosis; Albendazole

Core tip: The infection caused by the tapeworm *Echinococcus granulosus* (*E. granulosus*) leads to the development of hydatic disease. It is the most frequent mediterranean parasitic infection that commonly affects the liver and rarely involves multiple organs. The diagnosis is usually based on ultrasonography and serological markers. This paper reports an exceptional and confusing presentation of hepatopulmonary and splenic hydatidosis due to *E. granulosus* that caused diagnostic problems treated with chemotherapy, with favorable outcome.

Hammami A, Hellara O, Mnari W, Loussaief C, Bedioui F, Safer L, Golli M, Chakroun M, Saffar H. Unusual presentation of severely disseminated and rapidly progressive hydatic cyst: Malignant hydatidosis. *World J Hepatol* 2015; 7(3): 633-637 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i3/633.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i3.633

INTRODUCTION

Hydatidosis due to *Echinococcus granulosus* (*E. granulosus*) is an endemic parasitic zoonosis characterized by worldwide distribution particularly in Mediterranean countries. It remains a major public health problem in developing countries. The disease commonly affects the liver (> 65%), and less frequently the lungs (> 25%)[1]. Disseminated hydatidosis is an infrequent condition that usually results from the rupture of a liver cyst, with subsequent seeding of protoscolices in the abdominal cavity[2]. Hydatid disease may be asymptomatic or present with complications. Sometimes unusual locations as well as cyst metastasis can produce a diagnostic dilemma and make differential diagnosis from other abdominal cystic lesions sometimes difficult. The particularity of this case is related to its unusual clinical presentation. The purpose of this paper is to emphasize the fact that hydatid disease can involve any organ of the body and can have an unusual presentation; therefore, a high index of suspicion and correct diagnosis is justified in order to prevent complications and chances of recurrence.

CASE REPORT

A 70-year-old man had had intermittent abdominal pain in the right hypochondrium, for 2 wk, with no radiation, associated to fever that appeared one day before the admission. He had a medical history of diabetes and hypertension and was followed for benign prostatic hyperplasia. He had, in the past year, an abdominal ultrasound which was absolutely normal except for the prostatic hypertrophy. He was engineer in agriculture and he had been in Australia, for 6 mo, 30 years ago. There was no history of vomiting, weight loss, change in bowel habit, or urinary symptoms. Clinical examination revealed apyrexia, absence of icterus, normal blood pressure and heart rate, mild tenderness in the right hypochondrium with hepatomegaly, and a scar of a recent injury in the ankle. Hematological tests showed a slight anemia and a mild leukocytosis (10800/mm^3). Renal function and serum electrolytes tests were within normal limits. Liver function tests initially revealed anicteric cholestasis [alkaline phosphatase (ALP): 1.2N, gamma-glutamyl transferase (GGT): 19N] without cytolysis. The hepatic function was normal (Factor V: 100%). The C-reactive protein (CRP) was elevated to 85 mg/L. The abdominal computed tomography scan showed multiple thin wall cystic mass with homogeneous fluid density contains in the liver (Figure 1A). There was non wall enhancement. Basal chest sections revealed smaller cystic lesion in the lung (Figure 1B). Abdominal MRI confirmed the simple cystic nature of the hepatic lesions (Figure 1C). The liver cysts was then suspected as infectious or tumor. His tumor markers were normal. A serum Western blot and Elisa tests for hydatidosis *(Echinococcus granulosus)* were negative twice. Laboratory studies indicated negative amoebic antibodies. A guided puncture of cysts content was performed under scenographic control. The fluid aspirated from the hepatic cyst was limpid. The smears demonstrated the absence of protoscolices. Pathologic reports were negative for malignant cells. Bacteriological examination showed a gram positive Cocci. Then, the diagnosis of hepatic abscess secondary to staphylococcus septicemia from cutaneous wound in diabetic patient was retained. Thus, our patient was treated with antibiotics during 3 wk. The course of his disease was marked by the occurrence of fever, icterus and extensive edema. Upon examination, we noticed the increase of the hepatomegaly. Biological tests revealed icteric cholestasis [conjugated bilirubin (CB): 19 N, GGT: 30N]. CT scan control showed no improvement of hepatic lesions despite an appropriate treatment; otherwise, we noted a dissemination of pulmonary lesions and occurrence of spleen cysts. The volume of the liver was enlarged, causing compression of the inferior vena cava without thrombosis, leading to the diffuse edema. Therefore, we suspected a malignancy with hepatic and pulmonary metastasis despite the absence of typical radiological signs. Upper and lower endoscopy showed no tumor. The bone scintigraphy was normal. At this stage, considering the absence of formal radiological and biological features of hydatid cyst and the lack of solid arguments for tumor, we decided to enlarge the spectrum of antibiotics associated to anticoagulant treatment and lymphatic drainage, with close monitoring. The patient was followed by routine biochemistry, CRP, procalcitonin measurement with abdomino-pelvic ultrasonography. Another hydatic serology was performed, after 6 wk, as we have no idea about the origin of these lesions. This time, the serology was positive for *E.granulosus* with total serum antibodies by hemagglutination assay at the level of 2560 (n < 10), and Immunoglobulin G (IgG) by ELISA test at the level of 58 kU/L (n <10). Serology tests for *Echinococcus multilocularis* were negative. Then, the diagnosis of CE was confirmed, and chemotherapy was decided. Our patient was treated with oral albendazole (400 mg twice daily) in association with praziquentel 50 mg/kg per day during 15 d. This treatment was well tolerated and led to clinical and biological improvement with especially the decrease of cholestasis (GGT: 9N, CB: normal). After 3 wk of anti-parasitic chemotherapy, transabdominal ultrasound showed a significant reduction in the number and the size of hepatic cysts with appearance of detached membrane and daughter vesicles within some cysts (Figure 2A). These findings are typical of hydatid disease. On the 2 mo CT scan control; there was a significant regression of the hepatomegaly and the cystic liver involvement (Figure 2, Panel B). Currently, the condition of our patient is very well. He will be maintained on Albendazole with regular follow-up. He was discharged from the hospital after 4 mo.

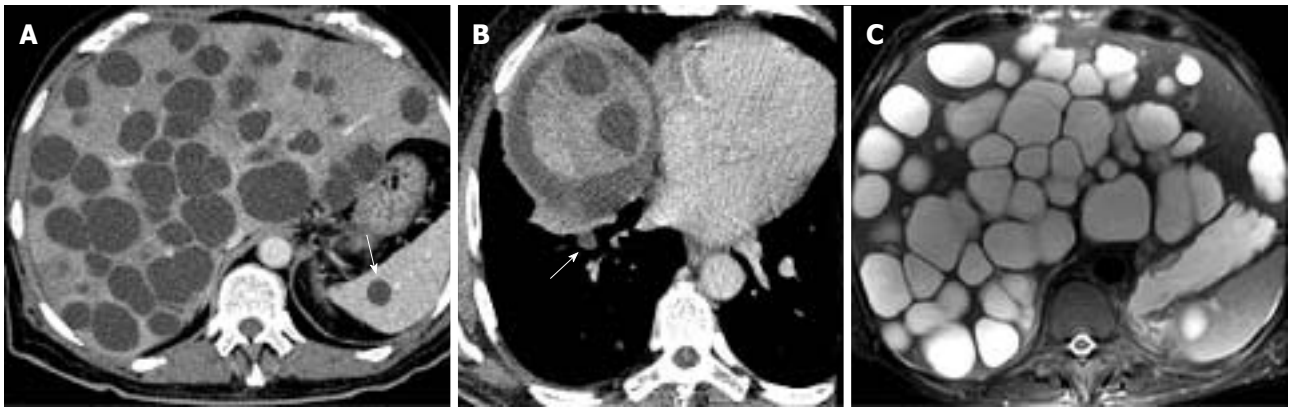

Figure 1 A: Abdominal computed tomography scan shows multiple cyst lesions in the liver and one cyst in the spleen (arrow); B: Axial scanning plane of the lung base shows a small cystic lesion in the right side (arrow); and C: magnetic resonance imaging of the liver with axial T2 weighted image shows homogeneous fluid signal intensity masses related to the cystic nature of hepatic lesions.

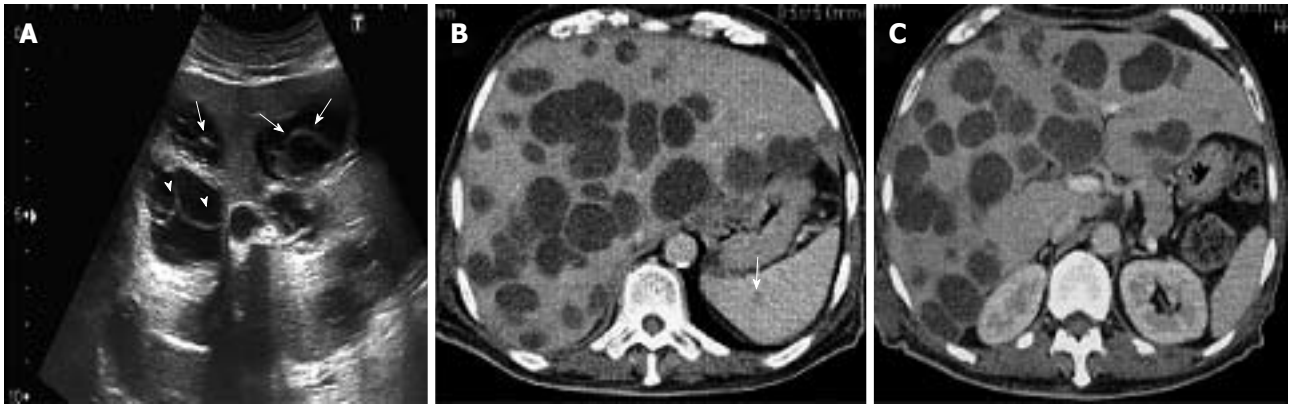

Figure 2 (A) Image from the liver ultrasound shows specific features of hydatid disease with detached membrane (arrows) and daughter vesicles (arrowheads); and (B and C) Images from the abdominal computed tomography scan demonstrate a significant regression of the number and the size of cysts in the liver. Note the almost complete regression of the spleen cyst (arrow, B).

DISCUSSION

Hydatid disease (HD) is a parasitic infection caused by *Echinococcus granulosus*[1] that represents a major public health problem in the Mediterranean region[3]. Humans are not included in the parasitic life cycle of *Echinococcus granulosus*. They can be intermediate hosts who become infected by accidental ingestion of food contaminated with eggs of *E. granulosus* shed by definitive hosts[4].

The liver is the major organ of echinococcal cysts (CE) involvement (60%-70% of cases) followed by the lungs (10%-25%), and less frequently, other organs such as brain[5], bones[6], and heart[7]. HD is usually asymptomatic and diagnosed incidentally, because cyst growth rate is commonly slow and progressive, ranging from 1 to 5 mm in diameter per year. Most primary infected patients have single cyst, but up to 20%-40% of them can develop multiple cysts[8]. Symptoms depend on organs affected, cyst size and number, the mass effect within adjacent organs and structures. The most frequent symptoms include asthenia and abdominal pain. Patients may also present jaundice, hepatomegaly or anaphylaxis due to cyst leakage or rupture. Simultaneous development of pulmonary and hepatic hydatid cysts is an unusual manifestation of hydatid disease that could be observed in less than 10% of cases[9]. It represents a specific entity called *hepatopulmonary hydatidosis* (HPH). The most common symptoms identified in patients with HPH are from the respiratory system, including cough, chest pain, dyspnea and hemoptysis[10]. Secondary splenic hydatid disease usually occurs after systemic dissemination or intraperitoneal spread complicating ruptured hepatic hydatid cyst.

Herein, we report an exceptional and confusing presentation of hepatopulmonary and splenic hydatidosis due to *E. granulosus* that caused diagnostic problems. The review of the literature revealed that it was the first case characterized by a fulminant dissemination of hydatid cysts through organs.

Appropriate clinical diagnosis of CE is necessary for early and adequate management of hepatic echinococcosis. It is generally approved that the combined use of ultrasonography and immunodiagnosis facilitate the distinction of echinococcal cysts from other hepatic cystic lesions[1]. The differential diagnosis of hepatic hydatid cyst includes abscess,

hemangioma, and non-parasitic cysts such as solitary bile duct cyst, polycystic disease, hepatobiliary cystadenoma. Unilocular hydatid cysts appear on ultrasound as anechoic unilocular fluid-filled space with imperceptible walls and posterior acoustic enhancement. Ultrasonography is not always able to differentiate hydatid cysts from other hepatic lesions, like tumors or liver abscesses, which reflect the lack of pathognomonic radiological signs. On ultrasound, a bile duct cyst appears as a well-circumscribed anechoic lesion with increased through-transmission of sound and no evidence of mural nodularity. There are no specific radiological features for hemangioma. They are typically well defined *hyperechoic* lesions. However, a small proportion are hypoechoic. They may or may not show peripheral feeding vessels. In autosomal polycystic liver disease, the numerous hepatic cysts of various sizes have identical features to those described for benign developmental hepatic cyst. On CT scan and MRI, the simple hydatid cyst is defined as a well-demarcated water attenuation lesion that does not enhance after the administration of intravenous contrast[11,12]. Ultrasound may detect detached endocyst membranes and daughter cysts (vesicles within the mother cyst), which are highly specific for hydatid disease. Detachment of the membranes inside the cyst is referred to as "the water lily" sign. Among serologic tests, immunoelectrophoresis has been reported to be the most specific for the primary diagnosis and postsurgical follow-up[13], and enzyme-linked immunosorbent assay (ELISA) represents a valuable diagnostic test for the initial screening[1]. In the present case, the first Serological tests were negative for specific IgG antibodies to *E. granulosus* by ELISA. The negative serology made the diagnosis of hydatid cysts more complicated. This test is limited by the high percentage of false-negative results and the large variability in its sensitivity and specificity between laboratories. Serologic tests do not supplant clinical or imaging investigations but they can, however, confirm the hydatid origin of a cyst. In doubtful cases, for example undetectable anti-*Echinococcus* antibodies or in patients whose hepatic cysts cannot be differentiated from liver abscess or neoplasms, ultrasonography-guided fine needle puncture may represent an additional diagnostic option. The demonstration of protoscolices or hydatid membranes or echinococcal antigens/DNA in the aspirated cyst fluid can confirm, in fact, the diagnosis of CE. In the current case, the content of the aspirated fluid showed no protoscolices with made the diagnosis more complicated. Anthelmintic coverage is important to reduce the risk of dissemination of CE: albendazole should be prescribed for 4 d before the procedure and continued for at least 1 mo after having punctured an *E. granulosus* cyst[14,15]. In the current case, the puncture of the cyst fluid, done without anthelmentic prophylaxis, did not show any protoscolices which made the diagnosis more difficult.

The main goal the treatment of hepatic hydatid disease should be the complete elimination of the parasite and prevention of recurrence of the disease with minimum morbidity and mortality risks. Three therapeutic modalities are validated in the treatment of hepatic CE: medical treatment, surgery (with open or laparoscopic approach) and percutaneous treatments (PTs)[16]. For many years, surgery was the first therapeutic option for hydatid disease[1]. Puncture, aspiration, injection, re-aspiration (PAIR) is a relatively recent and minimally invasive therapeutic option, introduced in the two past decades, and developed as an interesting alternative to surgery. It is a percutaneous treatment that consists of puncture of the cyst, aspiration of cyst fluid, injection of hypertonic saline and absolute alcohol that had a scolicidal effect, and re-aspiration of the cyst contents (PAIR) under sonographic guidance[17]. This treatment is considered in case of single or multiple cysts, which cannot be operated[1]. PAIR represents an effective and safe procedure[18] with low rates of complications[1] and is the unique method that provides direct diagnosis concerning the parasitic nature of the cysts in doubtful cases[18]. Chemotherapy based on benzimidazole carbamate compounds (albendazole and mebendazole) have, for a long time, remained the cornerstone of medical therapy for echinococcosis[1]. It has very effective results in patients with multiple or inoperable cysts and those with peritoneal hydatid cysts[19]. Albendazole, an oral benzimidazole antihelmintic agent, is a drug of choice for the medical therapy of echinococcal disease[20]. The dose of drug is 10 to 15 mg/kg per day in 2 divided doses given in cycles of 4 wk, without drug therapy for 2 wk. This regimen should be maintained for several cycles depending on the severity of disease or the improvement of patients[20,21]. Praziquantel, an isoquinoline anthelmintic, is a potent protoscolicidal agent *in vitro*[19,20]. The usage of albendazole with praziquantel together may be more effective than albendazole alone.

In conclusion, despite the progress made in imaging techniques and therapeutic modalities, the diagnosis of hydatid disease remains a problematic issue. The diagnosis of non complicated hepatic hydatidosis is based on clinical suspicion, combined to suggestive factors such as the patient's origin and occupation in order to identify high-risk patients. Many unresolved problems are still waiting for a solution; for instance, there is a need for prevention programs able to monitor and control parasite spreading. The present report describes clinical findings of very unusual and rapidly progressive case of malignant CE which caused a diagnosis dilemma.

COMMENTS

Case characteristics

Symptoms were common to other hepatic diseases, diagnsotic delay, rapidly

extensive hydatidosis.

Clinical diagnosis

Mild tenderness in the right hypochondrium with hepatomegaly.

Differential diagnosis

Abscess, hemangioma, solitary bile duct cyst, polycystic disease, and hepatobiliary cystadenoma are the main differential diagnosis. Ultrasonography and CT scan aspects help to distinguish them.

Laboratory diagnosis

Immunoelectrophoresis has been reported to be the most specific for the primary diagnosis. Enzyme-linked immunosorbent assay represents a valuable diagnostic test for the initial screening.

Imaging diagnosis

Ultrasound may detect detached endocyst membranes and daughter cysts (vesicles within the mother cyst), which are highly specific for hydatid disease.

Treatment

Three therapeutic modalities: chemotherapy (benzimidazole carbamate compounds), surgery and percutaneous treatments.

Related reports

This is the first case of very rapidly disseminated hydatid cyst with atypical presentation that caused diagnostic problems. It justifies that a high level of vigilance towards parasitic infections of the liver should be maintained especially in endemic countries.

Experiences and lessons

Hydatid disease is still a major public health problem in endemic regions. This diagnosis should be suspected in case of cystic lesions of the liver. Accurate diagnosis of this disease represents a challenge. It allows a prompt management of the disease and prevention of the recurrence.

Peer-review

Good.

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