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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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TOPIC HIGHLIGHT

2016 Hepatitis C Virus: Global view

Advances in hepatitis C therapy: What is the current state - what come's next?

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Abstract

Chronic hepatitis C virus (HCV) infection affects 80-160 million people worldwide and is one of the leading causes of chronic liver disease. It is only a few years ago that standard treatment regimes were based on pegylated interferon alpha and ribavirin. However, treatment of HCV has undergone a revolutionary change in recent years. The admission of the nucleotide polymerase inhibitor Sofosbuvir enabled an interferon-free regimen with direct antiviral agents (DAA). Meanwhile seven DAAs are available and can be applied in several combinations for 8 to 24 wk depending on HCV genotype and patient characteristics such as cirrhosis and chronic renal failure. High rates of sustained virological response (SVR) rates can be achieved with these novel drugs. Even in difficult to treat populations such as patients with liver cirrhosis, HCV-human immunodeficiency virus co-infections, after liver transplantion, or with chronic kidney disease comparable high rates of SVR can be achieved. The anticipated 2nd generation DAAs are strikingly effective in patients so far classified as difficult to treat including decompensated liver cirrhosis or post-transplant patients. These 2nd generations DAAs will have higher resistance barriers, higher antiviral effects and a pan-genotypic spectrum. This review highlights the current state of the art of antiviral treatment in hepatitis C and gives an outlook for upcoming therapies.

Key words: Hepatitis C virus; Direct antiviral agents; Sustained virological response; Liver transplantation; Renal impairment; Cirrhosis

Core tip: Treatment of chronic hepatitis C virus (HCV) infections has undergone a revolutionary change in recent years. This review highlights the current state of the art of antiviral treatment in chronic hepatitis C infections and gives an outlook for upcoming therapies. Difficult to treat populations such as patients with decompensated liver cirrhosis, HCV-human immunodeficiency virus co-infections, after liver transplantation and patients with renal impairment or on hemodialysis are highlighted.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the leading causes of chronic liver disease worldwide[1]. Worldwide 80-160 million people are estimated to be chronically infected with HCV[2]. The long-term follow of chronic HCV are highly variable, ranging from minimal histological changes to extensive fibrosis with or without cirrhosis[2].

The primary goal of HCV therapy is to achieve eradication of the HCV which is currently determined by a sustained virological response (SVR) as surrogate marker. SVR is defined as undetectable HCV RNA 12 wk (SVR 12) or 24 wk (SVR 24) after end of treatment.

In recent years antiviral therapy has experienced a tremendous progress. It is only a few years ago that standard treatment regimes were based on pegylated interferon alpha and ribavirin (P/R). These therapies were associated with many adverse effects, long treatment durations of usually 48 wk and low SVR rates[3,4]. In 2011, the two protease inhibitors boceprevir and telaprevir were approved for treatment of genotype 1. Due to their comparatively lower antiviral effectiveness and rapid resistance development both drugs were used only as triple-therapy regimen in combination with pegylated interferon alpha and ribavirin[5-8].

Since 2014 several direct acting antivirals (DAAs) have been approved enabling interferon-free antiviral treatments with high SVR rates.

The decoding of the HCV life cycle and the resolution of crystal structure of the relevant viral proteins enabled the development of many DAAs. The currently approved DAAs consist of three groups. The first group is directed against the viral protease NS3/4A (protease inhibitors; name ending on -previr), the second against the viral RNA-dependent RNA-polymerase NS5B (polymerase inhibitors, name ending on -buvir) and the third against the viral protein which is involved in the formation of the replicon complex NS5A (NS5A-inhibitors, name ending on -asvir)[9].

DAA SUBSTANCES

Sofosbuvir

Sofosbuvir (SOF) is an inhibitor of the NS5B-polymerase. As nucleotide analogue it causes chain termination during replication. SOF has a pan-genotypic effectiveness and a high resistance barrier. It is taken once daily with a good tolerability. No cross resistances with other substances have been reported. Drug interactions were described only for strong inductors of the gut transporters P-gp and BCRP (*e.g.*, rifampicin, St John's wort, carbamazepin, phenytoin)[10].

Simeprevir

Simeprevir (SMV) is a second-generation protease inhibitor. In addition to the activity against genotype 1 a simeprevir has clinically relevant antiviral effects against genotypes 4 and 6. Similar to boceprevir it needs to be taken only once a day. Adverse effects reported in clinical studies consisted mainly of skin lesions with or without itching, nausea and dyspnea. Of note, the variant (RAV) Q80K exhibits a preexisting resistance against simeprevir resulting in treatment failure of patients with genotype 1a[11].

As SMV is metabolized by hepatic CYP34A, inhibitors and inductors of CYP3A4 may affect plasma levels of SMV[12].

Daclatasvir

Daclatasvir (DCV) is a NS5A-inhibitor. DCV has a high antiviral activity against genotypes 1 to 4 *in vivo* and *in vitro* also against genotypes 5 and 6. On the other side the resistance barrier is relatively low. In case of treatment failure resistance-associated resistances (RAVs) may be selected, which remain detectable after end of treatment[13]. The influence of these RAVs on following therapies has not been systematically investigated so far. In studies using P/R the combination of DCV plus P/R showed no additional adverse effects[14]. Similar to SMV, DCV is also metabolized by CYP3A4.

Ledipasvir

Ledipasvir (LDV) represents another NS5A-inhibitor with antiviral activity particularly against genotype 1 and partially against other genotypes such as 4-6. LDV is only available in a fix dose combination with SOF. The most commonly reported adverse effects were headache and fatigue. As with DCV, RAVs have been detected during therapy and were not clinically relevant due to the strong antiviral effect of SOF[15].

In vitro, no detectable metabolism of ledipasvir was observed by human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Evidence of slow oxidative metabolism *via* an unknown mechanism has been observed. Biliary excretion of unchanged ledipasvir is a major route of elimination, with renal excretion being a minor pathway (approximately 1%).

Paritaprevir/ritonavir + ombitasvir ± dasabuvir

The combination of paritaprevir/ritonavir (PTV/r) + ombitasvir (OBV) ± dasabuvir (DSV) is referred to as 3D. PTV is an NS3/4A-inhibitor, which is boosted by r to optimize its pharmacokinetics. OBV is an NS5A-inhibitor, while DSV is a non-nucleotide polymerase-inhibitor. PTV/r and OBV are available at fix dose combinations and have antiviral activity against genotypes 1 and 4, while DSV is only effective against genotype 1. Registered adverse effects were pruritus in clinical study cohorts

Table 1 Current recommendations of antiviral regimens depending on the genotype

DAA-regime	HCV-genotype						
	1a	1b	2	3	4	5	6
SOF + R	(×)	(×)	×	×	(×)	(×)	(×)
SOF + SMV ± R	×	×			×		
SOF + DCV[1] ± R	×	×	(×)	×	×		
SOF + LDV ± R	×	×		(×)	×	×	×
OBV + PTV/r ± DSV ± R (3D)	×	×					
OBV + PTV/r ± R (2D)					×		

[1]DCV, no approval in the United States. DAA: Direct antiviral agents; LDV: Ledipasvir; SMV: Simeprevir; DCV: Daclatasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV: Paritaprevir; HCV: Hepatitis C virus; DSV: Dasabuvir; R: Ribavirin; r: Ritonavir.

without ribavirin and in very few patient increased transaminases as well as elevations of bilirubin. As PTV/r is metabolized by CYP3A4 interactions with several other drugs may occur. There are existing cross-resistances to other protease-inhibitors and NS5A-inhibitors while DSV has a low resistance-barrier[16].

DAA COMBINATION REGIMENS

Of the approved DAAs the following combinations have been studied in clinical trials (Table 1).

SOF is the so-called backbone of most combinations, as it has a high resistance barrier and a pan-genotypic activity. In contrast, the high antiviral activity is achieved by the combination of the various groups of substances in the 3D-regime. In all regimes the addition of R is possible and may be useful for defined patient groups.

SOF + R

This combination has high SVR-rates (86%-97%) in genotype 2 patients[10,17]. In genotype 1, however, SVR-rates were inconsistently in phase 2 studies. Especially in difficult to treat patients, *e.g.,* with cirrhosis, SVR-rates were unsatisfactory (SVR 10%-84%)[18]. Furthermore, genotype 3 treatment efficacy during a 12-wk regimen was low with SVR rates between 30% to 56%[10,17]. However, a significant increase in SVR-rates up to 85% could be achieved by extending the treatment duration to 24 wk in patients with genotype 3. Existence of cirrhosis was associated with poorer SVR-rates in genotype 3 patients (SVR 68% with cirrhosis and pretreatment with P/R *vs* SVR 91% without cirrhosis)[19]. Smaller studies treating genotype 4 patients for 12 and 24 wk, respectively, have shown SVR-rates in therapy-naïve patients of 79% and 100%, respectively, and in pretreated patients 59% and 87%, respectively[20].

SOF + SMV ± R

The COSMOS-study, a phase Ⅱ trial, analyzed the efficacy of SOF + SMV with and without R in patients with HCV genotype 1. This study consisted of two cohorts of which the first represented patients with null-response to P/R but without advanced fibrosis or cirrhosis. The second cohort included patients with advanced fibrosis (F3) or cirrhosis. Patients were treated for 12 or 24 wk with and without R. In the first cohort a cumulative SVR of 90% was observed, while in the second cohort an even higher SVR of 94% could be achieved. Neither the extension to a 24-wk treatment nor the addition of R were of any advantage in this study[12].

These results could be confirmed by two big observational-studies. The TRIO-trail was able to demonstrate a higher SVR in genotype 1b, compared to 1a (92% *vs* 80%)[21]. The TARGET-study with 883 genotype 1 patients (54% cirrhosis) treated with SOF + SMV ± R presented in an interim analysis a SVR4-rate of 93% in 98 patients without cirrhosis and in patients a SVR4 of 85% in 124 cirrhotics[22].

Meanwhile results of two phase-Ⅲ study entitled OPTIMIST 1 and 2 have been presented. In the OPTIMIST 1-study 310 naïve or pretreated genotype 1 patients without cirrhosis were treated with SOF + SMV for 8 or 12 wk. Patients treated for 12 wk achieved SVR-rates of 97% and those treated only for 8 wk 83%[23]. The OPTIMIST 2-study investigated 103 naïve or pretreated genotype 1 patients with cirrhosis being treated for 12 wk with SOF + SMV resulting in a SVR of 83%[24].

SOF + DCV ± R

The combination of SOF + DCV was investigated in treatment-naïve patients with genotypes 1, 2 and 3 without cirrhosis and in genotype 1-patients with treatment-failure of a protease-inhibitor based therapy. The treatment response in GT1 was investigated in different groups in a phase Ⅱ-study. The results showed high SVR-rates between 93%-100% regardless of treatment duration and addition of R. In pretreated genotype 1 patients only 24 wk of therapy were evaluated with or without R. This regimen resulted in SVR-rates of 95%-100%. The ALLY-1 study investigated SOF + DCV + R for 12 wk in patients with cirrhosis (n = 60) or after orthotopic liver-transplantation (OLT) (n = 53). For genotype 1, patients with cirrhosis achieved a SVR rate of 82%, whereas the SVR rate after OLT was even higher with 95%[25].

Initially only a 24-wk treatment was evaluated in genotype 2 and 3 patients. This lead to SVR rates of 92% in genotype 2 and 89% in genotype 3 patients[26]. In the ALLY-3 study genotype 3 patients were treated with SOF + DCV for 12 wk without R. Patients without cirrhosis achieved independent of pretreatment high SVR-rates of 97% in naïve and 94% in pretreated patients. In case of cirrhosis SVR rates were lower with only 58% to 69%[27]. The combination of SOF and DCV has an antiviral effectiveness in genotype 4 but results of studies are lacking.

SOF + LDV ± R

For this fixe dose combination profound phase 3 study data exists. Studies were performed on 1.952 patients

Table 2 Overview on clinical studies using the NS5B inhibitor sofosbovir

Study	Patient population	Therapy	Duration (wk)	SVR	Comments
ION-1	First-line therapy	SOF + LDV	12	99%	
		SOF + LDV + R	12	97%	
		SOF + LDV	24	98%	
		SOF + LDV + R	24	99%	
ION-2	Re-therapy	SOF + LDV	12	94%	86% cirrhotic
		SOF + LDV + R	12	96%	82% cirrhotic
		SOF + LDV	24	99%	100% cirrhotic
		SOF + LDV + R	24	99%	86% cirrhotic
ION-3	First-line therapy	SOF + LDV	8	94%	Only non-cirrhotic
		SOF + LDV + R	8	93%	Only non-cirrhotic
		SOF + LDV	12	95%	Only non-cirrhotic

SOF: Sofosbuvir; LDV: Ledipasvir; R: Ribavirin; SVR: Sustained virological response.

Table 3 Overview on clinical studies using the combination of paritaprevir/ritonavir + ombitasvir ± dasabuvir

Study	Patient population	Therapy	Duration (wk)	SVR	Comments
SAPPHIRE-Ⅰ	First-line therapy	OBV + PTV/r + DSV + R	12	96%	No cirrhosis
SAPPHIRE-Ⅱ	Re-therapy	OBV + PTV/r + DSV + R	12	96%	No cirrhosis
TURQUOISE-Ⅱ	Cirrhosis	OBV + PTV/r + DSV + R	12	92%	GT1a 89% GT1b 99%
		OBV + PTV/r + DSV + R	24	96%	GT1a 95% GT1b 100%
PEARL-Ⅱ	Re-therapy, GT1b	OBV + PTV/r + DSV	12	100%	No cirrhosis
		OBV + PTV/r + DSV + R	12	97%	No cirrhosis
PEARL-Ⅲ	First-line therapy, GT1b	OBV + PTV/r + DSV	12	99%	No cirrhosis
		OBV + PTV/r + DSV + R	12	99%	No cirrhosis
PEARL-Ⅳ	First-line therapy, GT1a	OBV + PTV/r + DSV	12	90%	No cirrhosis
		OBV + PTV/r + DSV + R	12	97%	No cirrhosis

OBV: Ombitasvir; PTV: Paritaprevir; DSV: Dasabuvir; R: Ribavirin; r: Ritonavir; SVR: Sustained virological response.

with genotype 1. The detailed results of the ION-studies are shown in Table 2. In all treatment-groups high rates of SVR were observed. In treatment-naïve patients there was neither advantage of treatment for more than 12 wk nor addition of R even in cirrhotic patients[28]. In contrast, pretreated patients with cirrhosis achieved higher SVR-rates after treatment for 24 wk. In these patients high SVR-rates could also be achieved by adding R to a 12 wk antiviral treatment[15].

Treatment-naïve genotype 1-patients without cirrhosis achieved a SVR of 94% after only 8 wk of treatment. The higher number of relapses in this cohort were patients with a viral load above 6×10^6 IU/mL[29].

For genotypes 3 and 6 data from a small study showed also a high antiviral effectiveness for SOF/LDV + R. Genotype 3 treatment-naïve patients with cirrhosis achieved 100% SVR, pretreated patients with cirrhosis 89% SVR. Without addition of R only 64% of the treatment-naïve cirrhotic patients achieved SVR[30,31]. In genotype 6 SOF/LDV without R resulted in SVR-rates of 96%[31].

OBV + PTV/r ± DSV ± R (3D)

The 3D-regimen achieved high SVR-rates in several phase Ⅲ-studies with a total of 1577 genotype 1 patients. The detailed results of these studies are shown in Table 3. The 3D-regimen achieved in genotype 1 patients without cirrhosis in the SAPPHIRE-study high SVR-rates of 96% regardless of a prior therapy[32,33].

Due to a weaker antiviral activity of 3D in genotype 1a the PEARL-studies (which did not include cirrhotic patients) were performed separately for both subtypes. The treatment-regimes differed regarding the addition of R. In genotype 1b nearly all patients achieved SVR without R regardless of pretreatment indicating that R can be omitted for this patient population[16,34]. In contrast, genotype 1a patients exhibited higher SVR rates by addition of R compared to those being treated without R (97% *vs* 90%)[16].

Genotype 1 patients with cirrhosis were investigated in the TURQUOISE-Ⅱ-study. Here, 3D + R was admitted for 12 or 24 wk. For genotype 1a extension of treatment from 12 to 24 wk resulted in higher SVR-rates (89% *vs* 95%), whereas for genotype 1b nearly all patients were cured by a 12-wk treatment[35].

In genotype 4 DSV was omitted due to lack of antiviral activity. Therefore, a combination of OBV + PTV/r with and without R was tested for 12 wk in the PEARL-I-study. Addition of R a resulted in a SVR rate of 100%, whereas without R only 91% of patients

achieved SVR[36].

Current treatment recommendations have been published in the AASLD and EASL guidelines. These take into account the specific conditions in different countries in terms of availability of DAAs[2,37].

So far there are currently sufficient IFN-free DAA-regimes with excellent SVR-rates, in particular for genotype 1 patients. Unresolved issues represent patients with relapse after DAA-regimen as they exhibit RAVs and cirrhotic patients with genotype 3 as SVR rates remain unsatisfactory for this population. Current antiviral studies address these challenges and in the near future we expect efficient regimens for the remaining difficult to treat HCV patients.

DIFFICULT TO TREAT POPULATIONS

Cirrhotic patients

Liver cirrhosis is the most important negative predictor of SVR in DAA therapies. In nearly all regimens treatment efficacy is lower compared to non-cirrhotic patients. In pivotal studies on cirrhotic patients for the 3D-regime and SOF/LDV SVR could be increased by treatment extension to 24 wk and addition of ribavirin. It should be noted that only compensated patients with Child Pugh stage A were included in these studies. Recently, in a prospective study 108 patients with decompensated cirrhosis with Child Pugh stage B and C were treated with SOF/LDV + R for 12 or 24 wk. SVR was achieved in 87% of patients with Child Pugh B and 89% with Child Pugh C indicating that this therapy regimen is safe and effective even in decompensated liver cirrhosis. Of note, an improvement of liver function was observed during and after therapy[38].

Liver transplantation

After liver transplantation of patients with chronic HCV infections a reinfection is common. Due to immunosuppression an accelerated progression of fibrosis in the transplant is often observed. Pre-treatment with SOF + R before transplantation in 61 patients with HCC within Milan criteria and compensated HCV-induced cirrhosis prevented a reinfection of the graft in 70%[39]. After liver transplantation a different study using the 3D-regime + R resulted in a SVR of 97% (33 out of 34 patients)[40]. Using SOF/LDV + R for 12 or 24 wk in 223 patients after liver transplantation similar high SVR rates could be achieved in patients without cirrhosis (96%-98%) or Child Pugh A cirrhosis (96%). However, in case of decompensated cirrhosis SVR rates were lower (Child Pugh B: 83%-85%, Child Pugh C: 60%-67%)[41].

Human immunodeficiency virus-HCV co-infection

Human immunodeficiency virus (HIV)-HCV co-infections result in a faster progression of fibrosis compared to mono-infections. In a variety of studies with similar designs comparable treatment responses were found. Thus, HIV-HCV co-infected patients can be treated equal to mono-infected patients. To give an example the combination-therapy with SOF/LDV achieved SVR-rate of 98% in GT1 first line therapy[42]. It should be noted that possible drug-drug interactions between HCV regimes and antiretroviral substances may occur.

Renal impairment

Due to its renal elimination SOF may only be given to patients with a glomerular filtration rate above 30 mL/min per 1.73 m^2. Patients with severe renal impairment (glomerular filtration rate < 30 mL/min per 1.73 m^2) or chronic renal failure undergoing dialysis therefore require other antiviral regimens.

Pharmacokinetic data showed the possibility of using OBV + PTV/r + DSV ± R (3D) in patients with severe renal impairment and chronic renal failure. Serum levels of the 3D substances were comparable to HCV-patients without renal impairment. SVR12 data are outstanding but all finished patients (10 out of 20) presented SVR4[43].

Another placebo-controlled study with Grazoprevir (100 mg) plus Elbasvir (50 mg) for 12 wk in 122 HCV GT 1 patients with renal impairment (75% under dialysis) presented SVR12 rates of 94%, representing a potential future treatment regimen in this difficult to treat patient population[44].

FUTURE HCV TREATMENT OPTIONS

Future developments consist of second generation DAAs. The protease inhibitors of the second generation will exhibit a better resistance barrier and broader spectrum of activity against various genotypes of HCV, in particular subtype 1a (*e.g.,* Grazoprevir, Sovaprevir) and pan-genotypic (*e.g.,* ABT-493, GS-9857). Moreover, these substances do not have complete cross-resistances against associated RAVs against first-generation protease inhibitors (*e.g.,* ABT-493)[45].

The second generation NS5A-inhibitors will have higher resistance barriers, higher antiviral effects and a pan-genotypic spectrum (*e.g.,* Elbasvir, Samatasvir, GS-5816, MK-8408, ABT-530). ABT-530 presented *in vitro* high antiviral effectiveness against frequent NS5A-RAVs[45]. Furthermore, novel drugs will be used as combination-regimes. Combinations of these protease inhibitors and NS5A-inhibitors could achieve similar SVR-rates than previous regimes based on nucleotide NS5B-polymerase inhibitors (NUC). It is likely that shorter treatment durations may be achieved using these regimens. A recently presented study using Grazoprevir/Elbasvir + SOF (C-SWIFT) in treatment-naïve genotype 1 and 3 patients with and without cirrhosis investigated the antiviral effectiveness in terms of treatment duration. In genotype 1 non-cirrhotic patients achieved SVR in only 33% after 4 wk of treatment. In contrast, an SVR-rate of 87% was achieved after 6 wk of treatment. Cirrhotic patients with HCV-genotype 1 achieved SVR in 80% after 6 wk and SVR in 94% after 8 wk of treatment. In genotype 3 non-cirrhotic patients were treated for 8 and 12 wk resulting in SVR-rates of 93% and 100%.

Table 4 Overview on clinical studies using future antiviral drugs and combinations

Substances (study)	RBV	Genotype	Population	Duration (wk)	Phase	Results (SVR)
SOF + GS-5816 (NS5A)[47]	±	1	TN, NCi	8	Ⅱ	81% - R; 90% + R; (*n* = 60)
	±	2	TN, NCi	8	Ⅱ	88% - R; 88% + R; (*n* = 52)
	±	3	TN, NCi	8	Ⅱ	96% - R; 100% + R; (*n* = 53)
	±	1	TE, Ci, NCi	12	Ⅱ	NCi: 100% (*n* = 38); Ci: 100% - R, 90% + R (*n* = 17)
	±	3	TE, Ci, NCi	12	Ⅱ	NCi: 100% (*n* = 53); Ci: 88% - R, 96% + R (*n* = 52)
SOF + ACH-3102 (NS5A) (PROXY)[48]	-	1	TN, NCi	6 and 8	Ⅱ	100% after 6 wk (*n* = 12) or 8 wk (*n* = 12)
SOF/LDV + Vedroprevir (SYNERGY)[49]	-	1	TN, NCi	6	Ⅱ	95% (*n* = 20)
SOF/LDV + GS 9669 (non-NUC-NS5B) (SYNERGY)[49]	-	1	TN, NCi	6	Ⅱ	95% (*n* = 20)
SOF + Grazoprevir + Elbasvir (C-SWIFT)[46]	-	1, 3	TN, NCi	4 or 6 (GT1)	Ⅱ	SVR8: GT1: 39% after 4 wk (*n* = 31); 87% after 6 wk (*n* = 30)
				8 or 12 (GT3)		GT3: 100% after 8 and 12 wk (*n* = 15/14)
			TN, Ci	6 or 8 (GT1)	Ⅱ	SVR8: GT1: 80% after 6 wk (*n* = 20); 89% after 8 wk (*n* = 21)
				12 (GT3)		GT3: 90% (*n* = 12)
Grazoprevir + Elbasvir (C-WORTHY)[50]	±	1	TN, NCi	8 or 12	Ⅱ	8 wk GT1a: 80%
						12 wk: 98% - R; 93% + R
			TN, Ci	12 or 18	Ⅱ	12 wk: 97% - R; 90% + R
						18 wk: 94% - R; 97% + R
			TE, Ci, NCi	12 or 18	Ⅱ	12 wk: 91% - R; 94% + R
						18 wk: 97% - R; 100% + R
SMV + Samatasvir (HELIX-1)[51]	+	1b, 4	TN, NCi	12	Ⅱ	SVR4: GT1b: 80% (*n* = 84)
						GT4: 100% (*n* = 9)
Asunaprevir + DCV + Beclabuvir (UNITY 1)[52]	-	1	TN, TE, NCi	12	Ⅲ	TN: 91%; TE: 89%
Asunaprevir + DCV + Beclabuvir (UNITY 2)[53]	±	1	TN, TE, Ci	12	Ⅲ	90% - R; 96% + R

TN: Therapy-naive; TE: Therapy-experienced; Ci: Cirrhosis; NCi: No cirrhosis; GT: Hepatitis C virus-genotype; SOF: Sofosbuvir; LDV: Ledipasvir; R: Ribavirin; SVR: Sustained virological response; SMV: Simeprevir; DCV: Daclatasvir.

Cirrhotic genotype 3 patients being treated only for 12 wk achieved a SVR-rate of 91%[46]. These future substances will enable a shortened treatment time and increase the antiviral activity in individual populations. A selection of future therapies and their phase Ⅱ - Ⅲ trial results are presented in Table 4[46-53].

As of mid 2016, the 2nd generations DAAs are expected to be available. These therapy regimes will have pan-genotypic and high antiviral effectiveness as wells as a better resistance profile. It is likely that R will become dispensable and treatment duration may be reduced to 6-8 wk for the majority of patients. In addition, difficult to treat populations (*e.g.*, genotype 3 with cirrhosis) may achieve higher rates of SVR.

However, in order to significantly lower HCV-induced morbidity and mortality, a higher proportion of infected patients would have to be treated. This will only be possible by an increased screening of risk populations and customized pricing.

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REVIEW

Management of immunosuppressant agents following liver transplantation: Less is more

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Abstract

Immunosuppression in organ transplantation was revolutionary for its time, but technological and population changes cast new light on its use. First, metabolic syndrome (MS) is increasing as a public health issue, concomitantly increasing as an issue for post-orthotopic liver transplantation patients; yet the medications regularly used for immunosuppression contribute to dysfunctional metabolism. Current mainstay immunosuppression involves the use of calcineurin inhibitors; these are potent, but nonspecifically disrupt intracellular signaling in such a way as to exacerbate the impact of MS on the liver. Second, the impacts of acute cellular rejection and malignancy are reviewed in terms of their severity and possible interactions with immunosuppressive medications. Finally, immunosuppressive agents must be considered in terms of new developments in hepatitis C virus treatment, which undercut what used to be inevitable viral recurrence. Overall, while traditional immunosuppressive agents remain the most used, the specific side-effect profiles of all immunosuppressants must be weighed in light of the individual patient.

Key words: Immunosuppression; Orthotopic liver transplantation; Metabolic syndrome; Acute cellular rejection; Hepatitis C virus

Core tip: The use of immunosuppressive agents is reviewed in the context of the modern post-orthotopic liver transplantation population. The side effects of mainstay immunosuppressive strategies exacerbate some patient pathologies, and combinations of different immunosuppressants could be more specifically tailored to patient needs. Acute cellular rejection and malignant complications are also discussed with respect to im-

munosuppressive strategies. Finally, hepatitis C virus and its impact on immunosuppression is re-evaluated in light of recent developments in viral clearance.

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INTRODUCTION

The use of immunosuppression in organ transplantation was revolutionary for its time, and its results were quickly embraced for their efficacy at suppressing host rejection of a graft.

However, given the increasing impact of metabolic syndrome (MS) as a public health issue[1], immunosuppression and its side effects may pose a greater risk to patients than rejection of a newly-transplanted organ. In fact, metabolic complications of immunosuppressive therapy were at one point the leading cause of morbidity and mortality for patients following orthotopic liver transplantation (OLT)[2]. Reduction of immunosuppression is a widely-recognized strategy to addressing this issue[3].

Cardiovascular disease (CVD) and renal disease account for 19.3% and 6.8% of nonhepatic causes of death in post-OLT patients, respectively[4]. In patients who survive at least 3 years, non-hepatic cause of death accounts for 58% of all-cause mortality post-OLT[5]. In their evaluation of liver transplantation as a cardiovascular risk factor, Madhwal *et al*[6] (2012) found that up to two-thirds of patients develop MS after OLT. Clearly, the trend towards post-transplant metabolic disturbances must be addressed; the first place to start is optimizing post-transplant immunosuppressive therapies[7].

Further, with recent advances in the treatment of hepatitis C virus (HCV)[8,9], long-term metabolic complications posed by immunosuppression must be weighed more heavily against the immediate issue of organ rejection. Just as the introduction of immunosuppression made longer-term complications a new focus in transplantation, eradication of this chronic liver infection leaves room to focus on metabolic issues.

BRIEF OVERVIEW OF LIVER IMMUNOLOGY: THE LIVER IS IMMUNOPRIVILEGED

From a physiological standpoint, the liver is one of the first organs exposed to the absorbed contents of the external environment. Handling of newly-acquired blood content immediately after absorption from the external environment necessitates that the liver maintain its own unique balance of immunity *vs* tolerance. The special status of the liver as immunoprivileged is well-recognized; for example, there is a paucity of hepatic B- and T-cell mediated autoimmune disease, and some autoimmune hepatitis liver markers are found in healthy and ill people alike[10]. Further, transplant tolerance is known to occur at greater frequency for liver transplant recipients, compared to other vascularized organ recipients[11]. At the same time, there remains much to be learned about liver immunology. For example, the role of humoral alloreactivity in ABO-compatible liver transplantation is still being elucidated[12-14].

The liver is rich with parenchymal hepatocytes, but also contains non-parenchymal immune cells that serve as a first barrier to antigens arriving from portal circulation. Hepatic nonparenchymal cells include the largest population of fixed resident macrophages in the body, Kupffer cells, as well as other reticuloendothelial cells[15]. The parenchymal hepatocytes further contribute to immunity by secreting 80%-90% of complement components and pathogen-recognition receptors (PRR), as well as synthesizing membrane-bound PRRs to catch portal antigens[16].

Along with antigens arriving from portal circulation, about 10^8 peripheral blood lymphocytes pass through the liver every 24 h[17]. These cells are squeezed through fenestrated capillaries that may open a window to T-cell priming[18]. The status of the liver as a major reservoir of immune cells has major implications, then, both as far as catching portal circulation antigens as well as immunomodulation.

For example, the privileged status of the liver might be used in the future to induce complete graft tolerance. As a promising example, animal models have shown a lifetime tolerance to liver grafts: In 20% of outbred pig liver recipients, lifetime tolerance can be achieved without the use of immunosuppressants[19]. The possibility of lifetime tolerance in humans, too, remains optimistic. Ramos *et al*[20] (1995) review their experience withdrawing immunosuppressive therapy after witnessing patient noncompliance with immunosuppression. They were able to accomplish immunosuppression-free tolerance in 16 patients for 3 to 19 mo, continuing efforts to completely wean 28 patients, but failed in 15 patients without any graft loss or demonstrable loss of function due to rejection.

Not only is the liver self-protective, but there is evidence of immunocompetence conferred to other organs: Simpson *et al*[21] (2006) showed that patients who receive combined same-donor liver and kidney transplants are immunoprotected compared to patients who receive kidney transplant after liver transplant. The authors speculate that the identical genotypes of the transplanted organs facilitates immunoprotective effects. There is, however, conflicting data: Katznelson *et al*[22] (1996), comparing incidence of acute rejection between 248 combined liver and kidney transplantations to a control group of 206 kidney-alone transplantations, found that 3-year survival rates are not significantly different.

Despite a key role in immunoregulation generally, human leukocyte antigen (HLA) histocompatibility has little clinical significance to liver allograft outcome[23,24]. On the other hand, HLA compatibility may play a more subtle role in OLT than we see clinically, where immunosuppressive regimens may paint broad enough strokes to obfuscate nuances. Neumann *et al*[25] (2003) report that HLA compatibility is associated with significantly less acute rejection, but no difference in graft survival. This peculiar behavior of the liver compared to other organs may further reflect the possibility that acute rejection is not as harmful to OLT as previously thought. A better understanding of the mechanisms of liver immunology is necessary to identify how to maximize the utility of histocompatibility[26].

IMMUNOSUPPRESSANT AGENT OVERVIEW

There are three signal pathways targeted by immunosuppressive agents. The first is calcineurin-mediated nuclear factor of activated T-cells activation *via* the T-cell receptor (TCR) and CD3 meeting an antigen presented on an major histocompatibility complex (MHC) protein; the second is a B7/CD28 costimulatory signal required for TCR-MHC complex synapsing; and the third signal is mediated by interleukin 2 (IL-2) as a ligand to CD25, through adaptor proteins JAK3 and PI-3K to mechanistic target of rapamycin (mTOR) regulation of cyclin-dependent kinases and cyclins to control the cell cycle[27].

Figure 1 depicts the process of an antigen-presenting cell (APC) travelling to a lymphoid organ, where it meets T cells in the paracortex. A native T-cell interacts with the APC, and if a set of several interactions and conditions are met, then the native T-cell replicates many times. This is called T-cell activation, and T-cell clonal expansion, and creates numerous effector T-cells that are specific to the antigen originally presented by the APC. These effector T-cells proceed to leave the lymph nodes and head back to the liver, where they can detect antigen and effect an immune response.

Many current immunosuppressive drugs target either those extracellular interactions or the intracellular signals that are highlighted in Figure 1. Calcineurin inhibitors (CNIs) prevent T-cell activation *via* an intracellular pathway, for example; on the other hand, the anti-IL-2 receptor antibodies basiliximab and daclizumab prevent IL-2 receptor activation[28].

Immunosuppressant drugs can function as depleting agents, or as non-depleting agents. Depleting immunosuppressive therapies cause destruction of T cells and/or B cells[27], whereas non-depleting agents affect the immune system by preventing immune cell proliferation.

CNIs have been the mainstay of immunosuppression since they were discovered, and increased 1-year patient and graft survival to greater than 80%[29,30]. CNIs serve to prevent transcription of the autocrine factor IL-2, preventing cell proliferation. These drugs halt the phosphatase activity of calcineurin, which is an intracellular signal transduction protein that mediates response to antigenic peptides. CNIs include cyclosporin A (CsA) and tacrolimus (TAC). There is no direct reduction in T or B cell count, so these agents are considered non-depleting.

Second, there is the non-depleting immunosuppressant, mycophenolate mofetil (MMF). MMF is a prodrug of mycophenolic acid (MPA), which inhibits inosine monophosphate dehydrogenase (IMPDH). Because IMPDH catalyzes the rate-limiting step of *de novo* guanosine synthesis, both genetic replication and transcription are inhibited. Further, MPA is five times more effective at inhibiting the type Ⅱ isoform of IMDPH - the isoform expressed in activated lymphocytes[31]. Because it is specific to the lymphocyte isoform of IMPDH, MPA prevents lymphocyte proliferation and transcription of activation-associated genes. As far as small molecules go, MMF is one of the more specific immunosuppressant agents. Side effects of MMF include nonimmune issues such as diarrhea and anemia[27], but, more importantly, exacerbation of cytomegalovirus infection[32].

Finally, the third major class of immunosuppressive drugs are called mTOR inhibitors. Found in soil from an Easter Island bacteria called *Streptomyces Hygroscopicus*, this class blocks IL-2-mediated autocrine leukocyte proliferation *via* inhibition of an intracellular signal transduction mechanism - thus, mTOR inhibitors are considered non-depleting agents[33,34]. Everolimus (EVR) and sirolimus (SIR) are the two best-known mTOR inhibitors.

Besides their use as immunosuppressants, another interesting property of mTOR inhibitors is their effect on longevity and age-related diseases. It is well-established that one way to increase longevity is through dietary restriction, and this effect is partially mediated by the mTOR pathway. Inhibition of mTORC1 has been associated with protection against neurodegenerative disease, heart disease, metabolic diseases, and a host of other age-related diseases[35]. Rapamycin administration, specifically, has repeatedly been shown to increase both mean and median lifespan in genetically heterogenous mice[36].

Immunosuppressive steroids such as methylprednisone are considered essential to graft tolerance induction, yet their use is highly associated with metabolic complications[37]. Several studies have examined outcomes of steroid-free or reduced-steroid immunosuppressive maintenance regimens[38,39]. In a prospective, randomized, placebo-controlled, double-blinded study, Lerut *et al*[40] (2014) report that in a cohort of 156 patients, patients treated with minimal or steroid-free immunosuppression displayed excellent outcomes over a period of 5 years. They report 5-year biopsy results, finding that histology presents similarly across both groups. In support of steroid withdrawal, other prospective studies of prednisone withdrawal post-OLT have demonstrated no difference in 2-year and 1-year survival of patients treated with or without

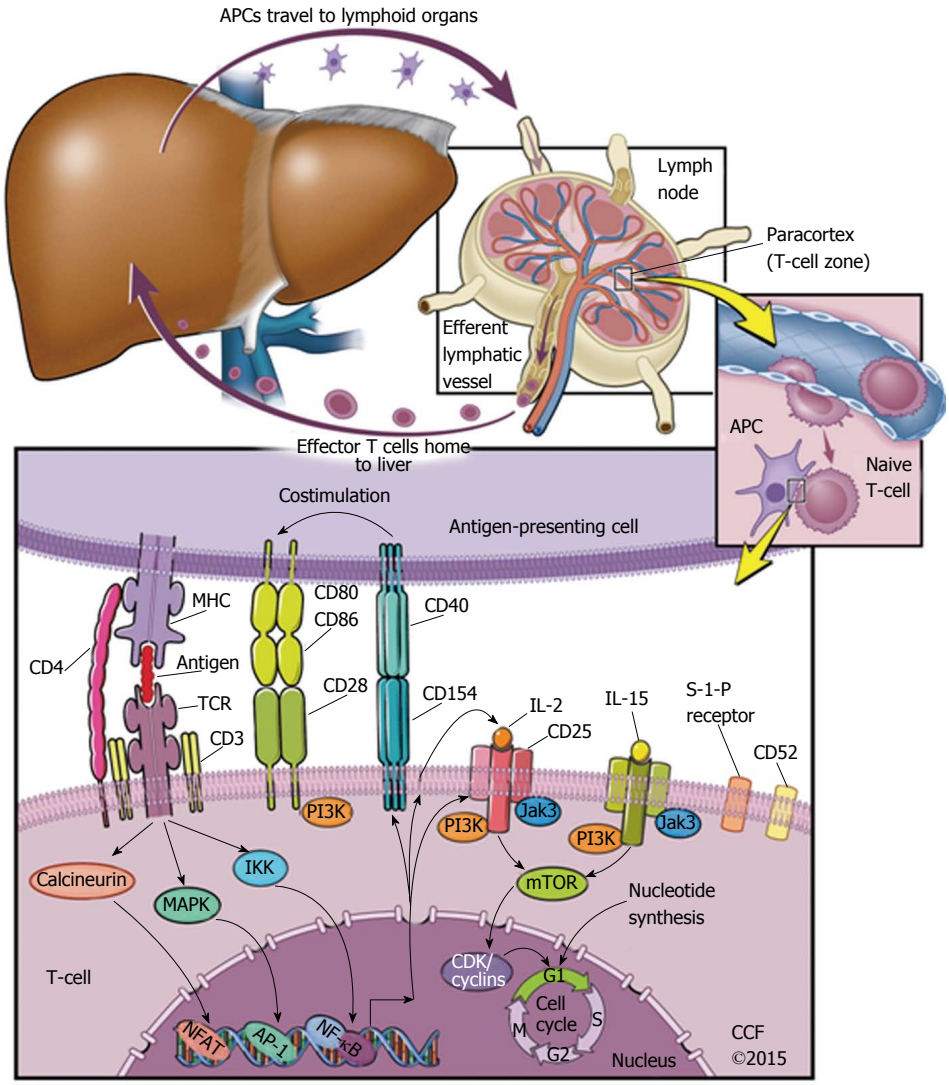

Figure 1 Signaling pathways targeted by modern immunosuppressive agents. Starting from the top left, an antigen-presenting cell (APC) migrates from local tissue to lymphoid organs. In the paracortex of the lymphoid organ, the APC meets a naive T-cell. If the naive T-cell has a T-cell receptor (TCR) that binds the antigen as it is presented by a Major Histocompatibility Complex on the APC, a set of other T-cell-APC interactions are likely to follow. This includes T-cell CD4 binding to the MHC on an APC, as well as costimulatory signals *via* extracellular receptors CD28 or CD40. After this set of T-cell-APC interactions begins, a set of intracellular signals follow towards the nucleus of the T-cell. The naive T-cell is then activated, and begins to replicate. This replication is called clonal expansion, and produces a population of T-cells that eventually migrate back to the tissue that contains antigen. AP-1: Activator protein 1; CD: Cluster of differentiation; CDK: Cyclin-dependent kinase; IKK: Inhibitor of kappa-B kinase; IL: Interleukin; Jak3: Janus-associated kinase 3; PI3K: Phosphatidyl inositide 3-kinase; MAPK: Mitogen-activated protein kinase; mTOR: Mechanistic target of rapamycin; NFAT: Nuclear factor of activated T-cells; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; TCR: T-cell receptor; MHC: Major histocompatibility complex.

prednisone[41,42]. Today, the use of steroids is generally limited to tolerance induction and treatment of acute cellular rejection (ACR); many studies have further investigated the possibility of steroid-free tranplantation[43-45].

Depleting immunosuppressant agents are mostly antibody-based. The first monoclonal antibody to be approved for use in humans was OKT3, an anti-CD3 antibody that modulates T-cell activation[46,47]. There are currently a host of biologics directed against T-cell proliferation, these are specific enough that their metabolic impact is significantly less than that of CNIs[48,49].

Issues associated with biological immunosuppression are unlike those of smaller molecules because they act extracellularly and are specific to their antigen[50], in contrast to small molecules such as tacrolimus that affect intracellular processes across a wider variety of cells. For example, one OKT3 side effect is called cytokine release syndrome, where widespread T-cell activation outweighs the antibody-mediated T-cell destruction that follows it[51]. This complication can become more severe if it leads to a positive-feedback loop, potentially causing a "cytokine storm"[52].

Besides OKT3, biologic agents have shown varying degrees of success and outcomes. For example, basiliximab is an IL-2 receptor antagonist that reduces rates of ACR at the expense of increased disease progression in HCV liver transplant patients[53]. Basiliximab, though, has been found to successfully treat graft-*vs*-host disease[54]. For those patients with

metabolic dysfunction, avoidance of steroid immunosuppression may be enough of a concern to warrant use of basiliximab.

Another biologic used to treat multiple sclerosis and Crohn's disease, natalizumab, is associated with significant liver injury as a side effect[55]. While the medical field is ripe for biologic development, implementation of biologics requires close evaluation.

On the other hand, some biologics open doors that might otherwise stay shut. Rituximab is an anti-CD20 monoclonal antibody that has been successfully used as immunological prophylaxis for ABO-incompatible (ABOi) liver transplantation[56]. Further, Yoshizawa *et al*[57] (2005) report that ABOi living-donor liver transplantation is possible without humoral rejection. Their protocol involves hepatic artery infusion and prophylactic use of rituximab, but, unlike previous attempts, does not involve splenectomy. Tanabe *et al*[58] (2010) later explain that ABOi has progressed to the point that it is as effective as ABO compatible transplantation, in part due to rituximab prophylaxis. Perfection of ABOi OLT will hopefully lead to other organ allocation advancements, such as humanized livers grown in non-human primates[59]. Immunosuppression in this context may become a hot topic in coming years.

ACR: DIAGNOSIS AND TREATMENT

ACR is most likely to occur within the first 6 wk of transplant, and is a very common event; in a study of 762 patients, the incidence of ACR post-transplantation is 64%. Several factors are associated with the occurrence of ACR, such as cold ischemic time of the organ, lower age of recipient, presence of edema, and HLA-DR mismatch[2].

There are three distinguishing features of ACR, each visible on histological examination. The first feature is portal triad inflammation, indicated by mixed inflammatory infiltrate; the second feature is damage to the bile ducts, specifically nonsuppurative cholangitis involving interlobular ductal epithelia; third, venous endotheliitis[60]. Venous endotheliitis is the most reliable diagnostic sign of ACR, but it is worth noting that phasic increase and decrease in lymphocyte aggregation may affect biopsy results[2].

In the clinic, ACR is suspected upon elevated liver function tests preceding jaundice and fever. Unfortunately, blood tests are neither sensitive nor specific for ACR diagnosis[61,62]. It follows, then, that liver biopsy is the gold standard of liver tissue evaluation[63]. To provide a level of standardization to biopsy evaluation, the Banff rejection activity index (RAI) assigns a score between zero and three to each characteristic of ACR[64].

Treatment for ACR includes high-dose steroids, optionally tapering off steroids and/or using biological immunosuppression[65]. Response to steroids is favorable; however, incidence of steroid-resistant rejection has been found to reach up to 14%[66].

ACR MORBIDITY AND MORTALITY

Timing of rejection is of major clinical significance in evaluating the potential impact of ACR[67,68]. While different studies have used different definitions, one commonly accepted cutoff defines early and late ACR as occurring within and after 90 d of transplantation, respectively. Several studies have found that early ACR is common and of lesser significance than late ACR[69]. For example, in a retrospective review of 231 histologically-confirmed cases of early ACR, Höroldt *et al*[64] (2006) report that neither total RAI score nor any of its components were correlated to steroid treatment response or graft survival. Indeed, Thurairajah *et al*[70] (2013), in a retrospective review of 970 patients, confirms that early acute rejection cases yield the best 10-year graft survival rates, at 85%.

In contrast to the minimal impact of early ACR, it appears that late ACR is associated with decreased graft survival. Uemura *et al*[69] (2008) found that of 1604 patients, 19.0% developed ACR later than 6 mo after the transplant; the only predictor of late ACR here was post-transplant lymphoproliferative disease. Thurairajah *et al*[70] (2013) found that 11% of patients developed late ACR, and that the highest rates of late ACR were found in patients with seronegative hepatitis, primary biliary sclerosis, and primary sclerosing cholangitis. Other studies have found that post-transplant lymphoproliferative disorder, decreased age, and increased medication level variability index are associated with and can predict late ACR[71].

Epidemiological evaluation of ACR is masked by its frequently subclinical character. Bartlett *et al*[72] (2002) explain that in a retrospective review examining 15 studies with a total of 1566 patients, 32% of standard protocol post-OLT biopsy samples showed evidence of ACR without any biochemical dysfunction. Since ACR is defined according to biopsy but not serum biomarkers, incidence of ACR may be higher than previously accepted. Another study, Tisone *et al*[42] (1999), explains that 80% of acute rejection episodes in their 45-patient cohort resolved spontaneously. The chances of clinically significant acute rejection, then, must be balanced against the risks of liver biopsy. In the future, metabolomic and other noninvasive studies could shed significant light on incidence of ACR[73-75].

Reduction of immunosuppression in light of ACR is not a new subject: Volpin *et al*[76] (2002), in a controlled study, evaluated two methylprednisolone regimens in the treatment of acute cellular rejection. They find that a 6-d taper regimen is safer than the higher-dose standard because ACR impact on graft rejection is minor, and the toxic effects of methylprednisolone outweigh the potential benefit of ACR suppression. Goddard *et al*[77] (2002) reviewed the Volpin study, concluding that immunosuppression therapies should be tailored to the individual patient after careful consideration of the interaction between past medical history and

immunosuppression side effects.

More recently, Rodríguez-Perálvarez *et al*[78] (2013) report that standard TAC trough concentrations are set too high (at 10-15 ng/mL), and that target TAC levels between 7 and 10 ng/mL are associated with longer graft survival while maintaining safety against rejection.

In contrast to the safety of simply reducing TAC, a randomized prospective trial of SIR monotherapy conversion regimen efficacy[79] found that liver transplant patients have no demonstrable benefit at 12 mo. Cumulative rates of graft loss or death were not significantly different, at 6.6% for the SIR group *vs* 5.6% for the CNI group. However, rates of acute rejection and discontinuation due to side effects were higher for patients treated with SRL. Then, one must consider the characteristics of the individual patient when designing an immunosuppressive regimen. For patients who are at great risk of end-stage renal disease (ESRD), the risk of acute rejection that is posed by conversion to SRL may be outweighed by the nephrotoxicity associated with the use of CNIs. For patients who are more concerned about acute rejection than nephrotoxicity, it makes sense to use CNI therapy.

MALIGNANT COMPLICATIONS POST-OLT

Recurrent and *de novo* malignancies are the top nonhepatic causes of late death in liver transplant patients, often listed alongside CVD. Some of the increased incidence in *de novo* malignancies in liver transplant recipients compared to the general population can be attributed to the use of exogenous immunosuppression[80,81].

The greatest incidence of post-transplant malignancies is associated with chronic viral infection. Specifically, Epstein-Barr virus-associated post-transplant lymphoproliferative disease, skin cancers, squamous cell carcinoma, and Kaposi's sarcoma are associated with status post-OLT[82]. Baccarani *et al*[82] (2009) find that 42 (12.8%) of patients undergoing OLT, out of 330, developed *de novo* cancers. Further, these patients had a lower 10-year survival rate than those who did not develop *de novo* cancer.

In hopes of reducing malignancy, current immunosuppression strategies focus on minimizing TAC with optional use of mTOR inhibitors or MMF. mTOR inhibitors are known for their antineoplastic activity[83], and CNI use can be associated with increased development of malignancy[84], making CNI reduction and replacement with mTOR inhibitors particularly favorable for patients at risk for malignancy.

MS

MS constitutes a number of symptoms that, when occurring simultaneously, indicate a primary clinical outcome of CVD. The criteria for MS is that a patient meet three of five components: Abdominal obesity and visceral fat, increased triglyceride (TG), decreased serum high-density lipoprotein, high blood pressure (HTN), insulin resistance and/or glucose intolerance[85]. Despite the wide range of systemic effects, each of these symptoms converges towards provoking CVD[86].

The Framingham study[87] found that 25% of all new-onset CVD could be predicted by presence of MS. More recently, Watt *et al*[4] (2010) report that causes of death more than 1 year after OLT have the following etiologies: 28% hepatic, 22% malignancy, 11% cardiovascular, 9% infection, and 6% renal failure. They conclude that modifiable risk factors such as diabetes, hypertension, and renal insufficiency can be used to improve long-term outcomes. Table 1 describes some of the interplay between MS components and immunosuppression. Because immunosuppressive strategies can sometimes be altered, relative risks and benefits should be weighed on a case-by-case basis.

Obesity: The boss of MS

Abdominal obesity is a prevalent characteristic of MS, and adipocyte dysfunction is hypothesized to underlie many metabolic disorders. Some explanations of adipocyte metabolism focus on the location of fat as a determinant of metabolic properties, such that visceral or abdominal fat might contribute more to MS than subcutaneous fat[88]; other explanations emphasize immunological modulation of adipocyte metabolism[89]. Whatever the underlying cause, obesity is a major public health issue[90], one that may be an environmental hit to a genetic predisposition.

Stegall *et al*[91] (1995) report that the incidence of obesity for adult liver transplant recipients 1 year after transplant was 41.9% for women, and 39.3% in men. In a more recent article, Richards *et al*[92] (2005) found that by one and 3 years after liver transplant, 24% and 31% of patients met criteria for obesity as body mass index (BMI) > 30 kg/m^2.

Despite clear evidence that obesity reduces graft and patient survival[93,94], there are studies that dispute its independent predictive power. Leonard *et al*[95] (2008) found that in a cohort of 1313 patients, obesity does not independently correlate to risk of graft or patient survival. Perhaps a measure of obesity is not enough, and there are more specific characteristics of excess adipose tissue that lead to it being a risk factor.

Després *et al*[96] (2006) explain that the deposition of visceral fat, as opposed to normal subcutaneous fat, can lead to adipose tissue overflow and hormonal imbalance. The net effect of these factors is an increase in ectopic fat to muscle, liver, and epicardial tissue. Compounding this issue, visceral adipocytes are less responsive to insulin, and thus not subject to the antilipolytic effects of insulin[97].

While the anatomical location of an adipocyte may be illuminating, physiologic factors also demonstrate the heterogeneity of metabolic dysfunction: Abnormal fat can accumulate as a result of defects in nuclear receptor genes involved in lipid sensing, synthesis, and

Table 1 Summary of immunosuppressant effects on metabolic syndrome

	Calcineurin inhibitors	Mycophenolate mofetil	mTOR inhibitors	Steroids
Body mass	Increase[92,103]	No change[105]	Less weight gain than CNIs[37]	Increase[144,145]
Dyslipidemia	Increase[104,121]	Less than CNIs[115]	Increase, but anti-atherosclerotic[144]	Increase[145]
Hypertension	Increase[107]	Less than CNIs[115,144]	No difference from CNI[146]	Increase[145]
Insulin resistance	Increase[128]	Potential benefit[145,146]	Potential benefit[149,150]	Increase[145]
Renal damage	Increase[112,113]	Less than both CNIs and mTOR inhibitors[147,151]	Decrease compared to CNIs[152]	Not significant
Note	Neoplastic[84]	Leukopenic[147,153,154]	Antineoplastic[155]	

CNIs: Calcineurin inhibitors; mTOR: Mechanistic target of rapamycin.

oxidation[98]. Supporting the ectopic fat hypothesis, Porter *et al*[99] (2009) report that analysis of 3000 Framingham study participants indicated that abdominal subcutaneous fat had no corresponding linear increase of obesity-associated risk factors.

Further complicating the issue is what type of lipids are most abundant in a dysfunctional metabolic state. In the context of non-alcoholic steatohepatitis/nonalcoholic fatty liver diseas, it has been suggested that hepatocyte TG accumulation may be protective against free fatty acid (FFA)-induced oxidative lipotoxicity[100]. As far as dietary fats go, there is evidence that unsaturated fats contribute to this lipotoxicity, whereas saturated fats are actually hepatoprotective[101].

Each of these issues - lipid distribution and lipid metabolism - is a qualitative issue that is outside the measure of BMI. Then, a more specific measure of adipose metabolism is necessary to further individualize treatment options for post-OLT patients. Given the heterogeneity of adipose metabolism, some authors suggest adipocyte transplantation as a treatment for metabolic issues such as diabetes, atherosclerosis, and nonalcoholic steatohepatitis[102].

Not only is obesity incident to the population of liver transplant recipients, it is exacerbated by the effects of immunosuppression[92]. A mainstay of immunosuppression, steroids, are well-known for effects on weight gain[103]. CNIs are also associated with weight gain: Ersoy *et al*[103] (2008) report that weight gain at 12 mo for renal transplant recipients prescribed TAC and CsA was 3.5 and 8.0 kg, respectively. As far as changing immunosuppressive regimens, it appears that using the mTOR inhibitor EVR in combination with a reduced dose of TAC can cause less weight gain than full-dose TAC immunosuppression[104]. If mTOR inhibitors are inappropriate for the specific patient, MMF is a different potential substitute that is not associated with post-transplant weight gain[105].

Hypertension and renal insufficiency

HTN and subsequent renal insufficiency are major concerns for post-OLT patients. Increased blood pressure and systemic vascular resistance is pathological, and can lead to hepatorenal syndrome. The use of immunosuppression, specifically CNIs, compounds the metabolic issues already present in liver transplant recipients[106,107].

The incidence of severe renal dysfunction can reach up to 18.1% at a mean of 13 years post-OLT[108]. Longenecker *et al*[109] (2015) recently reviewed renal function before and after OLT, finding that the overall rate of progression to ESRD is strongly associated with estimated GFR (eGFR) less than 60 mL/min × 1.73 m^2 and diabetes, but find that eGFR at OLT is not associated with 12-mo mortality.

For patients presenting with proteinuria as a result of HTN due to renal-insufficiency, treatment includes standard approaches such as angiotensin-converting enzyme inhibitors or angiotensin Ⅱ receptor blockers[86,110].

Aside from the standard of care for MS, the specific treatment of post-OLT patients with renal insufficiency should include reduction or withdrawal of CNI therapy as soon as possible because of the nephrotoxicity associated with CNIs[111,112]. Masetti *et al*[113] (2010) evaluated whether early withdrawal of CsA followed by initiation of EVR monotherapy preserves kidney function compared to their standard CsA regimen. The study found that incidence of stage 3 chronic kidney disease (< 60 mL/min GFR) at 1 year was significantly higher in the standard CsA group (55%) than in the group treated with EVR monotherapy (15.4%). Further, there was no difference in patient survival between the two groups.

Saliba *et al*[104] (2013) found that TAC reduction with addition of EVR were associated with increased estimated GFR, demonstrating a significant benefit to renal function. Tsai *et al*[114] (2009) confirm that, for renal allograft recipients, CNI minimization with the introduction of SRL reduces acute rejection and improves renal function and survival.

CNI withdrawal and replacement with MMF is another promising approach to post-OLT immunosuppressive management in patients who have renal insufficiency. Orlando *et al*[115] (2007) report success with MMF monotherapy as a means of reducing the toxic effects of CNIs. Of 42 post-OLT individuals who were weaned off of CNI therapy and placed on subsequent MMF monotherapy, renal function improved in 89% and arterial hypertension decreased in 80% of cases. In a separate study examining post-OLT patients with severe side effects from CNI therapy, Dharancy *et al*[116] (2009) found that a switch to MMF monotherapy could lead to increased eGFR without significant increase in rejection. Several studies have concluded that MMF is

less nephrotoxic, indicating that MMF could be used preferentially in patients with renal dysfunction[117,118].

Dyslipidemia

Incidence of dyslipidemia exceeds 70% and 40% for patients with and without pre-transplant MS, respectively[119]. Because MS affects a such great proportion of OLT patients[7], methods of preventing or reducing dyslipidemia could benefit a preponderance of patients.

In an evaluation of the metabolic impact of OLT, Luzi *et al*[120] (1996) reported that liver transplant recipients had abnormal FFA levels at 5 mo post-OLT. A follow-up at 26 mo found reduction in abnormal lipid and protein metabolism - in fact, plasma free fatty acids were reduced for transplant recipients with respect to the control group.

CNI therapy is associated with dyslipidemia post-OLT; but the level of dyslipidemia might be reduced upon use of mTOR inhibitors in combination with TAC[121]. Saliba *et al*[104] (2013) report that hyperlipidemia was more frequent in patients on EVR + reduced dose TAC compared to patients on only full dose TAC. In spite of an increase in dyslipidemia, mTOR inhibitors do seem to decrease arteriovascular plaque formation[122].

Orlando *et al*[115] (2007) found that CNI withdrawal and subsequent replacement with MMF improved dyslipidemia. Out of 41 patients, blood cholesterol decreased in 76% and blood TG decreased in 89%. Further supporting the use of MMF in patients who are at-risk for complications of atherosclerosis, Romero *et al*[123] (2000) also reported that MMF specifically reduces atherosclerosis in rabbits.

For patients with hyperlipidemia who are resistant to lifestyle changes, hydroxymethylglutaryl-CoA reductase inhibitors (statins) should be considered. Even with the potential for hepatotoxicity, the use of statins to counter immunosuppressive side effects can benefit patients[124]. Martin *et al*[125] (2007), in a retrospective review of 69 liver transplant patients, explain that there is a general tolerability and low incidence of adverse events in patients treated with lipid-lowering agents. Indeed, they report that there is no change in liver function tests.

Diabetes

Post-transplant diabetes mellitus (PTDM) is a well-recognized issue, and minimization of immunosuppression is currently the best treatment option for PTDM patients. The diabetogenic properties of immunosuppressive therapies seem to be intimately related to the signaling processes that are shared between pancreatic islets and leukocytes. Moreover, in contrast to the physiological proliferative signaling mechanisms used by white blood cells, renal calcineurin mediates glomerular hypertrophy and extracellular matrix accumulation[126].

Immunosuppressive regimens play a major role in new-onset diabetes, affecting patient and graft survival[127]. Rostambeigi *et al*[128] (2011) explain that beta cells exposed to TAC and CsA decreased insulin secretion and reduced mitochondrial density without affecting apoptosis rates, and posit that maybe there is a mitochondria-mediated dysfunction imposed by CNIs. Notably, the tacrolimus-exposed beta cells fared marginally better than their CsA counterparts. This is a reflection of the diabetogenic properties of TAC compared to CsA. On the other hand, some research finds no major metabolic differences between TAC and CsA post-OLT low-dose maintenance therapies[129,130].

HCV: FROM INVARIABLE TO INCONSEQUENTIAL

HCV recurrence after liver transplantation is nearly universal, immediate, and has an accelerated natural history[131]. However, the discovery of directly acting antiviral (DAA) protease inhibitors has dramatically reduced the impact of HCV. With SVR rates exceeding 90%[132], high safety[133], and a well-tolerated side-effects profile[134], Hepatitis C treatment will hopefully become a non-issue.

Still, there are OLT patients who experience HCV recurrence, and these patients deserve special consideration. Notably, in contrast to patients who do not have HCV, an episode of early ACR in post-OLT HCV patients is associated with a higher risk for mortality[135]. Despite the emphatic importance of treating ACR in this population, there has been a lack of consensus on the impact of using steroids - the front line of ACR treatment - in post-OLT HCV patients[136,137].

At the Cleveland Clinic Foundation, ACR treatment protocol first considers RAI of the HCV-positive post-OLT patient. For patients with RAI less than six, there is an increase in CNI dose and further monitoring for rejection before a bolus of steroids is administered. In contrast, patients who have HCV with a RAI greater than or equal to six are treated the same as patients who do not have HCV: 1 g of methylprednisone is administered daily for 3 d followed by steroid taper. Antibody therapy is used for steroid-resistant rejection.

Besides treatment of ACR, maintenance therapy for HCV-positive post-OLT patients can be nuanced. For example, use of the monoclonal antibody OKT3 is associated with early and severe post-OLT HCV recurrence, and must be approached with caution[138]. On the other hand, treatment with MMF and a 24-mo CNI taper appears to benefit liver function tests and presentation on histology for the hepatitis C patient[139].

With the introduction of DAAs, however, focus has shifted from accommodating immunosuppression to early HCV treatment for potential liver transplant recipients. Achieving SVR before the time of transplantation is ideal, and helps reduce risk of HCV recurrence post-OLT[140-142]. Still, the efficacy of recently-developed protease inhibitors must be evaluated specifically for post-OLT patients[143].

CONCLUSION

While the landscape of immunosuppressive medications

remains steadfast, temperamental clinical weather demands that clinicians stay up to date on best practices. The good news is that HCV is nearly subdued as a post-transplant complication, increasing graft survival, perhaps even decreasing allocation of organs to retransplantation for HCV. The bad news is that MS is increasingly harming patients in ways that are exacerbated by immunosuppression - an issue in sore need of revolution like that in HCV treatment. Finally, given the confluence of MS and immunosuppressive side effects, treatment of early ACR could be excessive and must be reevaluated in light of today's average patient.

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REVIEW

Innate immunity and hepatocarcinoma: Can toll-like receptors open the door to oncogenesis?

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Abstract

Hepatocarcinoma (HCC) is a highly prevalent cancer worldwide and its inflammatory background was established long ago. Recent studies have shown that innate immunity is closely related to the HCC carcinogenesis. An effective innate immunity response relies on the toll-like receptors (TLR) found in several different liver cells which, through different ligands and many signaling pathways can elicit, not only a pro-inflammatory but also an oncogenic or anti-oncogenic response. Our aim was to study the role of TLRs in the liver oncogenesis and as a consequence their value as potential therapeutic targets. We performed a systematic review of PubMed searching for original articles studying the relationship between HCC and TLRs until March 2015. TLR2 appears to be a fundamental stress-sensor as its absence reveals an augmented tendency to accumulate DNA-damages and to cell survival. However, pathways are still not fully understood as TLR2 up-regulation was also associated to enhanced tumorigenesis. TLR3 has a well-known protective role influencing crucial processes like angiogenesis, cell growth or proliferation. TLR4 works as an interesting epithelial-mesenchymal transition's inducer and a promoter of cell survival probably inducing HCC carcinogenesis even though an anti-cancer role has already been observed. TLR9's influence on carcinogenesis is also controversial and despite a potential anti-cancer capacity, a pro-tumorigenic role is more likely. Genetic polymorphisms in some TLRs have been found and its influence on the risk of HCC has been reported. As therapeutic targets, TLRs are already in use and have a great potential. In conclusion, TLRs have been shown to be an interesting influence on the HCC's micro-environment, with TLR3 clearly determining an anti-tumour influence. TLR4 and TLR9 are considered to have a positive relationship with tumour development even though, in each of them anti-tumorigenic signals have

been described. TLR2 presents a more ambiguous role, possibly depending on the stage of the inflammation-HCC axis.

Key words: Hepatocarcinoma; Carcinogenesis; Toll-like receptor; Innate immunity; Chronic inflammation

Core tip: The importance of hepatocarcinoma (HCC) is undeniable in the current medical perspective. However, a lot still remains to be understood in this context. Therefore, this review aims to present the significance of innate immunity in HCC through toll-like receptors as they have already shown interesting effects on tumour's microenvironment, influencing its progression or regression. As a result we also render some therapeutic usages of the established knowledge in this area.

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INTRODUCTION

Liver cancer is one of the most common cancers worldwide, with hepatocarcinoma (HCC) being, by far, the most frequent type[1,2].

Due to its close contact with gut, *via* portal vein, liver faces a continuous exposure to gut-derived bacterial products, toxics and many other agents[3]. In the presence of such pathogens or irritants and associated molecules our body is able to respond in a manner that aims to prevent injury and combat infection. This protection system is called inflammation which, despite its tremendous defensive and antiviral/antibacterial importance in the short term, starts to become deleterious when prolonged or exaggerated - chronic inflammation - possibly leading to fibrosis, cirrhosis and, ultimately, HCC[4].

Therefore, the idea that hepatic carcinogenesis arouses from an inflammatory basis is not new. Several studies already focused on the development of HCC and possibilities like the c-Myc elevation or the deregulated SRY and SGF29 pathways have been proposed[5]. However, just in the last few years we have become aware of the critical role of innate immunity in chronic liver diseases, including HCC[6,7].

Toll-like receptors (TLRs) are a family of pattern-recognition receptors (PRRs) that can be activated by either pathogen-associated molecular patterns (PAMPs) or danger/damage-associated molecular patterns (DAMPs), with their own importance in eliciting innate immunity, regulation of inflammation and tissue regeneration. To date, 11 human TLRs have been identified[8]. In recent years, activation of several TLRs have been associated with viral hepatitis, steatohepatitis (alcoholic or non-alcoholic) and to the progression of the inflammation-fibrosis-HCC axis[9-11]. However, data is somewhat contradictory and no clear conclusions have been made.

In this line of thoughts, this review aims to present an overview of the expression of TLRs in the liver, its influence on the development of liver carcinogenesis as chronic inflammatory inducers or potential oncogenes as well as possible therapeutic targets.

RESEARCH

Specific criteria were defined in order to guide this systematic review. Firstly, a query to obtain the articles related to the theme on PubMed was built: [(Hepatocarcinoma) OR (Hepatocarcinogenesis) OR (hepatic cancer) OR (hepatocellular carcinoma) OR (liver cancer)] AND [(toll like receptors) OR (toll like receptor)]. With this query we intended to embrace a wide range of articles until March 2015, which then would be carefully selected.

A total amount of 277 articles were obtained through the referred search. After discarding the duplicates and adding 28 articles obtained through cross-referencing, 305 articles were available to be screened. The following inclusion criteria were used: (1) studies that were published until the end March 2015; (2) the article should be written in English; and (3) studies relevant to the theme (presenting original data). As exclusion criteria we defined: (1) studies considered by the authors as unrelated to the theme; and (2) non-original studies. These criteria were applied by reading the title and abstract resulting in 227 articles excluded. After this step, the remaining 78 studies were selected for full-text reading. On a second level of eligibility, 18 more studies were excluded and 60 studies were selected, analysed and included in this revision (Figure 1).

Data about the defined topics were obtained from each article (Table 1) and the information was then summed up and organized in the present systematic review according to: The TLRs' expression in each liver cell; separately role of TLR2, TLR3, TLR4 and TLR9 in inflammatory-driven hepatocarcinoma; known TLRs' polymorphisms/genetic variations that influence the risk of hepatocarcinoma and lastly, TLRs modulators possibly used in hepatocarcinoma's therapeutics.

TLR EXPRESSION IN HEPATIC CELL POPULATION

The liver is a very special organ when it comes to dealing with pathogens. Due to its vascular links, contact with gut-derived bacteria is constant and, therefore, mechanisms not only to defend the organism from these pathogens but also to tolerate them, had to be

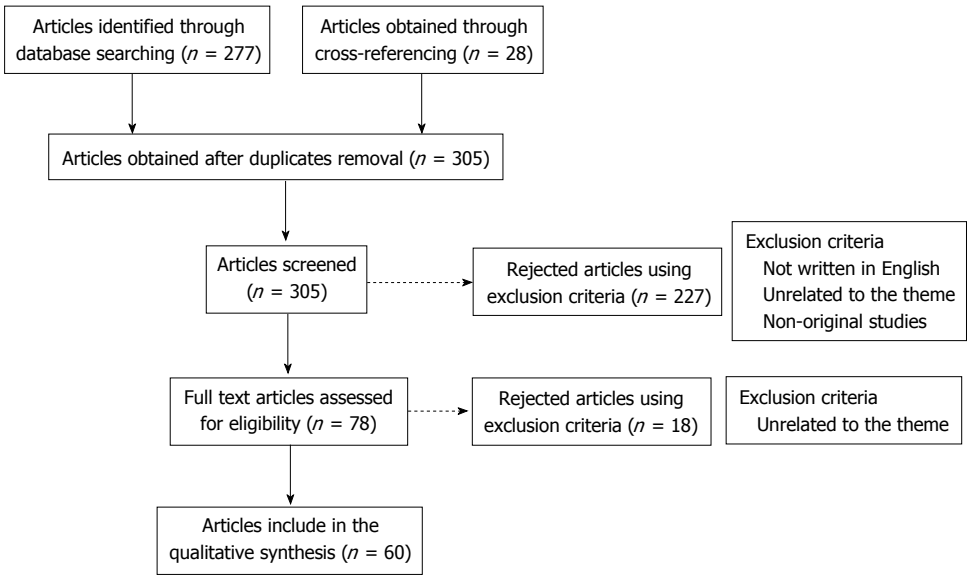

Figure 1 Methods' flowchart. A total amount of 277 articles were obtained, on PubMed, through the query [(hepatocarcinoma) OR (hepatocarcinogenesis) OR (hepatic cancer) OR (hepatocellular carcinoma) OR (liver cancer)] AND [(toll like receptors) OR (toll like receptor)]. After discarding the duplicates and adding 28 articles obtained through cross-referencing, 305 articles were available to be screened. The following inclusion criteria were used: (1) studies that were published until the end March 2015; (2) the article should be written in English; and (3) studies relevant to the theme (presenting original data). As exclusion criteria we defined: (1) studies considered by the authors as unrelated to the theme; and (2) non-original studies. These criteria were applied by reading the title and abstract resulting in 227 articles excluded. After this step, the remaining 78 studies were selected for full-text reading. On a second level of eligibility, 18 more studies were excluded and 60 studies were selected, analysed and included in this revision.

developed[8]. In this duality, TLRs play an interesting role as it is known that, in a healthy liver, mRNA levels of TLRs like TLR1, 2, 4, 6, 7, 8, 9 and 10 are decreased when compared to other organs[9].

In the liver, hepatocytes represent 60%-80% of the total cell population. Here, it can be found mRNA from all TLRs; however, only a response from TLR2 and TLR4, to their ligands, can be obtained[12]. Interestingly, only the response of TLR2 is up-regulated under inflammatory conditions[13].

Besides hepatocytes, it is possible to find, in the liver, non-parenchymal cells which consist of Kupffer cells (KCs), dendritic cells (DCs), Lymphocytes, hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs).

KCs not only express lipopolysaccharide (LPS)-responsive TLR4 but also TLR2, TLR3 and TLR9 that respond to their ligands[14]. These cells develop an inflammatory response to high levels of LPS but produce an anti-inflammatory cytokine (IL-10) in response to continuous low levels of LPS, known as LPS tolerance[15]. DCs represent a small population (< 1%). In humans, the plasmocytoid DCs subset expresses TLR1, TLR7 and TLR9 while other subsets carry all TLRs with the exception of TLR9[16]. When it comes to Lymphocytes population and TLRs relationship it is important to notice that differences can be found from one subpopulation to another. Natural killer (NK) cells contain TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR8 and TLR9[1,17] but T cells are only activated through TLR2 while B cells rely on TLR2, TLR4, TLR7 and TLR9[18]. HSCs, also in small proportion (< 1%), when activated are able to express TLR4 responsive to LPS which, in turn, enables inflammatory cytokines' secretion[19]. LSECs express mRNA from TLR1 to TLR9 despite not being able to respond to TLR5 ligands[20].

IMPORTANCE OF TLRS IN INFLAMMATORY-INDUCED HCC

HCC has long been considered a chronic-inflammation driven cancer independently of the possible risk factors; virus induced hepatitis, smoke, alcohol or metabolic diseases. Despite that, the liver is an organ with several mechanisms readily available to defend against carcinogenesis. Among these, we found PRRs, with special attention to TLRs which were already shown to exhibit different roles in the regulation of tumorigenesis and tumour progression. To date several works have documented its influence not only in specific types of cancer - breast, ovarian, prostate and lung - but also in processes directly linked with cancer - resistance to apoptosis, increased invasiveness and metastasis. This is the reflection of their actions on metalloproteases and integrins, tumour cell immune escape, among others[21-23]. However, the cellular and molecular effectors mediating the interplay between TLRs and HCC are still largely unknown.

TLR2

When the TLR2 signal is triggered, the downstream cascade initiates through a "Myd88 dependent pathway" with the activation of the apoptosis signal regulating kinase 1 (ASK1)/p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor kappa B (NF-κB), or through

Table 1 Table of original studies

Ref.	Year	Type of study	Methods	Limitations	Conclusions
Chew *et al*[1]	2012 December	Experimental	Natural killer cell activation and cytotoxicity were assessed *in vitro* after treatment with the TLR3 ligand poly (I:C). The effect of TLR in a spontaneous liver tumor mouse model and a transplanted tumor mouse model were determined by Immunohistochemistry and PCR	The effect of poly (I:C) on tumor growth was only analyzed in a transplanted, nonorthotopic model of HCC. The effect of poly (I:C) on human NK cells was assessed only with cells from healthy donors. Not all HCC cell lines undergo apoptosis after TLR3 triggering and the reason is not known	TLR3 is an important modulator of HCC progression and is a potential target for novel immunotherapy
Mohamed *et al*[2]	2015 March	Experimental	Tissue microarrays containing liver samples from patients with cirrhosis, viral hepatitis and HCC were examined for expression of TLR7 and TLR9. Proliferation of human HCC cell lines was studied following stimulation of TLR7 and TLR9 using agonists (imiquimod and CpG-ODN respectively) and inhibition with a specific antagonist (IRS-954) or chloroquine. The effect of these interventions was confirmed in a xenograft model and DEN/NMOR-induced model of HCC	Before translation to the clinical arena, it is important to further characterize the exact mechanisms through which TLR7 and TLR9 exert their actions and determine what effects their inhibition may have on the immune system	Inhibiting TLR7 and TLR9 with IRS-954 or chloroquine could potentially be used as a novel therapeutic approach for preventing HCC development and/or progression in susceptible patients
Dapito *et al*[3]	2012 April	Experimental	TLR2-deficient mice, TLR4-deficient mice, TNFR1-/IL-1R1-double deficient and C57Bl/6 mice were used. HCC was induced by intraperitoneal injection of DEN. Gut-sterilization was done using a combination of ampicillin (1 g/L), neomycin (1 g/L), metronidazole (1 g/L) and vancomycin (500 mg/L) in drinking water. Samples from patients with features of alcoholic hepatitis were used. Liver biopsies were obtained from mice and from cadaveric donners or resection of liver metastases	Clinically feasible methods of targeting the intestinal microbiota or TLR4 need to be established. The quadruple combination of antibiotics employed is not suitable for long-term treatment due to known side effects in patients with advanced liver disease	Gut sterilization restricted to late stages of hepatocarcinogenesis reduced HCC, suggesting that the intestinal microbiota and TLR4 represent therapeutic targets for HCC prevention in advanced liver disease
Eiró *et al*[8]	2014 July	Experimental	The expression levels of TLR3, TLR4 and TLR9 were analyzed from 30 patients with HCC and correlated with various clinicopathological findings and with overall survival	In the scoring system, after immunostaining analysis, when setting of the threshold for positive staining and the determination of the intensity different observers can set different thresholds and intensity levels	An association between TLR3, TLR4 and TLR9 expression and tumor aggressiveness and poor prognosis in HCC has been observed
Liu *et al*[12]	2002 July	Experimental	Cultures of primary mouse hepatocytes were incubated with LPS to assess its effects on the global gene expression, hepatic transcription factors, and MAP kinase activation	Using hepatocytes' cell lines loses the capacity to observe the importance of a direct response to LPS by hepatocytes	NF-κB activation was reduced in TLR4-mutant or - null hepatocytes compared to control hepatocytes
Matsumura *et al*[13]	2000 October	Experimental	PCR analysis of mice's hepatocytes and an murine hepatoma cell line Hepa 1-6	Murine hepatoma cell line Hepa 1-6 may have reached an overquantitative level after stimulation	LPS and proin-flammatory cytokines differentially regulate gene expression of TLR2 and TLR4 in murine hepatocytes, which may lead to pathologic and host defense reactions in the liver
Thobe *et al*[14]	2007 March	Experimental	Wester blotting and cytokine analysis in a cell culture. Evaluation of Kupfer cells response after a trauma-hemorrage procedure	Does not explain if the increase in MAPK-activity is due to TLRs' overexpression	Kupffer cell TLR signaling employs different MAPK pathways in eliciting cytokine and chemokine responses following trauma-hemorrhage

Knolle *et al*[15]	1995 February	Experimental	Human Kupffer cells were isolated by collagenase perfusion followed by centrifugal elutriation and analyzed for cytokine secretion after 3 d in culture	Only IL-10 and IL-6 were analysed	The important role for IL-10 in the regulation of the local immune response in the liver sinusoid after Kupffer cells exposure to to lipopolysaccharide
Edwards *et al*[16]	2003 April	Experimental	Splenocyte reparations were enriched for $D11c^+$ and for $Ly6C^+$ cells using magnetic selection. Four populations were routinely isolated and TLR's mRNA was amplified by PCR	To analyze the functional significance of TLR mRNA expression in DCs subsets it was only used ligands for TLR7 and TLR9	mRNA for most TLRs is expressed at similar levels by murine splenic DC sub-types. TLR expression between plasmacytoid and non-plasmacytoid DC is not conserved between species
Sawaki *et al*[17]	2007 March	Experimental	Total RNA was extracted, and mRNA for TLR1, 2, 3, 4, 5, 6, 7, 9 and b-actin was determined by reverse transcription-PCR. Nuclear localization of NF-κB was determined and cytokines and chemokines were measured by a commercially available kit	It was not evaluated precise roles of NK cell responses *in vivo*	Upon microbial infection, macrophages produce IL-12 that renders NK cells highly responsive to TLR agonists to produce IFN-γ and chemokines, which might in turn recruit and fully activate macrophages
Meyer-Bahlburg *et al*[18]	2007 December	Experimental	It was compared the TLR response profile of germinal center after immunization *vs* naive mature B cell subsets, using real time PCR, ELISA and Western Blotting to evaluate MyD88 pathway	TLRs' role in B-cells immune response was only accessed in splenic B cells from MyD88 WT, Het, or KO, being studied only the MyD88-dependent pathway	B cell-intrinsic TLR signals are not required for antibody production or maintenance
Paik *et al*[19]	2003 May	Experimental	LPS-associated signalling molecules in culture-activated HSCs and HSCs isolated from patients with hepatitis C virus-induced cirrhosis was evaluated by NF-κB-dependent luciferase reporter gene assays, electrophoretic mobility shift assays and *in vitro* kinase assays	It does not fully explain why only full activated HSCs respond to LPS. It was not evaluated the activation of TLR4 downstream molecules like MyD88	Human activated HSCs utilize components of TLR4 signal transduction cascade to stimulate NF-κB and JNK and up-regulate chemokines and adhesion molecules
Wu *et al*[20]	2010 March	Experimental	Isolated Kupffer cell and liver sinusoidal endothelial cells from wild-type C57BL/6 mice and examined their responses to TLR1 to TLR9 agonists. Characterization of cell surface protein expression was done by flow cytometry and quantification of mRNA was done by reverse transcription-polymerase chain reaction	The *in vitro* assay does not explore the organ-specific regulation of immune responses. For the identification of TLR-induced antiviral cytokine(s) only TLR3 and TLR4 were used	Non-parenchymal cells display a restricted TLR-mediated activation profile when compared with "classical" antigen-presenting cells which may, at least in part, explain their tolerogenic function in the liver
Huang *et al*[21]	2012 July	Experimental	TLR expression in BLE-7402 cells was assayed by RT-PCR, real-time PCR and FCM. To investigate the function of TLR2 in hepatocarcinoma growth, BLE-7402 cells were transfected with recombinant plasmids expressing one TLR2 siRNA	Only the effect on tumour volume is evaluated after tumour implantation in nude mice	TLR2 knockdown inhibit proliferation of cultured hepatocarcinoma cells and decrease the secretion of cytokines
Kim *et al*[22]	2009 January	Experimental	LLC cells were implanted in mice. Metastasis enhancing factors were identified on a QSTAR XL qQTOF mass spectrometer. Gene and protein expression were monitored by Q-PCR and immunoblot analysis. Tumors were analyzed by immunohistochemistry and indirect immunofluorescence	It does not explain if the interaction between versican and TLR2 is direct or depends on a versican's ligand	By activating TLR2:TLR6 complexes and inducing TNF-α secretion by myeloid cells, versican strongly enhances lewis lung carcinoma metastatic growth
Lin *et al*[24]	2013 January	Experimental	A DEN injection was done in $TLR2^{-/-}$ and WT mice. Than they were sham-treated or treated with interferon-gamma. TUNEL, heterochromatin and SA b-gal staining were performed	The mechanism by which TLR2 signaling participates in the regulation of cellular senescence to maintain growth arrest and promote programmed cell death remains inconclusive	Loss of immune networks may play a role in the failure of initiating and maintaining cellular senescence and autophagy flux in the TLR2-mutant liver tissue

Lin *et al*[25]	2013 October	Experimental	WT mice were pre-treated with anti-TLR2 antibody and a subset of $TLR2^{-/-}$ mice were pre-treatment with NAC (antioxidant) or physiological saline. Both were submitted to DEN. Histology was submitted to western blotting, ROS assay, immunohistochemistry and immunofluorescence	It does not report any results about the effects on non-parenchymal cells like Kuppfer cells. It does not reveal interactions that regulate the signal from TLR2 activation to suppression of oxidant and ER stressors in HCC	A TLR2 activity defends against hepatocarcinogenesis through diminishing the accumulation of ROS and alleviating ER stress and unfold protein response
Li *et al*[26]	2015 March	Experimental	WT and $Tlr2^{-/-}$ mice were used. Flow cytometry, Histopathological analysis and Immunofluorescence, Western blot and ELISA were performed. MDSC induction *in vitro* and functional T cell suppression assay and knockdown of IL-18 and caspase-8 in hepatocytes with quantitative PCR were also done	The exact role of IL-18 in MDSC generation is still unknown. It does not reveal the levels of TLR2 that determine the possible use of IL-18 as a therapeutic target	TLR2 deficiency accelerates IL-18-mediated immunosuppression during liver carcinogenesis, providing new insights into immune control that may assist the design of effective immunotherapies to treat HCC
Soares *et al*[27]	2012 October	Analytic - cross sectional	It was used samples from patients with hepatitis, cirrhosis and hepatocarcinoma. mRNA isolation and quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2 were performed. Immunohistochemical evaluation of TLR2 and TLR4 was also done	Most patients included in the reference group have evidence of NAFLD and it was demonstrated that NAFLD is associated with increased hepatic TLR2 and TLR4-mRNA expression. the hepatitis, cirrhosis and hepatocarcinoma groups included both patients with HBV infection or HCV infection. Included only patients with virus-induced chronic hepatitis. The method used for quantification of protein expression was semi-quantitative	Increased expression of TLR2 and TLR4 in hepatitis and cirrhosis and maintained expression in hepatocarcinoma. Up-regulation of TLR2, TLR4 and their pro-inflammatory mediators is associated with virus-induced hepatic IFC sequence
Dolado *et al*[31]	2007 February	Experimental	WT and $p38a^{-/-}$ were used. Growth in soft agar was evaluated. Intracellular ROS levels were determined, immunoblot Analysis was performed. To induce p38 MAPK activation, cells were treated with H_2O_2, sorbitol and cisplatin	The tumorigenesis enhanced by ROS is not evaluated on hepatocarcinoma	Oxidative stress sensing plays a key role in the inhibition of tumor initiation by p38alpha
Kang *et al*[32]	2011 November	Experimental	For transposon-mediated intra-hepatic gene transfer mice received a transposon- to transposase encoding vector (30 mg total DNA). DNA was administered by hydrodynamic tail vein injection. Immunohistochemical analyses were performed	It was not investigated if factors secreted from pre-malignant senescent hepatocytes also contribute to the oncogenic transformation of neighbouring cells	Indicates that senescence surveillance represents an important extrinsic component of the senescence anti-tumour barrier, and illustrates how the cellular senescence program is involved in tumour immune surveillance
Ogata *et al*[34]	2006 December	Experimental	Electron microscopic analysis was performed using neuroblastoma SK-N-SH cells exposed to ER stressors. GFP-LC3 fluorescence was used to monitor autophagy in cells transiently transfected with an expression vector for GFP-LC3. Then was performed an Amino acid uptake assay and autophagosome formation was evaluated	A signalling pathway other than the IRE1-JNK pathway may also play important roles in the activation of autophagy signalling after ER stress. The detailed signalling pathway for activation of the autophagy induced by ER stress is still unknown	Disturbance of autophagy rendered cells vulnerable to ER stress, suggesting that autophagy plays important roles in cell survival after ER stress
Pikarsky *et al*[36]	2004 September	Experimental	The possibility that NF-κB activation is involved in Mdr2-knockout hepatocarcinogenesis was investigated by RelA (p65) immunostaining. Hystological analysis was performed. To study the relationship between the	It does not explain how the inflammatory process in Mdr2-knockout mice is maintained in the double mutants as it is independent of hepatocyte NF-κB activity	NF-κB is essential for promoting inflammation-associated cancer, and is therefore a potential target for cancer prevention in chronic inflammatory diseases

			TNF-α-producing cells and NF-κB activation in the hepatocytes, liver sections were stained for both TNF-α and p65		
Gong *et al*[37]	2013 September	Experimental	BALB/c mice were used and inoculated with H22 hepatocarcinoma cells into the hind thigh muscle. They were treated with TLR2/4 ligands, HSP70 and HMGB1. The main tumor nodules were measured and satellite tumor nodes counted. To downregulate HMGB1, RAGE or Beclin-1 in tumor cells, cells were transduced with short interfering RNA	It does not explain the mechanisms responsible by the NF-κB's phosphorylation in the first 30 min. It was observed only one of the pathways responsible for the involvement of HMGB1/RAGE in the NF-κB signaling	Activation of NF-κB was indispensable for the effect of HSP70. HSP70 induced a positive feedback loop involving Beclin-1/HMGB1 production, causing re-phosphorylation of NF-κB
Shi *et al*[38]	2014 October	Experimental	Human hepatocellular carcinoma cell lines were used. Into the cell lines were transfected small-interfeering-RNAs and at 48 h after transfection, the TLR2-siRNA-transfected group, scramble control group, and blank group were treated with recombinant-HMGB1. Evaluation included real time PCR, Western blot, MTT assay, Transwell assay and Flow cytometry assay	It does not explore the signaling pathway that regulates NF-κB through TLR2 inhibition or stimulation with recombinant-HMGB1	TLR2-siRNA could effectively inhibit the growth, migration, invasion, and expression of NF-κB/P65, and HMGB1 promoted HCC progression *via* TLR2
Wu *et al*[39]	2012 April	Experimental	It was used mice and HCC cell lines. Eukaryotic expression vectors psTLR2 and psTLR4 were created. An adhesion assay, a tumor cell proliferation assay, a flow cytometric analysis, an apoptosis analysis, an analysis of gene expression by RT-PCR and a western blot analysis were performed	More than one signaling pathways activated by HSPA1A might be required for the survival of tumor cells. The effect of eHSPA1A was only evaluated in one cell line. Injection of HSPA1A suppressed tumor growth in early stage of tumor development, but promoted tumor growth in later stage	Extracellular HSPA1A functions as endogenous ligand for TLR2 and TLR4 to facilitate tumor growth
Yoneda *et al*[41]	2008 November	Experimental	HCC cell lines and 74 HCC samples were used. Poly (I:C), cycloheximide and actinomycin were included in the study. Profiling analysis of TLRs recognized by viral components, flow cytometric analysis, immunohistochemical staining, Detection of TLR3 by immunofluorescence, Detection of cell viability and apoptosis assays, Detection of apoptosis-related proteins by immunoblotting, NF-κB activity assays and measurement of IFN-β were also performed	Further evaluation of the possible roles and the type of regulation associated with TLR3 needs to be undertaken	Intracellular TLR3 signalling is involved in cell death, while in contrast, the cell surface TLR3 signalling is responsible for activation of NF-κB
Zorde-Khvalevsky *et al*[42]	2009 July	Experimental	It was used TLR3-WT mice and $TLR3^{-/-}$ mice. Partial hepatectomy was done followed by immuonhistochemistry stainings, plasma aminotransferase activity assay, measurements of serum cytokine levels, semi-quantitative reverse-transcription polymerase chain reaction, Western blotting, caspase-8 immunopurification and injection with poly (I:C) or saline solution	It is not explained what happens to the levels of ALT in mice's serum before the 10-h time point following 70% PHx. Cytokine evaluation only includes IL-6 and IL-22	TLR3 plays an inhibitory role in the priming of liver regeneration, thus reinforcing the role of the innate immune system in balancing tissue regeneration
Khvalevsky *et al*[44]	2007 April	Experimental	Various cell lines and plasmids pTLR7, pTLR8, and pTLR9, carrying the respective human *TLR* gene, were used. Transfection	The role of TLR3 signaling in normal hepatocytes requires further investigation *in vivo*. It is not specified the degree of	Preferential induction of the apoptotic pathway over the cytokine induction pathway by TLR3 signaling in

			assays, RNA quantification, immuno-staining and flow cytometry, were performed	NF-κB activation obtained from the overexpression of TLR3 nor the degree of this overexpression that is needed	hepatocellular carcinoma cells with potential implications for therapeutic strategies
Chen *et al*[45]	2012 July	Experimental	The human HCC cell line HepG2.2.15 was used. After treating HepG2.2.15 with BM-06 or poly (I:C), NF-κB activity was checked by dual luciferase reporter gene kit. Then it was performed a nuclear and cytoplasmic extraction, Western blot analysis, a cell proliferation assay, cell invasion assays and flow-cytometry was used to determine the apoptotic rate	The role of TLR3 in the antiviral defense against HBV was not analyzed according to differences in the type of viruses, the type of cells that are infected, the viral load, its model of infection (endoplasmic *vs* cytoplasmic), and stage of infection	BM-06 inhibited the proliferation, invasion and secretion of HBV, and induced apoptosis in HepG2.2.15 cells. In addition, the antitumor effects of BM-06 were superior to poly (I:C)
Guo *et al*[46]	2012 February	Experimental	Cell cultures were used and submitted to BM-06 and poly (I:C) treatment. RNA isolation and one-step quantitative real-time PCR were performed. Analysis included detection of TLR3 by immunocytochemistry, luciferase reporter assays, Endothelial cell tube formation assay, rat aortic ring assay, annexin V/PI for cell apoptotic analysis and Cell migration assays	It does not evaluate the molecular mechanisms after TLR3 stimulation that lead to modulation of endothelial tube-forming activity of HUVECs and vascular sprouting or enhanced apoptosis	TLR3 agonists not only affect tumor microenvironment by suppressing angiogenesis but also directly induce tumor cell apoptosis and inhibit tumor cell migration
Bergé *et al*[47]	2010 December	Experimental	It was injected transgenic mice developing HCC with either control siRNAs or siRNA targeting neuropilin-1. The study used antibodies (goat anti-TLR3 and rabbit anti-tubulin antibody), Western Blotting, and Immunofluorescence Analysis. Real-time RT-PCR, ELISA, MTT assay and three-dimensional collagen assay were also performed	It is not known why INF-γ does not inhibit cells' functions in the *in vitro* study despite the high levels in HCC. *In vivo* evaluation was not performed	Synthetic siRNAs inhibit target-independently HCC growth and angiogenesis through the activation of the innate interferon response and by directly inhibiting endothelial cell function
Xu *et al*[48]	2013 October	Experimental	Thirty rats were used, all 30 were fed with 2-acetylaminofluorene to establish the HCC model. Two animal groups were treated, respectively, with the drug candidate (BM-06) and poly (I:C). It was performed a H and E staining, an Immunohistochemical staining, a Western blot analysis	It does not explore the pathway though which BM-06 and poly (I:C) are capable of inducing cell death. It is not evaluated TLR3's downstream molecules to explain the signalling pathway responsible for these results	Treatment with BM-06, showed a decrease in tumor growth and cell proliferation, and an increase in apoptosis compared with that in a phosphate-buffered saline control group
Wang *et al*[49]	2013 August	Experimental	Fifty-three HCC and ten normal liver specimens were analyzed by immunohistochemistry, and three cell lines were used for *in vitro* studies. Lipopolysaccharide was used to activate TLR4 signaling. Cell survival, proliferation and invasion were examined	Only a specific amount of LPS has shown to have an effect on the mRNA expression of IL-6, EGFR and HB-EGF. Opposing to HL-7702 cell line, PLC/PRF/5, with a moderate level of TLR4 expression, was not affected by inhibiting p38	Indicate that TLR4 signaling in cancer cells promotes cell survival and proliferation in HCC
Liu *et al*[50]	2015 March	Experimental	Two HCC cell lines and a splenic vein metastasis of the nude mouse model were used. A total of 88 clinical samples from HCC patients were used. A fluorescence activated cell sorting system and flow cytometry analysis were performed. Nude mouse splenic vein metastasis assay, immunohistochemistry analysis, real-time quantitative PCR, Western blot analysis, immunofluorescence and cell apoptosis assay were also done	More pathological specimens should be enrolled to verify the tendencies of association between TLR4 expression and malignant characteristics of HCC found in this study. A particular signaling pathway involved in the relationship between TLR4 expression and stem cell features remains elusive	There is a relationship between TLR4 expression and CSC's features, TLR4 may act as a CSC marker, prompting tumor invasion and migration, which contributes to the poor prognosis of HCC

Li *et al*[52]	2014 October	Experimental	A HCC cell line was used where a Scratch assay was performed. Invasion assay, Western blot analysis, quantitative real-time reverse transcription PCR and siRNA knockdown of *TLR4* gene expression were also done	It does not reveal the time needed for induction of epithelial-mesenchymal transition after LPS stimulus. Does not explore influence of LPS on TLR2	TLR4/JNK/MAPK signaling is required for LPS-induced EMT, tumor cell invasion and metastasis, which provide molecular insights for LPS-related pathogenesis and a basis for developing new strategies against metastasis in HCC
Jing *et al*[53]	2012 August	Experimental	Four HCC cell lines and a splenic vein metastasis of the nude mouse model were used and stable TLR4-expressed and knocked-down cell lines were generated. 106 clinical samples from HCC patients were also used. Quantitative real-time PCR, Western-blot analysis, Immunofluorescence, FACS Analysis and IHC analysis were performed	HCC development is a multifactorial and complicated process, which has a close association with various risk factors. Many gene alterations and cytokines also could induce EMT. HCC cells with low expression or even a lack of TLR4 are not susceptible to LPS, they might perform EMT induced by other TLR4-independent mechanisms	TLR4 signaling is required for LPS-induced EMT, tumor cell invasion and metastasis, which provide molecular insights for LPS-related pathogenesis and a basis for developing new strategies against metastasis in HCC
Xu *et al*[54]	2014 October	Experimental	HCC and adjacent tissues were obtained from 84 patients. HCC cell lines were used and a PLV-PTPRO-GFP plasmid was constructed. Real-time PCR, immunofluorescence, Western blot analysis and cell proliferation assay were performed	It does not specify the doses of NF-κB specific inhibitor needed to result in a decreasing of PTPRO's levels in HuH7 cells stimulated with LPS	The effect of PTPRO on TLR4 signaling is dependent on NF-κB pathway, suggesting an interesting PTPRO/TLR4/NF-κB signaling feedback loop in HCC carcinogenesis and progression
Wang *et al*[55]	2015 January	Experimental	It was used LPS-induced human hepatocellular carcinoma cell lines. Cell viability was assessed using the MTT assay. Double staining for annexin V-FITC and propidium iodide was performed. Inflammatory mediators were evaluated through a specific ELISA kit. Immunoprecipitation and Western blot analysis were also used	Only one type of cell line is used to observe the effect of CXC-195. It does not reveal the level (high or low) of TLR4 expression. It does not explore the influence of LPS in TLR2	Treatment with CXC195 could attenuate the TLR4-mediated proliferation and inflammatory response in LPS-induced HepG2 cells
Yu *et al*[56]	2010 October	Experimental	Rats and mice were used, including TLR4-deficient mice. Immunohistochemical analysis and bone marrow transplantation were performed	It does not explore the effect of modulating gut flora. It does not evaluate the effect of different LPS' levels	Sustained LPS accumulation represents a pathological mediator of inflammation-associated HCC and manipulation of the gut flora to prevent pathogenic bacterial translocation
Lin *et al*[58]	2012 September	Experimental	It was used wild-type and TLR4-deficient mice. A flow cytometry analysis and Isolation and Culture of $CD4^+$ cells were performed	TLR4 knockout showed decreased liver injury induced by Con A, contrarily to what was expected. It is needed to determine whether the regimen with antiendotoxin effects will prove beneficial in preventing or delaying T cell-mediated hepatitis and hepatitis-induced HCC	Gut-derived LPS and TLR4 play important positive roles in Con A-induced hepatitis and modulation of the gut microbiota may represent a new avenue for therapeutic intervention
Chen *et al*[60]	2013 July	Experimental	It used HCV Tg mouse models and patients with HCC functional cDNA. Then, functional cDNA screening for oncogenes was performed. *In vitro* and *in vivo* oncogenic activities were evaluated. It was also done a liver TIC engraftment *via* splenic injection	The degree of attenuation of TLR4 expression in TICs by Nanog, implying a feedback loop is not shown. Besides this, the underlying mechanisms are not known	TLR4/NANOG oncogenic pathway is linked to suppression of cytostatic TGF-β signaling and could potentially serve as a therapeutic target for HCV-related HCC
French *et al*[63]	2013 August	Experimental	Liver biopsies from patients diagnosed with alcoholic hepatitis, with or without cirrhosis were selected. Double Immunohistochemistry was performed	The antibody stain was only against TLR4	The Mallory-Denk-bodies forming cells expressed two additional progenitor cell markers. These markers were CD49f and TLR4

Machida *et al*[64]	2014 November	Experimental	An immunostaining of liver tumor sections from alcohol-fed Ns5a mice was performed along with TLR4 silencing with lentiviral short-hairpin RNA	LPS-independent mechanisms of TLR4 activation in TICs remain to be elucidated. The oncogenic role of TLR4 is explored only around the synergism alcohol-HCV	TLR4-dependent mechanisms of TIC generation actually contribute to or at least promote the initiation of HCC
Yan *et al*[65]	2012 June	Experimental	Human HCC liver samples and mice were used. Stable HMGB1-expressing cells and HMGB1 knockdown cells were established. immunoblotting analysis, RNA Interference by short interfering RNA, enzyme-linked immunosorbent assay, confocal microscopy exam, caspase-1 colorimetric assay, cell migration and invasion assays and metastatic potential exam were all performed	Mechanisms by which caspase-1 affects tumor cancer progression remain incompletely understood	In hypoxic HCC cells, HMGB1 activates TLR4- and RAGE-signalling pathways to induce caspase-1 activation which, in turn, promote cancer invasion and metastasis
Xu *et al*[67]	2008 February	Analytic - cross sectional	52 patients were studied. The protein and mRNA levels of TLR7 and TLR9 were evaluated using real-time PCR, Western blot analysis, and flow cytometry. We also detected the serum viral load of HBV in the patients and analyzed the correlation between HBV-DNA copies and the TLR expression	The statistical analysis indicated no difference in the TLR9 levels among the HCC and LC groups. If the sample size was enlarged, the results may be different. The expression of TLR7 was not different among the groups of patients, suggesting that TLR7 has no correlation with HCC	There are downregulations of TLR7 expression and TLR9 mRNA in PBMC of HBV-infected patients, but an increased TLR9 expression at the protein level
Tanaka *et al*[68]	2010 October	Experimental	HCC cell lines and 42 HCC tissues were used. The type C CpG oligonucleotide was used as TLR9 ligand. Flow cytometric analysis, Immunohistochemical staining, Cell proliferation assay, Immunoblotting, NF-κB activity assays and expression analysis of IRF-7, RNA extraction and oligonucleotide microarray and Microarray data analysis were all performed	Despite being present both intracellular or extracellular TLR9's intracellular function is not observed with TLR9 ligands and its function is not known	Functional cell surface expression of TLR9 in human HCC may play an important role in tumorigenesis and cancer progression
Liu *et al*[69]	2015 February	Experimental	C57BL6 mice were injected with Hepa1-6 cancer cells. TLR9 and HMGB1 were inhibited using shRNA or direct antagonists. HuH7 and Hepa1-6 cancer cells were investigated *in vitro* to determine how the interaction of HMGB1 and mtDNA activates TLR9 signaling pathways	The contribution of TLR9 to cancer pathophysiology remains incompletely understood. The regulation of TLR9 signaling and the physiological ligands which may induce TLR9 mediated tumor growth remain poorly characterized	Reveals a novel mechanism by which the interactions of HMGB1 and mtDNA activate TLR9 signaling during hypoxia to induce tumor growth
Zhang *et al*[70]	2014 December	Experimental	It was used HCC cell lines to where was transfected CpG oligodeoxynucleotide and poly (I:C). Proliferation analyses, Detection of apoptosis with an Apoptosis Detection Kit, quantitative real-time PCR analysis, Western blot analysis and Fluorescence microscopy were also performed	The precise molecular interactions that likely occur between CpG ODNs and poly (I:C) to block poly (I:C) entry, remain to be established. Poly (I:C) may be influenced by many molecules in the microenvironment	When combining poly (I:C) and CpG ODN for cancer therapy, these agents should be used in an alternating rather than simultaneous manner to avoid the blocking effect of phosphorothioate-modified TLR9 ligands
Zhang *et al*[71]	2014 April	Experimental	Human hepatoma cell lines were used. Cells were transfected with CpG ODNs or small interfering RNAs targeting TLR9. Reverse transcriptase polymerase chain reaction assay, Proliferation measurements, cell cycle analysis, detection of apoptosis, quantitative real-time PCR analysis, Western blot analysis were all performed. An *in vivo* study was also done	Apoptosis induced by ODN M362 Ctrl and ODN M362 occurred independently of TLR9 stimulation. TLR9- and MyD88-independent mechanisms in ODN-stimulated immune cells, including B lymphocytes and neutrophils may exist	Phosphorothioate-modified TLR9 agonist ODN M362, and its control, elicit antitumor activity in HCC cells and may serve as a novel therapeutic target for HCC therapy

Bubici *et al*[74]	2004 December	Perspective			Induction of FHC and Mn-SOD represents an additional, indirect means by which NF-κB controls proapoptotic JNK signaling
Liu *et al*[75]	2009 April	Experimental	Cell cultures were used. Immunocytochemistry stain for TLR9, a Cell proliferation assay, reverse transcriptase PCR for TLR9 and real-time reverse transcriptase PCR for DNMT-1 and Bcl-2, NF-κB activation measurement and Cellular apoptosis analysis were all performed	L-02 cells were used to allow *in vitro* studies but cells may behave differently *in vivo*. Future *in vivo* models are needed	Identified a possible novel mechanism that indicates how CpG DNA of HBV DNA may contribute to the malignant transformation of benign liver cells
Nischalke *et al*[76]	2012 March	Analytic - cross sectional	A total of 197 patients with HCV-associated HCC, 192 HCV-infected patients without HCC and 347 healthy controls were included. HCV antibodies were detected for diagnosis. Determination of TLR2-196 to -174 del/ins polymorphism was performed by LightCycler real-time PCR. *In vitro* induction of TLR2 expression and IL-8 was performed	Analysis of the functional role of TLR2-196 to -174 del/ins alleles with respect to TLR2 expression was based on *in vitro* stimulation studies but it is not known if an *in vivo* analysis would have the same results	TLR2-196 to -174 del allele to affect HCV viral loads and to increase the risk for HCC in HCV genotype 1-infected patients
Junjie *et al*[77]	2012 February	Single center-based case-control	SNaPshot method was used to genotype sequence variants of TLR2 and TLR9 in 211 patients with HCC and 232 subjects as controls	Despite the SNP rs3804099 and rs3804100 were out of HWE (P = 0.01-0.02), they were retained in the analyses	TLR2 rs3804099 C/T and rs3804100 C/T polymorphisms were closely associated with HCC. In addition, the haplotypes composed of these two TLR2 synonymous SNPs have stronger effects on the susceptibility of HCC
Jiang *et al*[79]	2014 December	Single center-based case-control study	426 HCC subjects and 438 cancer-free control subjects were used. SNP genotyping was performed. A Vector was constructed and luciferase reporter assays were done. TLR4 mRNA levels were evaluated and Western blotting was done	The hypothesis that the overexpression of TLR4 induced by the rs1057317 polymorphism miRNA-disrupting function may influence the development of hepatocellular carcinoma is possible but still not proved. More studies in this area are needed	The risk of hepatocellular carcinoma was associated with a functional variant at miR-34a binding site in *TLR4* gene. miR-34a/TLR4 axis may play an important role in the development of HCC
Minmin *et al*[80]	2011 April	Analytic-case-control	A systematic genetic analysis of sequence variants of TLR4 by evaluating ten single-nucleotide polymorphisms was performed from 216 hepatocellular carcinoma cases and 228 controls	The contribution of the SNPs in TLR4 to HCC is modest. More studies are needed to validate this finding in independent populations and to understand the mechanism by which TLR4 sequence variants affect the pathological role of TLR4 in the signaling pathways that control carcinogenesis	The risk of hepatocellular carcinoma was associated with TLR4 sequence variation. TLR4 single nucleotide polymorphisms may play an important protective role in the development of hepatocellular carcinoma
Kawamoto *et al*[82]	2008 April	Experimental	Mouse cells were used together with plasmids containing TLRs. Cells were submitted to LPS and TAK-242. Nitrite and TNF-α were measured. Reporter gene assay for ligand-dependent signaling by TLRs, Reporter gene assay for ligand-independent signaling by TLR4, CD4-TLR or adaptors and Western blot analysis were performed	Human studies are needed as the interacting affinity of TAK-242 with TLR4 may be affected by a subtle difference in the amino acid sequences of TIR between humans and mice	TAK-242 selectively suppresses TLR4-signaling mediated by the intracellular domain
Matsunaga *et al*[83]	2011 January	Experimental	293 cells of human embryonic kidney and murine resident peritoneal macrophages were used. They were subited to TAK-242 and LPS. Vectors for FLAG-TLR4 and FLAG-TLR2 were cloned. Measurement of nitrite and	To fully understand the physical basis whereby TAK-242 disturbs signaling complex formation and intracellular signal transduction, a crystal structure analysis of the TLR4-TAK-242 complex is needed	TAK-242 binds selectively to TLR4 and subsequently disrupts the interaction of TLR4 with adaptor molecules, thereby inhibiting TLR4 signal transduction and its downstream

			cytokine concentrations in culture supernatants, radiolabeling of the cells, immunoprecipitation, Western blot analysis and autoradiography, reporter gene assay and *in vitro* IL-1 receptor-associated kinase-1 kinase assay were all performed		signaling events
Xu *et al*[84]	2013 November	Experimental	Four dsRNAs were designed and synthesized. The expression of proteins was compared. The migration, proliferation and apoptosis of HepG2.2.15 cells were evaluated in presence of BM-06, sorafenib alone or in combination of both. The similar treatments were also applied in an SD rat primary HCC model	Since synthetic siRNAs must be transfected into the target cells through a vector, such as Lipofectamine™ 2000 reagent, they always exhibit cytotoxicity, which may limit their use in clinic	dsRNA alone was capable of inhibiting the proliferation of HepG2.2.15 cells and tumor growth of orthotopic HCC SD rats, but the effect of combination of dsRNA with sorafenib was more prominent
Behm *et al*[85]	2014 December	Experimental	Rabbits were randomised to receive RFA, CpG B, their combination or no therapy, further tested by rechallenging a separate group with intravenously injected VX2 tumour cells after 120 d. Animals were assessed for survival, tumour size and spread, and tumour and immune related histological markers after 120 d. Peripheral blood mononuclear cells were tested for tumour-specific T cell activation and cytotoxicity. Immune modulatory cytokines were measured in serum	Lack of antibody reagents for the VX2-tumour model in rabbits. It was not possible to elucidate in depth histopathological changes	The combination of TLR9 stimulation with RFA resulted in a potentiated antitumour T cell response and cytotoxicity in the VX2 tumour model. Only this combination prevented subsequent tumour spread and resulted in a significantly improved survival

TLR: Toll-like receptor; PCR: Polymerase chain reaction; HCC: Hepatocarcinoma; LPS: Lipopolysaccharides; TNF-α: Tumour necrosis factor α; DEN: Diethylnitrosamine; NAC: N-acetyl cysteine; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear transcription factor kappa B; ER: Endoplasmic reticulum; MDSC: Myeloid-derived suppressor cells; NAFLD: Non-alcoholic fatty liver disease; JNK: Junamino-terminal kinase; HMGB1: High mobility group box 1; INF-γ: Interferon gamma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HSCs: Hepatic stellate cells; ODN: Oligodeoxynucleotides; NMOR: N-nitrosomorpholine; TNFR1: Tumor necrosis factor receptor 1; IL: Interleukine; DC: Dendritic cells; NK: Natural killer; HSCs: Hematopoietic stem cells; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; SA b-gal: Senescence-associated beta-galactosidase; ELISA: Enzyme-linked Immunosorbent Assay; COX: Ciclo-oxigenase; IFC: Inflammation-fibrosis-carcinoma; ROS: Reactive oxigen species; HMGB1: High mobility group box 1; RAGE: Receptor for advanced glycation endproducts; HSP: Heat shock protein; HUVECs: Human umbilical vein endothelial cells; CSC: Colony stem cells; EMT: Epithelial-mesenchymal transition; TICs: Tumor-initiating cells; IRF: Interferon regulatory transcription factor; SNPs: Single nucleotide polymorphisms; HWE: Hardy-Weinberg equilibrium.

a "Myd88 independent manner/toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta (TRIF) dependent" with the extracellular signal-regulated kinase (ERK)/Junamino-terminal kinase (JNK) and PI3K/Akt pathways[24]. Besides this, TLR2 signal is also involved in processes like autophagy and senescence in response to oxidative stress and DAMPS release[25].

Diethylnitrosamine (DEN) is a chemical carcinogen capable of inducing HCC through accumulation of reactive oxygen species (ROS) and endoplasmatic reticulum (ER) stress. It was found that when a TLR2-deficient ($TLR2^{-/-}$) mouse was submitted to DEN treatment the ROS and ER stress were abundantly accumulated, even though less apoptosis was observed[25].

In fact, Lin *et al*[24] demonstrated, in two separated works, that both $TLR2^{-/-}$ and wild-type (WT) mice developed HCC after being submitted to a DEN-treatment. However, the $TLR2^{-/-}$ mice revealed earlier tumours (every $TLR2^{-/-}$ mouse developed HCC at 6 mo after DEN treatment *vs* only 68% WTs)[24] that were, not only significantly increased in number and in volume, but also less differentiated[24,25]. This increase reached the 3 fold (20.1% ± 4.5% *vs* 6.4% ± 1.0%, $P < 0.01$) in the tumour area and 5 fold in visible tumour nodules (29.1% ± 2.8% *vs* 5.5% ± 0.9%, $P < 0.001$)[24]. Meanwhile, in the WT mice, is possible to attenuate HCC development if a TLR2 agonist is used[26]. Ultimately, $TLR2^{-/-}$ mice had shorter mean survival times with HCC than WTs[24]. Moreover, similar scenery was observed when a WT was pre-treated with an anti-TLR2 antibody[25]. Indeed, when observing liver samples from patients in different stages of liver diseases it is notorious that, in patients with HCC, not only the mRNA levels of TLR2 are lower but also TLR2 immunohistochemical expression grade and intensity are reduced, when compared to patients with hepatitis or cirrhosis[27].

A ROS-generation reaction in cytochrome p450 2E1 is responsible for DEN metabolism. In spite of not finding any significant difference in cytochrome activity, $TLR2^{-/-}$ mice still revealed enhanced accumulation of ROS in their liver tissue[24].

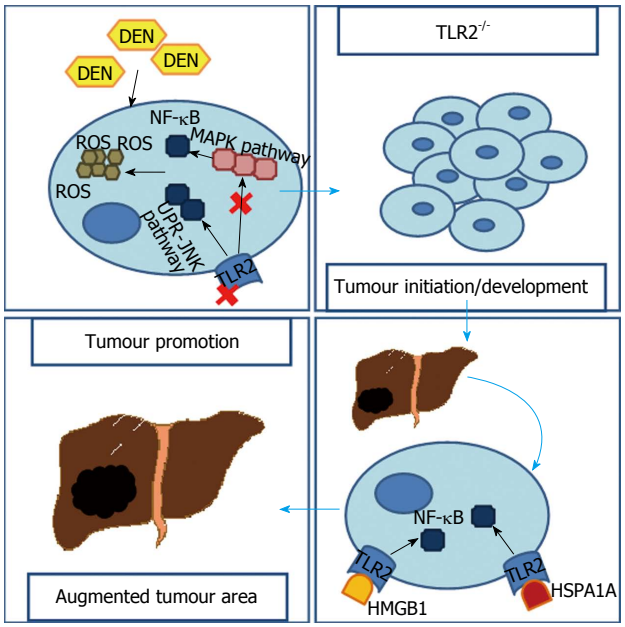

Figure 2 Toll-like receptor 2's signalling pathways contributing to hepatocarcinoma. In the absence of TLR2, cells are incapable of responding to an increasing ROS when submitted to DEN. This is the result of an absence of MAPK/NF-κB pathway and an up-regulation of the UPR-JNK pathway. Consequently, cells containing higher ROS and DNA damages have more chances to survive and, HCC develops. In a second stage, where HCC is already established, HMGB1 and HSPA1A released by tumour's dying cells, through TLR2 stimulation, lead to an NF-κB up-regulation which, in this contest, seems to contribute to tumour's growth. HCC: Hepatocarcinoma; TLR2: Toll-like receptor 2; ROS: Reactive oxigen species; DEN: Diethylnitrosamine; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear transcription factor kappa B; UPR: Unfold protein response; JNK: Junamino-terminal kinase; HMGB1: High mobility group box 1; HSPA1A: Heat shock protein A1A.

Generation of ROS results in oxidative stress, which is often the source of DNA mutation or a direct link with chronic inflammation[28-30]. The ASK1/p38 MAPK/NF-κB pathway is one of the major sensors for ROS accumulation contributing to induced senescence cell death when risk of mutation is present[31]. However, in TLR2$^{-/-}$ mice submitted to DEN treatment, it is possible to assist to an attenuation of this major pathway[25] together with a suppression of biomarkers of autophagy-associated cell death and cellular senescence, like β-galactosidase[24]. Moreover, unlike the WT, TLR2$^{-/-}$ mice fail not only, to induce other important channels to premature cellular senescence like the p16-pRb/p21 pathway[24], but also to activate DNA damage repair mechanisms[32].

Furthermore, ER-stress is augmented after DEN-treatment in TLR2$^{-/-}$ mice as a result of ROS accumulation[25]. This leads to an enhanced unfold protein response (UPR) and activation of UPR-JNK pathway[33], necessary for autophagy activation under ER-stress which, paradoxically, plays a dominant pro-survival role[34]. Lin *et al*[25] noticed that, in livers from TLR2$^{-/-}$ mice there was an increased JNK activity.

Overall this data indicates that in the absence of TLR2, a down-regulation of common ROS neutralizing mechanisms, due to supressed activation of ASK1/p38 MAPK/NF-κB, results in HCC cells containing higher ROS and DNA damages that, because of an up-regulated UPR-JNK pathway, have more chances to survive.

However, other pathways relating to TLR2 and hepatocarcinogenesis exist. Li *et al*[26] focus their work on IL-18, which was found to be fundamental to carcinogenesis in TLR2$^{-/-}$ mice. In these mice, HCC developing after DEN treatment was capable of inducing IL-18 up-regulation in a caspase-8-dependent manner, therefore contributing to promotion of angiogenesis and suppression of NK cell arm of tumour immunosurveillance[26].

Another perspective is related to the High mobility group box 1 (HMGB1), a nuclear protein released from dead/dying cells or even from cancer cells. It has the ability to bind to TLR2 and, with that, successfully activate NF-κB[35] which, in turn, can have an important role as a tumour promoter in inflammation-associated cancer[36]. Up-regulation of HMGB1 in an HCC cell line can result in increased matrix metalloprotease 9 and satellite tumour nodules in the liver, while blocking it supresses tumour growth[37]. A recombinant HMGB1 (rHMGB1) was used by Shi *et al*[38] in order to simulate TLR2 activation in an HCC cell line. Interestingly, rHMGB1 not only reduced cell apoptosis but also accelerated the tumour's growth and enhanced the ability of migration and invasion. Additionally, rHMGB1 activity significantly declined when HCC cells were pre-treated with a TLR2 inhibitor[38].

Similarly to HMGB1, HSPA1A - a member of the HSP70 family - is also a TLR2's ligand released by the tumour's necrotic cells. With a resembling pathway based on up-regulation of NF-κB, HSPA1A is capable of promoting the proliferation and survival of tumour cells[39].

TLR2 clearly represents an important modulator of cells' response to stress situations. It has influence in mechanisms like autophagy, apoptosis or even DNA damage repair, possibly contributing to a protective role against HCC. However, it is, also, important to notice that these pathways may not be, already, clearly understood as studies reveal that TLR2's ligands like HMGB1 and HSPA1A, can result in tumour enhancement (Figure 2).

Taken altogether this data suggests that TLR2 activation may slow down initiation and development of HCC (anti-oncogenic potential) in the earlier phases of HCC carcinogenesis. However, at later stages its activation may influence the progression of inflammation and fibrosis (pro-oncogenic potential). Therefore, new studies are required in order to understand the exactly pathways through which this receptor is able to work and to conclude if its role in HCC carcinogenesis is different or not depending on the stage of the Inflammation-fibrosis-carcinoma axis.

TLR3

Several studies have already shown that TLR3 is expressed in many cancer cells such as colonic adenocarcinoma, lung cancer, breast cancer and melanoma.

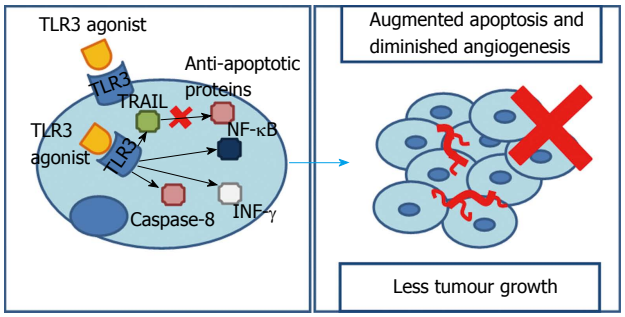

Figure 3 How toll-like receptor 3 stimulus works against hepatocarcinoma. Stimulation of intracellular TLR3 is able to elicit cell apoptosis in a TRAIL-dependent manner that synergistically accompanies a down-regulation of anti-apoptotic proteins. Additionally, TLR3 stimulus can promote either an inflammatory or an apoptotic response. The first one pending on NF-κB, the second in caspase-8 activation and INF-γ release. As a result, TLR3 works as a protector against cancer which stimulation results in diminished tumour growth. TLR3: Toll-like receptor 3; TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand; INF-γ: Interferon gamma; NF-κB: Nuclear transcription factor kappa B.

In HCC it was found that 17-fold longer median survival accompanied patients with higher intratumoral TLR3 expression[40]. However, Yoneda *et al*[41] observed that 52.7% of the HCC tissues and 34.8% of the HCC metastasis studied expressed TLR3. Furthermore, the receptor was not only present in the cytoplasm, but also in the membrane, particularly in the exterior, suggesting a cell surface recognition mechanism for TLR3 agonists[41].

Other works have already implied that the TRIF-dependent pathway of TLR3 signalling could have a special contribution to a tumours response. In fact, this adaptor molecule can promote either an inflammatory or an apoptotic response. The first one pending on NF-κB, the second in caspase-8 activation and interferon-γ (INF-γ) release[42]. Experiments showed that using synthetic TLR3 agonists resulted in a rise in NF-κB. In fact, as it was seen with TLR2 signalling, NF-κB is normally associated with augmented tumour necrosis factor α (TNF-α) responsible for cells' growth and proliferation[43]. However, here, an NF-κB rise is responsible for affecting the tumour microenvironment and driving HCC and endothelial cells to apoptosis[44], accompanied by a significantly decreased tumour invasiveness and angiogenesis/vascular endothelial growth factor (VEGF) levels[45-47]. Thus, it seems that, whether NF-κB promotes or inhibits hepatocarcinogenesis depends on the presence of inflammation and the degree of NF-κB inhibition/promotion[3].

Moreover, INF-γ - a potent inhibitor of endothelial cell proliferation/angiogenesis - and caspase-8/caspase-3 - inhibitors of hepatocytes proliferation - were found to be significantly increased in HCC cell lines pre-treated with TLR3 agonists[48].

However, it is important to notice that, when stimulated through polyinosinicpolycytidylic acid, the surface TLR3 is only able to induce apoptosis if a protein synthesis inhibitor or a RNA synthesis inhibitor are present[41]. This might indicate that, in an HCC cell line, endogenous suppressors of TLR3-mediated apoptosis are present. Curiously, stimulation of intracellular TLR3, even without protein or RNA synthesis' inhibitors, was able to elicit cell apoptosis in a tumour necrosis factor-related apoptosis-inducing ligand-dependent manner that synergistically accompanies a down-regulation of anti-apoptotic proteins[41].

Notably, despite overall tumour growth could be reduced through TLR3 activation (from a 3-fold increase, when no TLR3's stimulus is present, to an only 1.9-fold increase after TLR3's agonists being used), the number of tumour nodules increases even after eliciting TLR3 signalling, leading to the conclusion that it does not affects the incidence but limits their growth[47].

Interestingly, it appears that in HCC carcinogenesis TLR3 is a TLR that works as a protector against cancer. This is possible through molecules, downstream to TLR3, such as caspases, INF-γ or NF-κB, influencing crucial processes like angiogenesis, cell growth or proliferation (Figure 3).

TLR4

It is known that, despite being present in multiple liver cells, TLR4 expression is relatively low in this organ[49]. However, following liver damage and inflammation it is possible to assist to an up-regulation of this receptor[50]. Emerging evidence associates TLR4 to several types of tumours, enlightening its role in carcinogenesis, metastasis and cancer progression[51]. Observation of human's livers detected a high expression of TLR4 in cancer cells of HCC patients[49].

Bacterial LPS is capable of initiating TLR4 signalling and subsequently activating NF-κB and MAPK signalling pathways - p38, ERK, JNK. In fact, in a HCC cell line incubated with bacterial LPS both TLR4 expression[52-54] and MAPK signalling pathways are significantly augmented[52]. However, it was found that, in contrast with a normal hepatocytes cell line, in a HCC cell line, the cellular growth was augmented and the cytotoxicity induced by LPS was decreased and dependent on TLR4 expression (higher expression is equal to less cytotoxicity)[49]. Additionally, these effects are reduced after inhibiting TLR4 signalling[55].

The explanation of these results is based on two perspectives. One based on the fact that, in TLR4-overexpressing cells, ERK and JNK's activity is promoted[49] contributing to cell survival and proliferation. Nonetheless, loss of TLR4 results in a substantial decrease in proliferating hepatocytes as well as in a reduced duration of JNK and ERK mitogenic signals[56]. This pro-survival effect, when facing LPS, can also be blocked by down-regulating this TLR4-downstream molecules - ERK and JNK[49].

A second and slightly opposing situation relies on p38 - capable of inducing cell cycle arrest and apoptosis - and NF-κB - capable of stimulating pro-inflammatory cytokines (IL-1, -6, -10, TNF-α)[57] - that were inhibited by LPS, in a TLR4-overexpressing HCC cell line, allowing

cell proliferation[49]. In fact, after stimulating TLR4, either blocking[49] or augmenting[39,55] NF-κB have been reported to promote tumour's survival. Once more we face an ambiguity in interpreting NF-κB values. However, in this situation, the explanation can rely on the degree of the stimuli/block and the underlying inflammation[3].

Consequently, we are able to conclude that increased expression of TLR4 may protect HCC cells from LPS-induced cytotoxicity and promote cell HCC survival and proliferation.

This pro-tumorigenic effect of TLR4 is confirmed by the fact that, in TLR4$^{-/-}$ mice subjected to DEN, tumour incidence is 25% lower and diameters are smaller accompanied by less inflammation, proliferation as well as enhanced apoptosis[56]. Moreover, using antibiotics to reduce the LPS levels results in diminished activation of T helper 1 cells[58] and consequently less liver damage, and lower cell proliferation in tumour mass[56].

However, Xu *et al*[54] presented a different vision when reported increased expression of protein tyrosine phosphatase receptor type O (PTPRO) in TLR4-over-expressing HCC cell lines after LPS treatment. Here, cell proliferation was inhibited and apoptosis was augmented as a result of the tumour suppressor capability of PTPRO[54]. To that end, it was found that, contrarily to the effects on LPS-induced cytotoxicity, TLR4-overexpression might also have a protective role through PTPRO and thus, worth being subjected to new studies.

Li *et al*[52] also observed that, with TLR4 over-expression, came a gradual disappearance of epithelial cell markers and increased mesenchymal ones, suggesting an epithelial-mesenchymal transition (EMT). This EMT is considered to be the molecular basis of tumour cell infiltration and metastasis[59] and can, actually, be induced by two possible pathways related to TLR4 and LPS stimulus. On one hand, the TLR4 - MAPK/JNK pathway, confirmed by the fact that, blocking directly MAPK/JNK or indirectly through TLR4, lead to inhibition of LPS-induced EMT[52]. On the other hand, Snail, a transcription factor handled by NF-κB and a major inducer of EMT[53].

For this reason, LPS, *via* activation of TLR4 signalling pathway and consequently MAPK/JNK pathway activation or NF-κB up-regulation, can significantly induce EMT.

This EMT phenotype is conveyed by cancer stem cells[60] which, in turn, are thought to be involved in processes like formation and progression of cancer, being, inclusively, responsible for chemotherapy resistance, metastasis and postoperative recurrence[61,62]. Recent studies revealed that TLR4 positive cells exhibit a series of stem cells characteristics[50,60]. These cells not only display a higher invasive ability, when compared to TLR4 negatives, but also express many stem cell markers (CD133 increase 85% when TLR4 is overexpressed[60]) as well as a stronger colony forming ability and increased chemotherapy/apoptosis resistance[50].

In agreement with these results, Chen *et al*[60] proposed that TLR4 could work as a proto-oncogene which aberrant expression/activation leads to induction of pluripotency genes and genesis of tumour-initiating stem-like cells (TICs). This process is possible through activation of a TLR4/NANOG pathway[60,62-64] and consequent inhibition of the transforming growth factor β (TGF-β)[60,62,64].

NANOG is *per se* a core transcription factor found in pluripotent stem cells[62]. TGF-β is an effective proliferation inhibitor and an apoptosis promoter that, when down-regulated, is able to initiate tumorigenesis *via* stemness gene induction in an epithelial tissue such as liver[60]. In fact, knockdown of TLR4 attenuated the induction of stem cell genes as well as DNA synthesis of TICs in 50% to 80% and blocking NANOG, results in a tumour growth reduction of 60% to 75%[60].

However, some cancer cells grow efficiently *in vitro* without addition of LPS but this growth is still reduced by TLR4 knockdown, suggesting LPS-independent mechanisms of TLR4 activation in these cells[64]. One possibility includes non-LPS ligands influencing tumorigenesis through TLR4 signalling.

Yan *et al*[65] observed that hypoxia was also responsible for TLR4 up-regulation in an HCC cell line. Hypoxia is a hallmark of several solid tumours, including HCC, and an important factor in tumour progression[66]. A possible explanation of this relationship may involve hypoxia-induced HMGB1 release, capable of activating TLR4 signalling and consequently augment caspase-1. This one is, in turn, related with maturation of pro-inflammatory cytokines and consequent tumorigenesis and tumour progression. After TLR4 blockage, caspase-1 expression diminished significantly[65]. Additionally, caspase-1 blocking was capable of decreasing HCC cell invasiveness[65]. This suggests that hypoxia-induced caspase-1 activation, as well as caspase-1-mediated tumour progression, can depend on TLR4 signalling.

In spite of several evidences attributing a pro-tumorigenic role to TLR4, the pathways to that end are many and still not fully understood. Diminished apoptotic-response to LPS, EMT-induction or caspase-1 up-regulation through TLR4 were already proposed but, opposing effects mediated by tumour suppressors like PTPRO were also found (Figure 4). According to these results new studies are suggested to clarify not only how each work, but also how they are related. However, contrarily to TLR2 most data suggests that TLR4 activation not only has an important role in inflammation and fibrosis but also in HCC initiation and progression.

TLR9

A possible relationship between TLR9 and carcinogenesis came to light when its high expression levels of TLR9 were found in samples of lung and breast cancer cell lines[67]. HCC cells exhibit a broad repertoire of TLRs, also including TLR9. This receptor plays a crucial role in cell survival as it recognises several bacterial and viral components, including unmethylated CpG-DNA. Different works revealed that there is an augmented TLR9 positivity in human HCC cells[8,68,69] with Eiró *et al*[8]

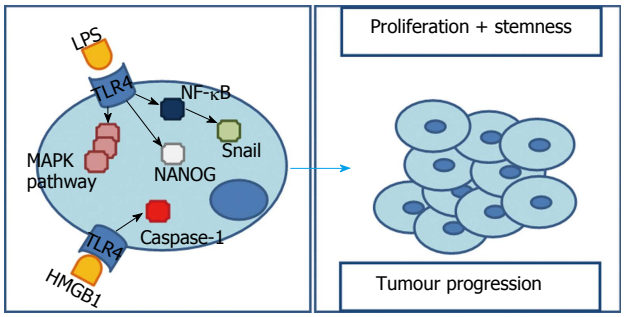

Figure 4 Toll-like receptor 4's signalling pathways influencing hepatocarcinogenesis. Bacterial LPS is capable of initiate TLR4 signalling and subsequently activate NF-κB and MAPK signalling pathways. In one hand, in a HCC cell line incubated with bacterial LPS both TLR4 expression and MAPK signalling pathways are significantly augmented, contributing to cell survival and proliferation. On the other hand, TLR4 over-expression contributes to an EMT through MAPKs pathway and Snail. Additionally, NANOG induces TICs' formation. Both are considered to be the molecular basis of tumour cell infiltration and metastasis. HMGB1's stimulation of TLR4 with caspase-1 activation is related with maturation of pro-inflammatory cytokines and consequent tumorigenesis and tumour progression. TLR4: Toll-like receptor 4; LPS: Lipopolysaccharides; HCC: Hepatocarcinoma; MAPK: Mitogen-activated protein kinase; EMT: Epithelial-mesenchymal transition; TICs: Tumour-initiating stem-like cells; NF-κB: Nuclear transcription factor kappa B; HMGB1: High mobility group box 1.

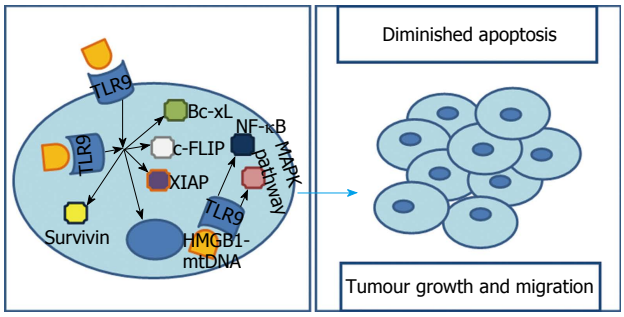

Figure 5 Toll-like receptor 9's signalling pathways influencing hepatocarcinoma. TLR9 is present not only in the cytoplasm but also on cells' membrane. However, it is still controversial whether cytoplasmatic stimulation results in increased cell viability. Independently, membrane receptors' stimulation results, not only, in up-regulation of apoptosis inhibitors such as survivin, Bcl-xL, XIAP and c-FLIP, but also, in a modulation of oncogenic genes with a major contribution in tumorigenesis and cancer progression. Additionally, a cytoplasmatic HMGB1-mtDNA interaction was proved to be capable of activating TLR9 and MAPK pathway as well as NF-κB leading to augmented survival, growth, proliferation, differentiation and migration of cancer cells. TLR9: Toll-like receptor 9; XIAP: X-linked inhibitor of apoptosis protein; c-FLIP: Cellular FLICE-Like inhibitory protein; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear transcription factor kappa B; HMGB1: High mobility group box 1.

showing a TLR9's prevalence of 60% (in a population of 30 cases) and Tanaka *et al*[68] reaching the 85.7% (in a population of 42 cases) in their works with human samples of HCC. Moreover, in the later, 7 of 8 cases of HCC metastasis presented TLR9 positivity[68]. Additionally, it was found that, in both HCC cell line or HCC human samples, TLR9 was present not only in the cytoplasm but also on cells' membrane[68]. However, is important to notice that, possibly, only the stimulation of membrane receptors could result in increased cell viability as transfecting a TLR9 agonist, CpG-oligodesoxynucleotide (CpG-ODN), which stimulates intracellular TLR9 receptors, may not affect proliferation and survival[68]. The explanation for this tumour-promoter role of TLR9 comes from the fact that, after TLR9 stimulation, a HCC cell line is able to, not only up-regulate apoptosis inhibitors such as survivin, Bcl-xL, XIAP and cFLIP, but also, to closely modulate oncogenic genes with a major contribution in tumorigenesis and cancer progression[68] (Figure 5).

Although, this data is not that linear, and, somehow, different from what Zhang *et al*[70,71] stated in their studies. Here, transfecting a TLR9 agonist into a HCC cell line lead to a marked increase in IFN-α, IFN-β, TNF-α, IL-6 and IL-8 without activating NF-αB. As a result a cell-proliferation's inhibition rate was increased approximately 50% and apoptosis was augmented[70,71].

The contradictory findings about the influence on tumour's environment of intracellular TLR9 agonists could be explained by the fact that the phosphorothioate-modified backbone of CpG-ODN are able to form a complex with or cause conformational changes in other compounds, like Poly (I:C) that, normally would result in enhanced apoptosis but, when together with CpG-ODN, are unable to act[70]. Moreover is important to look at the protocols used, as CpG-ODN induces HCC cell apoptosis in a dose-dependent manner, at concentrations below 0.5 μg/mL. In contrast, high concentrations of this agonist (*e.g.*, 5 μg/mL) had no effect on HCC cells[71].

Additionally, this pathway from TLR9 signalling to carcinogenesis is supported by HMGB1. We have already seen that HMGB1 and hypoxia could influence tumorigenesis through different TLRs. Interestingly, they are, also, both involved with TLR9. It was seen that along with TLR9 overexpression, hypoxic cancer cells accumulate structurally and functionally abnormal mitochondria, which release mitochondrial DNA (mtDNA) to the cytosol, and induce translocation of HMGB1 from nucleus to cytoplasm[69]. The role of HMGB1 as a promoter of invasion, metastasis and angiogenesis when its location is extracellular is not new[72]. However, Liu *et al*[69] revealed that, on top of this, an cytoplasmatic HMGB1-mtDNA interaction is required for complete activation of TLR9 signalling cascade and therefore essential for HCC cells to proliferate under hypoxic conditions. The underlying mechanism in this pro-tumorigenic pathway lies in MAPKs activation - fundamental in growth, proliferation, differentiation and migration[73] - and also in NF-κB signalling - capable of suppressing apoptosis in response to stress[74] - after the interaction between HMGB1/mtDNA and TLR9[69,75] (Figure 5).

TLRS GENETIC POLYMORPHISMS AND VARIANTS AND HCC SUSCEPTIBILITY

Several authors have already focused their studies on the relationship between TLRs' genetics and carcinogenesis, approaching different cancers such as non-Hodgkin lymphoma, endometrial cancer, cervical cancer, non-cardiac gastric cancer, among others.

Genetic studies on the *TLR2* gene have shown a number of polymorphisms capable of interfering with host defenses and disease progression[76]. In fact, it was already seen that inherited variation in TLR2 influence the risk of HCC. Genetic TLR2 analysis revealed that two single nucleotide polymorphisms (SNP), rs3804099 and rs3804100, had a significantly different distribution between HCC patients and the healthy controls[77]. Interestingly, in what is concerned to these SNPs, Junjie *et al*[77] suggested that, TLR2 gene variation could play an important protective role in HCC as the heterozygous genotype comprise lesser HCC risk (OR from 0.331 to 0.759, $P < 0.001$) when compared to wild-type homozygous genotype. In fact, individuals carrying the TT haplotype had a significantly decreased risk of HCC [odds ratio (OR) = 0.524, 95%CI: 0.394-0.697, $P < 0.001$]. Contrarily, the CC haplotype had greater risk (OR = 2.743, 95%CI: 1.915-3.930, $P < 0.001$). Unfortunately, the authors do not reveal the real influence of the referred SNPs on the TLR2's activity and more studies are suggested to clarify this information.

Moreover, the frequency of a -196 to -174 deletion allele was, also, significantly higher in HCC patients than in healthy controls (22.5% *vs* 15.3%) and HCV-infected patients without HCC (22.5% *vs* 15.6%)[76]. Nischalke *et al*[76] observations indicate that the -196 to -174 deletion allele possibly augment the risk of HCV-induced HCC, probably as a result of diminished TLR2 signalling and thus increased viral loads. This -196 to -174 deletion not only had greater viral loads than -196 to -174 ins/ins but also, contribute to a 3-fold increase in HCC risk relatively to this -196 to -174 ins/ins when both are compared to healthy controls or a 1.5 fold increase when both are compared to hepatitis C patients without HCC[76].

Researchers have already studied the possible presence of polymorphisms in the area of TLR3. It was found that, at least in the chinese population, a +1234CT polymorphism is present which might contribute to increased susceptibility to HCC (specially 1234CT and TT genotypes)[78]. The presence of this SNP is responsible for a markedly diminished TLR3 function, which may result in up-regulated vasculature remodelling and tumour growth and, in that way, contributing to HCC[78].

The TLR4 is probably the more extensively studied TLR and therefore, not an exception when it comes to having polymorphisms or variants capable of influence carcinogenesis. Growing evidence has shown that TLR4 polymorphisms are related to chronic inflammation and inflammatory-related cancer. As a matter of fact, a polymorphism in microRNA-34a binding site in TLR4 (rs1057317) was significantly associated with higher HCC risk, especially in HBsAg (+) patients and in the AA homozygous genotypes[79]. MicroRNA-34a is capable of inducing apoptosis, G1 arrest and senescence explaining why its down-regulation may be associated with malignancy. However, there are not only polymorphisms related to augmented risk. Indeed, some mutations of *TLR4* gene - four SNPs in 5'-UTR (rs10759930, rs2737190, rs10116253 and rs1927914) and one intron polymorphism (rs1927911) - may allow a two-fold decrease in HCC risk, especially in heterozygous genotypes when compared to wild-type homozygous[80]. The justification can rely on the fact that 5'-UTR is involved in regulation of proteins concerned with growth and differentiation in normal tissues and these SNPs may, therefore, exert regulator effects in these proteins[80]. Therefore, according to the TLR4's polymorphism observed, an augmented or diminished risk of HCC is possible, even though its magnitude is small.

TLRS AS THERAPEUTIC TARGETS FOR HCC

So far we have seen that different TLRs could work as specific modulators of HCC. Therefore it is logical to think that its use as therapeutic targets could open the door to new promising strategies in the fighting against HCC. In fact, the modulation of TLRs' signalling, by targeting either the TLRs or their adaptors or downstream signalling molecules, is not new and they have already proved to be useful in ovarian, colorectal or head and neck cancer[81].

TLR4 modulators seem to be important chemo-therapy adjuvants that enhance chemotherapy efficacy and prolong survival[81]. TAK-242 is a TLR4 ligand capable of selectively supress both ligand-dependent and independent signalling *via* the intracellular domain of TLR4, disrupting the TRAM and TIRAP interactions with TLR4[82]. This small molecule is, therefore, able to down-regulate NF-κB and consequently diminish inflammatory mediators such as nitric oxide, TNF-α, IL-1, -6 and with that, reduce the proliferation/invasion activity induced by LPS in the liver cancer cell lines[57,82,83]. Furthermore, TAK-242 might also show an efficacy against inflammation mediated by excessive expression of TLR4, what, in fact, has been shown to happen in HCC[82]. Independently, new studies are still required for better evaluating effects, doses and other characteristics of TAK-242.

To date, we still lack an effective systemic curative therapy for advanced cases of HCC and, in most cases the only alternative is palliative treatment.

Even though, sorafenib, a multi-kinase inhibitor, represents an important chemotherapeutic drug in the treatment of this type of cancer. Xu *et al*[84] found that a combination of sorafenib with a TLR3-synergist (BM-06) results in a superior inhibition of tumour growth in HCC cell lines or rat models when compared to the two different agents alone. Their results were based on a significantly reduced proliferative capacity, invasion ability, tumour volume and an increased apoptotic rate[84]. Therefore, BM-06 emerges as a possible adjuvant agent in the therapeutic against HCC.

In the TLR9 domain several studies were already conducted. It was reported that using TLR9 antagonists

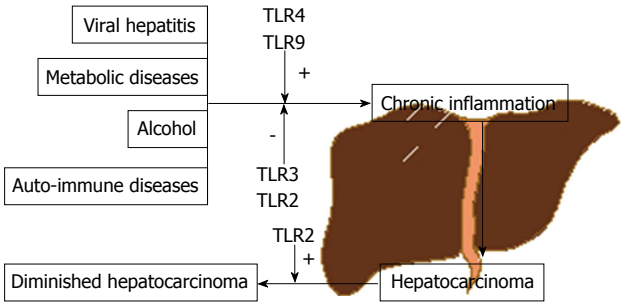

Figure 6 Toll-like receptors influence on the pathway to hepatocarcinoma. Several factors are known to contribute to the carcinogenic process including viral hepatitis, alcohol, auto-immune or metabolic diseases, among others and the link between all this factors is chronic inflammation which, in turn, is an important hepatocarcinoma's percursor. Moreover, innate immunity represents an important player in this equation with TLRs such as 4 and 9 having, mainly, a positive contribution to hepatocarcinogenesis and TLR3, essentially, a negative/protective one. TLR2 still presents an ambiguous role, possibly depending on liver's stage in the inflammation-cirrhosis-carcinoma axis to exert its pro-tumorigenic or anti-tumorigenic capacity. TLR: Toll-like receptor.

like chloroquine could be useful in several autoimmune diseases[2]. In fact, a markedly reduced proliferation is seen in a HCC cell line when TLR9 is inhibited by chloroquine[2]. This antimalaric agent works as a direct TLR9 antagonist, being proposed that its activity on HCC cells may be brought about *via* its effects on the protein kinase AKT, tumour-associated angiogenesis factor VEGF as well as NF-κB. Moreover, the same tumour growth restriction, followed by smaller volume and reduction in tumour's markers of aggressiveness was seen when this treatment was used in HCC cell lines intrahepatic implanted in mice[2].

However, as it was said, sometimes, the only option is the palliation and radiofrequency ablation (RFA) which has already established an important role in this setting. Behm *et al*[85] successfully demonstrated that, in a rabbit model, TLR9 agonists could work together with RFA in an anti-tumour response through a strong cytotoxic immune response mediated by increased tumour-specific lymphocytes. In fact, it was not only a good predictor of containment of tumour growth and spread but also of prolonged survival[85].

Moreover, some studies focus on the use of TLRs as vaccine's adjuvants against HCV or HBV mediated hepatocarcinogenesis. There are also some proposals for using TLR4's antagonists in patients with septic shock[86]. Besides this, the use of TLR4's antagonists is being investigated in the prevention of alcoholic liver injury and Non Alcoholic Steato-Hepatitis[9].

Despite the good results, when it comes to using TLRs as a novel HCC therapeutic it still has a long run before every mechanism is understood. TLRs' signalling pathways are too many and effects remain controversial but a lot is to be expected from these innate immunity receptors.

CONCLUSION

HCC occupies the third place when it comes to mortality in cancer[1]. Several factors are known to contribute to the carcinogenic process including viral hepatitis, alcohol, auto-immune or metabolic diseases, among others. The link between all these factors is inflammation or, more precisely, chronic inflammation.

However, despite all this malignant potential or this knowledge around the inflammatory causality, most of the pathways of carcinogenesis are still unknown or, at least, not entirely known.

TLRs' role in the tumour formation is part of a more recent concept that involves innate immunity but, despite all the advances, a lot is still waiting to be studied. The reasons for this lack of information include the range of responses that can be obtained from a single TLR signalling. Humans dispose of 11 TLRs capable of initiating a signal cascade from only five molecular adaptors. Consequently, some can be used by more than one receptor. Moreover, each of this activated adaptor molecules, depending on the initial TLR, elicit a response based on the production of several effectors, pro-inflammatory or anti-inflammatory cytokines, interferons and many others with countless results.

To understand the role of TLRs we must remember that HCC comes from an inflammatory background where a complex and progressive process appears with fibrosis and cirrhosis until the last stage, HCC, is reached. This review tried to show that, the path taken can be closely influenced by innate immunity/TLRs (Figure 6). TLR2 was shown to be an important stress manager so that, in its absence, an attenuated ASK1/p38 MAPK/NF-κB pathway and an increased JNK activity result in a larger and less differentiated HCC. Contrarily, TLR2's stimulation through HMGB1 and HSPA1A also indicates a tumour-promoter role. TLR3 may be responsible for driving HCC and endothelial cells to apoptosis and decreasing invasiveness and angiogenesis by mediating NF-κB, caspase-8 and INF-γ up-regulation. TLR4 is closely related to LPS cytotoxic, which is diminished in TLR4-overexpressing HCC due to promoted ERK's and JNK's activity and limited NF-κB and p38 activation. Moreover, this receptor is tightly involved in EMT and progression of cancer based on non-LPS ligands like NANOG and HMGB1. TLR9 activity is different whether the membrane or the intracellular receptor is activated. The first promotes HCC through apoptosis inhibitors and oncogenic genes. The second augments apoptosis by increasing Interferons and interleukines. Consequently, initial findings attribute a pro-tumorigenic role to TLR4 and TLR9 and a protective capacity to TLR3. When it comes to TLR2, the available data suggests that its influence may go both ways (pro-tumorigenic and protective) depending on the liver's stage in the inflammation-cirhosis-carcinoma axis. However this is not that simple or linear as a closer look easily reveals studies with interesting but opposing conclusions from the ones before.

Independently of this lack of knowledge, one thing is certain; TLRs can have a determining influence on the cancer's progression. Therefore, the usage of TLRs

as therapeutic targets has already been established, especially as adjuvants to other agents currently in use. However, the possibilities are many and with a deeper insight over the mechanisms involved new ways of dealing with HCC are expected to emerge.

In conclusion, we cannot say that TLRs came to facilitate our understanding of HCC mechanisms. Instead they came to open the door to a new reality and, with that, to possible new approaches, perhaps in a closer future than we might know.

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REVIEW

Sofosbuvir treatment and hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is a serious problem worldwide. The use of interferon-based therapy has made HCV eradication challenging. The recent appearance of direct-acting antiviral agents (DAAs) has changed HCV therapy. Combining the use of DAAs with peginterferon and ribavirin has improved treatment efficacy. Furthermore, the combination of different orally administered DAAs has enabled interferon-free therapy with much higher efficacy and safety. In particular, sofosbuvir, a nucleotide-based NS5B inhibitor, prevents HCV RNA synthesis by acting as a "chain terminator". Treatment with sofosbuvir has attained an extremely high rate of sustained virologic response. The current review summarizes the efficacy and safety of sofosbuvir therapy.

Key words: Hepatitis C virus; Interferon; Interferon-free; Genotype; Sofosbuvir

Core tip: Sofosbuvir, a nucleotide-based NS5B inhibitor, is an effective treatment against pangenotypic strains of hepatitis C virus (HCV). Sofosbuvir-containing regimens have attained extremely high rates of sustained virologic response. Because regimens including sofosbuvir result in fewer adverse events than interferon-based regimens, sofosbuvir has taken a central role in HCV treatment.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health problem. Approximately 130 to 170 million people experience chronic HCV infection, which has a global prevalence of 2%-3%[1,2]. In 2002, worldwide, 27% of 783000 deaths from cirrhosis and 25% of 619000 deaths from hepatocellular carcinoma were attributed to HCV infection[3].

The use of interferon-based therapy has made HCV eradication challenging, especially for patients infected with HCV genotype 1. Treatment with peginterferon plus ribavirin induces only about 50% of patients infected with HCV genotype 1 at high viral loads to achieve sustained virologic response (SVR)[4], while about 80% of patients infected with HCV genotypes 2 and 3 achieve SVR[5].

The appearance of direct-acting antiviral agents (DAAs), which specifically target HCV proteins, has provided insights into the current situation. The use of protease inhibitors, such as telaprevir, boceprevir, simeprevir, faldaprevir and vaniprevir, in combination with peginterferon and ribavirin has improved treatment efficacy in treatment-naïve patients (70% to 80% achieve SVR) and in patients infected with HCV genotype 1 who have relapsed post-treatment[6-10]. However, SVR rates in patients who exhibited no responses to previous treatments remain low[11,12]. Furthermore, patients who are ineligible for or intolerant to treatment with peginterferon plus ribavirin are contra-indicated from receiving the above treatment.

The use of combinations of different orally administered DAAs has enabled the realization of interferon-free therapy. DAA-based therapies generally have higher treatment efficacy and lower risk for adverse effects compared to regimens using interferon. In particular, sofosbuvir, a pyrimidine nucleoside analog inhibitor of HCV pangenotype NS5B polymerase, has produced extremely high rates of SVR when used in combination with other DAAs. In the current review, we discuss the use of treatment regimens that include sofosbuvir to combat HCV.

STRUCTURE OF HCV GENOME AND DAAS

HCV is an enveloped, positive-stranded RNA virus. Its genome spans 9600 nucleotides in length and contains a 5′ non-translated region (5′ NTR), a single open reading frame and a 3′ NTR. A single polyprotein is translated from HCV genome and is then cleaved into structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins[4]. The serine protease NS3 plays a key role in processing the HCV polyprotein. The phosphoprotein NS5A and RNA-dependent RNA polymerase NS5B are essential to the replication of HCV RNA. DAAs against HCV target these proteins and strongly inhibit HCV replication.

The first-generation NS3/4A protease inhibitors telaprevir and boceprevir are typically used in combination with peginterferon and ribavirin. This triple regimen has improved SVR rates in HCV genotype 1-infected patients[6,7] but often produces serious adverse events[13]. The second-generation NS3/4A protease inhibitors simeprevir, faldaprevir and vaniprevir have further improved treatment efficacy while resulting in fewer adverse effects[8-10].

In Japan, daclatasvir, a first-in-class NS5A inhibitor, enabled effective interferon-free therapy for the first time. Interferon-free dual oral therapy with daclatasvir and asunaprevir, a NS3/4A protease inhibitor, led to high SVR rates (87.4% in interferon-ineligible or -intolerant patients, 80.5% in non-responders, 89.1% in treatment-naïve patients, and 95.5% in relapsers)[14,15]. However, the evolution of drug-resistant HCV variants has created new problems. For example, Y93H variants of the NS5A protein have markedly decreased SVR rates to as low as 43.3%[14].

Sofosbuvir

Sofosbuvir (formerly known as GS-7977; Gilead Sciences, Foster City, CA, United States) is a nucleotide NS5B inhibitor[16]. Sofosbuvir is converted into a pharmacologically active form (GS-461203) within hepatocytes[17]. GS-461203 inhibits RNA-dependent RNA polymerase activity by competing with uridine and prevents HCV RNA synthesis by acting as "chain terminator". Because the catalytic site of the NS5B protein is highly conserved, sofosbuvir is believed to have pangenotypic activity[18].

Another favorable characteristic of sofosbuvir is its high genetic barrier. In an HCV replicon study, a S282T substitution in NS5B was reported to impart drug resistance[19]. However, in clinical trials, this NS5B variant was detected in only one patient after treatment with sofosbuvir monotherapy[16,20-22]. Foster *et al*[23] reported that sofosbuvir treatment led to the emergence of drug-resistant variants in 9/78 (12%) of patients. An L159F variant was present both at baseline and at the time of virologic failure in 1 patient and at the time of virologic failure in 7 additional patients. The V321A variant emerged at the time of virologic failure in 2 patients.

Sofosbuvir also possess a noteworthy safety profile and high tolerability. In phase 3 trials of sofosbuvir, the frequency of serious adverse events ranged from 1% to 8% and the rate of treatment discontinuation because of adverse events range from 0% to 4.4%[24,25].

CLINICAL EFFICACY OF TREATMENT REGIMEN CONTAINING SOFOSBUVIR

More than 3000 patients have been assessed in clinical

Table 1 Summary of phase 3 study for hepatitis C virus genotype 1-infected patients

Study name	Population	Treatment	Duration (wk)	*n*	LC (%)	SVR12 (%)		
						All	Non-LC	LC
NEUTRINO	Naïve	SOF/PEG-IFN/RBV	12	291	-	89	-	81
ION-1	Naïve	SOF/LDV	12	210	16	99	100	97
		SOF/LDV/RBV	12	216	15	97	97	100
		SOF/LDV	24	214	15	98	99	97
		SOF/LDV/RBV	24	214	17	99	99	100
ION-3	Naïve	SOF/LDV	8	214	0	94	94	-
		SOF/LDV/RBV	8	216	0	93	93	-
		SOF/LDV	12	216	0	95	95	-
Japanese study	Naïve	SOF/LDV	12	83	16	100	100	100
		SOF/LDV/RBV	12	83	14	96	97	92
ION-2	Experienced	SOF/LDV	12	20	94	95	86	20
		SOF/LDV/RBV	12	20	96	100	82	20
		SOF/LDV	24	20	99	99	100	20
		SOF/LDV/RBV	24	20	99	99	100	20
Japanese study	Experienced	SOF/LDV	12	32	100	100	100	32
		SOF/LDV/RBV	12	25	100	100	100	25

LC: Liver cirrhosis; SVR12: Sustained virologic response at 12 wk; Naïve: Treatment-naïve; Experienced: Treatment-experienced; SOF: Sofosbuvir; LDV: Ledipasvir; PEG-IFN: Peginterferon; RBV: Ribavirin.

studies of treatment regimens containing sofosbuvir. Below, we describe the efficacy of a sofosbuvir-containing regimen.

HCV genotype 1-specific virologic response of sofosbuvir

The results from phase 3 clinical trials evaluating the use of treatment regimens containing sofosbuvir against HCV genotype 1 are shown in Table 1. The NEUTRINO study was the first phase 3 trial to evaluate sofosbuvir-containing therapy[26]. In this single-group, open-label study, 291 treatment-naïve patients infected with HCV genotype 1 were treated with sofosbuvir plus peg-interferon and ribavirin for 12 wk. The patients attained a high rate of SVR at 12 wk after completion of therapy (SVR12) (89%) and had a low rate of treatment discontinuation (2%) compared with historical controls. This study demonstrated the efficacy and safety of combining sofosbuvir with peginterferon and ribavirin; however, patients contraindicated for peginterferon or ribavirin were excluded.

Based on high rates of SVR in phase 2 studies, phase 3 ION studies (ION-1, ION-2 and ION-3 studies) were conducted to assess a fixed-dose combination of sofosbuvir and ledipasvir[27-30]. In the ION-1 trial, 865 treatment-naïve patients infected with HCV genotype 1 were randomly divided into four groups and received either 12 or 24 wk of sofosbuvir and ledipasvir with or without ribavirin. High SVR12 rates were attained in all groups (range: 97%-99%), and no patients in either 12-wk group discontinued therapy because of adverse events[28].

The ION-2 trial was conducted to evaluate the combination of sofosbuvir and ledipasvir with or without ribavirin in treatment-experienced patients. In this trial, 440 patients were divided into groups and treated with either 12 or 24 wk of sofosbuvir and ledipasvir with or without ribavirin. As in the ION-1 trial, high SVR12 rates were attained in all treatment groups (range: 94%-99%). No cases of treatment discontinuation owing to adverse events were reported[29].

The ION-3 trial was conducted to evaluate the feasibility of shorter duration therapy in previously untreated patients without cirrhosis. The noninferiority of an 8-wk regimen was demonstrated by the similar SVR12 rates in all groups (range: 93%-95%)[30]. The results from the ION-3 trial also indicated that an 8-wk regimen of sofosbuvir plus ledipasvir is not generally equal to 12 wk of treatment; however, international guidelines recommend that an HCV RNA threshold of 6 million IU/mL is maintained.

Among Asian countries, Mizokami *et al*[25] reported a phase 3 study conducted to evaluate Japanese patients. In this study, 166 treatment-naïve and 175 treatment-experienced patients received 12 wk of sofosbuvir and ledipasvir with or without ribavirin. High SVR12 rates (range: 96%-100%) were also attained in this study. This is noteworthy, as Japanese patients tend to be older, have more advanced fibrosis and are more frequently treated with previous therapy than patients in other countries. In the above studies, the inclusion of ribavirin in the treatment regimens produced no additional benefit. Furthermore, adverse events were more common in groups treated with ribavirin than in those without ribavirin.

The SOLAR-1 and SOLAR-2 studies showed the usefulness of sofosbuvir plus ledipasvir for decompensated cirrhosis[31,32]. In patients with cirrhosis and moderate or severe hepatic impairment who had not undergone liver transplantation, 86%-89% SVR12 rates were achieved[31]. In patients who had undergone liver transplantation, 96%-98%, 85%-88%, and 60%-75% SVR12 rates were achieved in patients without cirrhosis or with well-compensated cirrhosis, patients with

Table 2 Summary of phase 3 study for hepatitis C virus genotype 2-infected patients

Study name	Population	Treatment	Duration (wk)	*n*	LC (%)	SVR12 (%)		
						All	Non-LC	LC
FISSION	Naïve	SOF/RBV	12	70	-	97	-	-
POSITRON	IFN-ineligible/intolerant	SOF/RBV	12	109	15	93	92	94
VALENCE	Naïve	SOF/RBV	12	32	-	97	97	100
Japanese study	Naïve	SOF/RBV	12	90	9	98	97	100
FUSION	Experienced	SOF/RBV	12	36	28	86	96	60
			16	32	-	94	100	78
VALENCE	Experienced	SOF/RBV	12	41	-	90	91	88
Japanese study	Experienced	SOF/RBV	12	63	14	95	96	89

LC: Liver cirrhosis; SVR12: Sustained virologic response at 12 wk; Naïve: Treatment-naïve; Experienced: Treatment-experienced; SOF: Sofosbuvir; IFN: Interferon; RBV: Ribavirin.

moderate hepatic impairment, and patients with severe hepatic impairment, respectively[31]. All 6 patients with fibrosing cholestatic hepatitis achieved SVR12. Response rates in the 12- and 24-wk treatment groups were similar[31]. The use of sofosbuvir plus simeprevir for 12 and 8 wk in HCV genotype 1-infected patients without cirrhosis resulted in 83% and 97% SVR12 rates, respectively[33]. Likewise, the use of sofosbuvir plus simeprevir for 12 wk resulted in an 83% SVR12 rate in HCV genotype 1 - infected patients with cirrhosis[34].

HCV genotype 2-specific virologic response of sofosbuvir

The results from phase 3 clinical trials evaluating the use of treatments containing sofosbuvir against HCV genotype 2-infected patients are shown in Table 2. Three phase 3 trials have evaluated previously untreated patients (the FISSION, POSITRON and VALENCE studies)[23,29-31]. In the FISSION study, 12 wk of sofosbuvir plus ribavirin treatment and 24 wk of Peg-interferon α-2a (Peg-IFNα2a) plus ribavirin treatment produced comparable results in treatment-naïve patients. The SVR12 rate was 97% in a group of 70 patients who received sofosbuvir plus ribavirin and 78% in a group of 67 patients who received Peg-IFNα2a plus ribavirin. The noninferiority of the sofosbuvir plus ribavirin regimen relative to interferon-based therapy was shown. Furthermore, adverse events were less frequent with the sofosbuvir plus ribavirin regimen[26,35].

In the POSITRON study, 109 patients for whom treatment with Peg-IFN was not an option received 12 wk of sofosbuvir plus ribavirin, and 78% of these patients achieved SVR12[36]. These results were confirmed by the VALENCE study, in which 97% of 32 treatment-naïve patients achieved SVR12[37].

In addition, the FUSION study[36] and the other arm of the VALENCE study evaluated the use of a sofosbuvir plus ribavirin regimen in treatment-experienced patients. In the FUSION study, 103 patients received sofosbuvir plus ribavirin for 12 wk, and 98 patients received the treatment for 16 wk. The rates of SVR12 were 86% and 94% in the 12 and 16-wk groups, respectively[36].

Omata *et al*[38] reported the efficacy and safety of a 12-wk sofosbuvir plus ribavirin treatment in Japanese patients. The rates of SVR12 were 98% and 95% in treatment-naïve and previously treated patients, respectively. According to these trials, the use of sofosbuvir plus ribavirin has become a standard of care for the treatment of HCV genotype 2-infected patients.

HCV genotype 3-specific virologic response of sofosbuvir

HCV genotype 3 has become the most difficult genotype to cure in the era of interferon-free therapy. Although sofosbuvir is considered to have pangenotypic inhibitory activities, its treatment efficacy against HCV genotype 3 is lower than against the other genotypes. The results from phase 3 clinical trials evaluating treatments containing sofosbuvir to combat HCV genotype 3 are shown in Table 3.

In the FISSION study, SVR12 occurred in 56% of patients who received 12 wk of sofosbuvir plus ribavirin and in 63% of patients who received 24 wk of peginterferon plus ribavirin. These results were poor, as 97% of patients infected with HCV genotype 2 achieved SVR12 following treatment with sofosbuvir plus ribavirin in the same study[26,35].

The results of the POSITRON study were similar to those of the FISSION study. In the FISSION study, 61% patients infected with HCV genotype 3 achieved SVR12 compared to 93% of patients infected with HCV genotype 2. The rate of SVR12 in patients with cirrhosis was especially low in the HCV genotype 3 group (21%)[36].

Although the above results are somewhat disappointing, extending the duration of treatment might improve sofosbuvir's efficacy against HCV genotype 3. In the FUSION study, 16 wk of sofosbuvir plus ribavirin therapy resulted in higher SVR12 rates than 12 wk therapy (37% *vs* 63% in patients without cirrhosis; 19% *vs* 61% in patients with cirrhosis)[36].

In addition, the VALENCE study was conducted to assess the efficacy of 24 wk of sofosbuvir plus ribavirin treatment in 250 HCV genotype 3-infected patients[37]. The rates of SVR12 were 95% and 92% in treatment-naïve patients with and without cirrhosis, respectively, and 87% and 62% in previously treated patients with and without cirrhosis. A longer treatment duration

Table 3 Summary of phase 3 study for hepatitis C virus genotype 3-infected patients

Study name	Population	Treatment	Duration (wk)	*n*	LC (%)	SVR12 (%)		
						All	Non-LC	LC
FISSION	Naïve	SOF/RBV	12	183	-	56	-	-
POSITRON	IFN-ineligible/intolerant	SOF/RBV	12	98	15	61	68	21
VALENCE	Naïve	SOF/RBV	24	105	12	93	95	92
FUSION	Experienced	SOF/RBV	12	64	39	30	37	19
			16	63	-	62	63	61
VALENCE	Experienced	SOF/RBV	24	145	32	79	87	62

LC: Liver cirrhosis; SVR12: Sustained virologic response at 12 wk; Naïve: Treatment-naïve; Experienced: Treatment-experienced; SOF: Sofosbuvir; IFN: Interferon; RBV: Ribavirin.

Table 4 Summary of phase 3 study for hepatitis C virus-human immunodeficiency virus co-infected patients

Genotype	Study name	Population	Treatment	Duration (wk)	*n*	LC (%)	SVR12 (%)
1	PHOTON-1	Naïve	SOF/RBV	12	114	4.4	76
	PHOTON-2	Naïve	SOF/RBV	24	112	15	85
2	PHOTON-1	Naïve	SOF/RBV	12	26	-	88
		Experienced	SOF/RBV	24	24	-	92
	PHOTON-2	Naïve	SOF/RBV	12	19	5	89
		Experienced	SOF/RBV	24	6	33	83
3	PHOTON-1	Naïve	SOF/RBV	12	42	-	67
		Experienced	SOF/RBV	24	17	-	94
	PHOTON-2	Naïve	SOF/RBV	24	57	5	91
		Experienced	SOF/RBV	24	49	47	86
4	PHOTON-2	Naïve	SOF/RBV	24	31	26	84

LC: Liver cirrhosis; SVR12: Sustained virologic response at 12 wk; Naïve: Treatment-naïve; Experienced: Treatment-experienced; SOF: Sofosbuvir; RBV: Ribavirin.

was not associated with a higher frequency of adverse events or treatment discontinuation. However, further studies will be necessary to improve treatment efficacy in previously treated patients with cirrhosis.

Foster *et al*[23] reported a 93% SVR12 rate following 12 wk of treatment with peginterferon, sofosbuvir and ribavirin *vs* an 84% SVR12 rate following 24 wk of treatment with sofosbuvir plus ribavirin *vs* an 88% SVR12 rate following 12 wk of treatment with peginterferon, sofosbuvir and ribavirin in cirrhotic patients. Treatment with daclatasvir plus sofosbuvir for 12 wk led to a 96% SVR12 rate in HCV genotype 3-infected patients without cirrhosis[39].

Other HCV genotypes-specific virologic response of sofosbuvir

In the NEUTRINO study, 35 patients infected with HCV genotypes 4 through 6 were treated with sofosbuvir, ribavirin and peginterferon for 12 wk. The rates of SVR12 were 96% and 100% in patients infected with HCV genotypes 4 and 5-6, respectively[26]. A treatment regimen that contains sofosbuvir might therefore be efficient against these genotypes.

The use of a ribavirin- and interferon-free regimen of ledipasvir/sofosbuvir for 12 wk resulted in an SVR4 rate of 93% in genotype 4 and genotype 5 HCV-infected treatment-naïve and treatment-experienced patients with or without cirrhosis[40].

HIV co-infection

The PHOTON-1 and -2 studies were conducted to evaluate the use of sofosbuvir plus ribavirin therapy to combat HCV-human immunodeficiency virus (HIV) co-infection[41,42]. The results of these trials are summarized in Table 4.

In the PHOTON-1 study, in patients infected with HCV genotype 1, 12 wk of sofosbuvir plus ribavirin led to a 78% SVR12 rate. Extending treatment to 24 wk tended to result in higher rates of SVR (85%)[41].

The use of a longer treatment duration also appeared to be effective in treatment-naïve patients infected with HCV genotype 3. Similar to the results from the study against HCV genotype 3 infection alone, 12 wk of sofosbuvir plus ribavirin therapy attained low rates of SVR12 compared with other genotypes in the PHOTON-1 study (67%)[32]. Extending treatment from 12 to 24 wk improved the rate of SVR12 in the PHOTON-2 study (91%)[42]. Among patients infected with other HCV genotypes, high rates of SVR12 (83%-92%) were attained after 12 wk of sofosbuvir plus ribavirin treatment in treatment-naïve patients and 24 wk of the above treatment in previously treated patients[41,42].

The ALLY-2 study examined the use of daclatasvir plus sofosbuvir in patients co-infected with HIV and HCV genotype 1. The results showed that SVR rates were 96.4% following 12 wk of treatment and 75.6% following 8 wk of treatment in treatment-naïve patients.

The SVR12 rate was 97.7% in treatment-experienced patients following 12 wk of treatment with the above regimen[43].

The ION-4 study evaluated the use of ledipasvir plus sofosbuvir for 12 wk in HIV co-infected patients. The SVR12 rates were 96% in HCV genotype 1a-infected patients, 96% in HCV genotype 1b-infected patients, and 100% in HCV genotype 4-infected patients[44].

CONCLUSION

Sofosbuvir, a first-in-class NS5B inhibitor, has rapidly become the standard of care for the treatment of numerous HCV genotypes. However, its efficacy against HCV genotype 3, especially in patients with cirrhosis, has not been satisfactory. The optimal duration of treatment and use of novel combinations with other DAAs should be examined in the future.

Patients with severe renal impairment (estimated glomerular filtration rate < 30 mL/min per 1.73 m^2) and on hemodialysis are contraindicated for sofosbuvir-containing regimens. This limitation of sofosbuvir should be recognized. Nevertheless, sofosbuvir is an important drug that possesses high efficacy and safety. Sofosbuvir-containing therapy has become a standard of care for the majority of patients with HCV infections.

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MINIREVIEWS

Ablation techniques for primary and metastatic liver tumors

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Abstract

Ablative treatment methods have emerged as safe and effective therapies for patients with primary and secondary liver tumors who are not surgical candidates at the time of diagnosis. This article reviews the current literature and describes the techniques, complications and results for radiofrequency ablation, microwave ablation, cryoablation, and irreversible electroporation.

Key words: Liver; Ablation; Hepatocellular carcinoma; Metastasis; Radiofrequency; Microwave; Cryoablation

Core tip: Innovative ablation techniques, including radiofrequency ablation, microwave ablation, cryoablation and irreversible electroporation have become accepted as treatment modalities for patients with early stage tumor or for single metastases. This review paper describes the available ablation techniques and summarizes the evidence supporting the use of each modality.

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INTRODUCTION

The liver is a common site for both primary malignancy and metastatic disease. Hepatocellular carcinoma (HCC) remains the fifth most common malignancy in the world and its incidence is rising[1,2]. Traditionally, the first line therapy for hepatic tumors has been surgical resection or transplantation. However, many patients are not surgical candidates at the time of diagnosis[2]. For this reason interest in minimally invasive, ablative treatment methods has grown[3]. Percutaneous ablative techniques include radiofrequency ablation (RFA), microwave ablation, cryoablation, and irreversible electroporation (IRE).

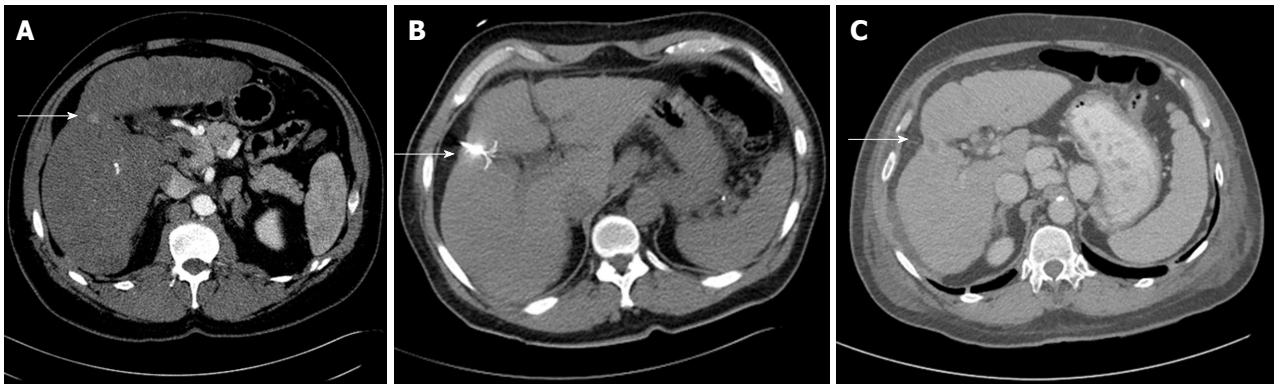

Figure 1 Sixty-eight-year-old male with hepatitis C and cirrhosis. A: Contrast enhanced CT shows a 16 mm HCC; B: RFA probe covering the lesion; C: Post contrast follow up CT shows capsular retraction at the site of the RFA and no residual tumor. HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; CT: Computed tomography.

This review focuses on the use of percutaneous ablative techniques in the treatment of HCC, as well as of metastatic disease from colorectal, neuroendocrine, and breast carcinomas.

TECHNIQUE AND COMPLICATIONS

RFA

RFA is a low risk alternative treatment for HCC and liver metastases in patients who cannot undergo surgery or transplant[4]. Unlike other non-surgical strategies (TACE, Y90), the goal of RFA is curative[4].

Technique

RFA creates a closed loop circuit which results in an alternating electric field causing agitation of ions within the target tissue[5]. The circuit is created using an RF generator, an electrode, grounding pads, and the patient[3]. The resultant ionic agitation creates heat leading to cell death from coagulative necrosis[6]. In order to ensure tumor destruction, the mass needs to be treated to a temperature of 50 ℃-100 ℃ for approximately 4-5 min[6]. Temperatures higher than 100 ℃ can cause gas formation, also known as carbonization, which can reduce ablation effectiveness, and char adjacent tissues[7].

In order to achieve primary technical success, the entire tumor must be ablated as well as a sufficient margin around the tumor. Similar to surgical techniques, a 1 cm margin in all planes is needed to minimize the risk of residual disease or local recurrence[3]. Therefore, the planned target ablation diameter should be 2 cm larger than the tumor diameter[3]. If the tumor is small enough, this can be accomplished with one electrode (Figure 1). However, if the tumor is too large, multiple ablations can be performed[8], although there is a risk of local recurrence due to inadequate tumor destruction from the error inherent in positioning electrodes[3]. Other causes of inadequate tumor ablation include heterogeneous tissue composition (*i.e.,* fibrosis, calcification) and adjacent blood flow, known as a "heat sink", which can cool the tissue and reduce the maximum achieved temperature[9].

RFA can be performed with guidance by ultrasound (US), computed tomography (CT), or magnetic resonance imaging (MRI) depending on lesion visibility and operator experience. Patients typically receive either conscious sedation or general anesthesia to control pain and minimize patient movement during the procedure. The decision to administer prophylactic antibiotics is somewhat controversial and institution dependent. A longer course of antibiotics may be warranted in patients who are at increased risk of liver abscess, including patients with a history of biliary-enteric anastomosis, biliary stents, or sphincterotomy[6]. This is thought to be due to retrograde movement of bacteria into the ablation cavity as a result of altered anatomy[10].

Complications

RFA has a low rate of major complications. The largest study on RFA complications by Koda *et al*[11] evaluated 13283 patients (16346 treated lesions) with a total of 579 complications (3.5%) and 5 deaths (0.04%). The rate of liver injury was 1.69% (276 patients) which included 75 (0.47%) hepatic infarcts, 32 (0.19%) liver abscesses, 110 (0.67%) bile duct injuries, and 37 (0.23%) bile leaks[11]. A more recent study from Lee *et al*[1] reported a similar major complication rate of 3.1% in 169 treated lesions, including 2 bile duct injuries. The overall reported complication rate ranges from 2.2% to 9.5%[6].

The rate of extrahepatic injury is also extremely rare. Koda *et al*[11] reported a total of 113 (0.69%) extrahepatic complications including, in order of decreasing frequency, pleural effusions, skin burns, pneumothorax, gastrointestinal injury, diaphragmatic injury, gallbladder injury, and cardiac tamponade. The risk of extrahepatic injury can be reduced by a technique called "hydrodissection", which involves injecting D5W to create space between adjacent organs. Saline infusions are not used for hydrodissection due to the theoretical risk of conduction of the electrical current through this type of fluid. Another potential complication is seeding, either in the peritoneum or along the ablation track. The

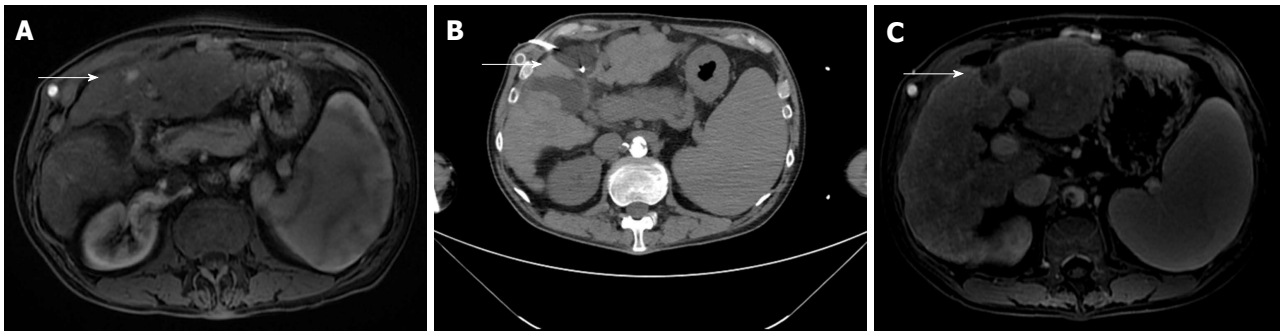

Figure 2 Sixty-one-year-old with a history of alchohol abuse and cirrhosis. A: MRI demonstrates a 13 mm HCC in the left lobe; B: Two cryoablation probes covering the lesion; C: Post contrast follow up MRI shows capsular retraction at the site of the cryoablation and no residual tumor. MRI: Magnetic resonance imaging; HCC: Hepatocellular carcinoma.

reported risk of tumor seeding ranges from 0.04%[11] to 0.6%[12]. The risk (0.95%) of tumor seeding has been described to be slightly increased when concomitant biopsy is performed[12].

Cryoablation

Cryoablation involves rapid cooling of a cryoprobe resulting in cell death[13]. Cryoablation has been historically used for both HCC (Figure 2) and hepatic metastases.

Technique

Cryoablation works by passing high pressure argon gas through a probe resulting in cooling of the metallic. As the probe cools, surrounding tissues are also cooled by convection and conduction[14]. Helium gas is then forced through the probe causing warming of the probe and thawing of the adjacent tissues. The cooling and subsequent thawing of the probe results in cell death by a variety of methods. The initial cooling results in intracellular ice crystal formation leading to cell membrane damage and death[15]. Larger ice crystals also form during slow thawing, resulting in a shearing effect and additional cell death[16]. Lastly, ice crystals develop in the small blood vessels feeding a tumor, leading to ischemia[16]. Like the other ablative techniques, cryoablation can be performed percutaneously or laparoscopically. Percutaneous cryoablation can be performed with CT, MRI or US guidance.

Although cryoablation has many uses for tumor ablation, including renal and osseous lesions, its utility in the liver is somewhat limited. The disadvantages of cryoablation include variable ablation size (resulting in the need for multiple cryoprobes), reduced cooling effect due to a heat sink from hepatic vessels, and the risk of major complications. An advantage of cryoablation over other ablative techniques is that the ice ball can be visualized during the procedure under both CT and ultrasound guidance, allowing for better adjustment.

Complications

The risk of complication within the liver is higher with cryoablation compared to RFA. Complications include hemorrhage, injury to adjacent organs, biliary injury, and "cryoshock". Hemorrhage results from ice ball formation within the liver leading to shearing injury to the liver parenchyma and nearby blood vessels. Shearing forces can also cause biliary injury which can lead to late hemorrhage or hepatic abscess formation. Cryoablation of lesions near the liver edge risks damage to adjacent organs, usually bowel, kidney or adrenal glands. A complication unique to cryoablation is "cryoshock" which occurs due to the release of cytokines, resulting in a systemic syndrome characterized by fever, tachycardia, and tachypnea. A retrospective study by Adam *et al*[17] found increased complication rates among patients treated with cryoablation (29%) compared to those who underwent RFA (8%). Additional studies have found similar results including a study demonstrating a 41% complication rate for cryoablation patients compared to 3% in patients who underwent RFA[18].

However, a large study by Yang *et al*[19] found very low rates of major complications with cryoablation. In this study of 300 patients who underwent cryoablation, the major complication rate was 6.3%[19]. Major complications included cryoshock (6 patients), extensive hemorrhage (5 patients), gastric bleeding (4 patients), liver abscess (1 patient), intestinal fistula (1 patient), and liver failure (2 patients)[19]. The risk of minor complications is reported to be 48.6%[19]. These include fever, pain, skin frostbite, pleural effusion, and arterial-portal venous fistula. Pneumothorax is rarely reported in treated tumors located near the diaphragm[19].

MICROWAVE ABLATION

Background/indications

Microwave ablation is an emerging technology with particular applicability in treating hepatic tumors in patients who are not surgical candidates. It has been used for larger tumors than those treated by RFA[20].

Technique

Microwave ablation utilizes an antenna to locally deliver a high frequency (915 MHz or 2.45 GHz) oscillating electromagnetic field to induce rapid realignment of polar molecules (typically water molecules) in a lesion

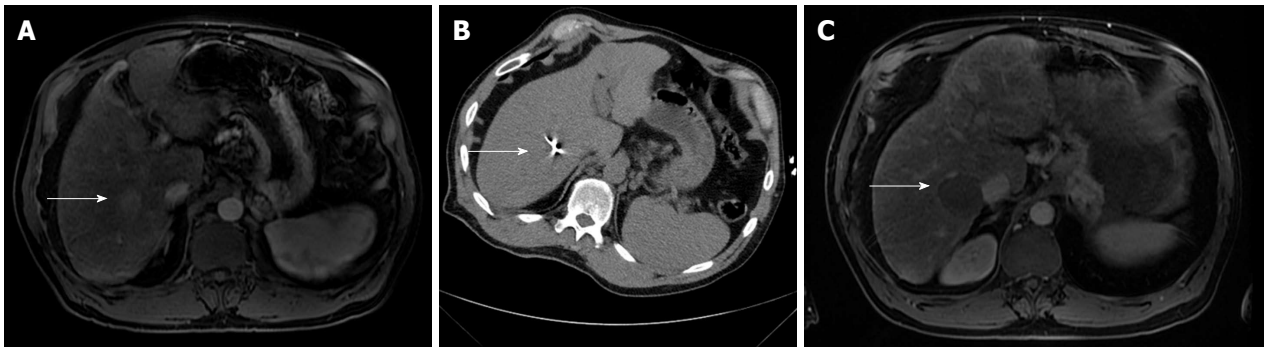

Figure 3 Sixty-two-year-old with hepatitis C cirrhosis. A: MRI shows an arterial enhancing lesion consistent with hepatocellular carcinoma; B: CT guided microwave ablation of the right hepatic lobe lesion; C: MRI shows an ablation zone and no evidence of residual tumor. MRI: Magnetic resonance imaging; CT: Computed tomography.

(Figure 3). This results in markedly increased kinetic energy and subsequent tissue heating[21]. Tissues with a larger concentration of water, such as tumors, are particularly susceptible to microwave heating[21].

Microwave ablation can be performed with one or multiple antenna probes. Multiple antenna probes in close proximity allow for electrical and thermal synergy. Multiple probes can also be powered simultaneously which is not possible with RF ablation. Recent developments in microwave technology have produced high-powered water cooled systems which allow for smaller applicators and increased power.

Compared to RF ablation, microwave has several advantages. Microwave is capable of producing very high temperatures (greater than 150 ℃) much faster than RF. In addition, microwave is more effective in propagating heating through charred and desiccated tissues which allows for a large ablation zone. Microwave does not require grounding pads or other similar devices[15]. Microwave ablation is not as susceptible to heat sink phenomena as RF ablation. This is particularly useful in the liver, which has a rich vascular supply. A recent study demonstrated larger zones of ablation and faster heating with microwave compared to RFA[22]. Additional studies have demonstrated larger and more consistent ablation zones with microwave without significant influence from adjacent hepatic vessels[23,24]. Ablation time is often less than 10 min, typically averaging 2-5 min, which improves overall efficiency and reduces anesthesia time.

Although microwave ablation is promising, several disadvantages have limited its widespread adoption. Compared to RFA, microwave power is more difficult to generate safely, mostly due to larger cables which are prone to heating issues[21]. In addition there remains still uncertainty about the size and shape of ablation zones with microwave[21].

Microwave ablation is typically performed under general anesthesia to reduce patient discomfort and for better control of patient breathing and motion. As with RF ablation, microwave can be performed under CT or ultrasound guidance. Ultrasound allows for real time monitoring of the ablation and shorter procedure time. CT guidance allows for localization of lesions which are difficult to visualize, and for better evaluation of adjacent structures. Hydrodissection can be used to displace adjacent structures, typically bowel or diaphragm.

Complications

A systematic review of the literature by Lahat *et al*[25] evaluated the safety of ablative techniques including microwave ablation. In the review of 16 studies, they reported a major complication rate of 4.6% for microwave ablation compared to 4.1% for RFA. The pooled mortality rate for microwave was 0.23% compared to 0.15% for RFA. The most common major complication was hemorrhage requiring blood transfusion. Additional complications included portal vein thrombosis, bile leak/biloma, liver abscess, pleural effusion, and tumor seeding.

IRE

IRE is a relatively new non-thermal ablative technique approved by the Food and Drug Administration in 2006 for soft tissue ablation[26]. It has been used for liver, pancreas, kidney and lung ablations. IRE has several advantages over current, more proven ablative techniques.

Technique

IRE utilizes multiple electrodes to deliver high voltage (2-3 kV) direct current pulses lasting microseconds to milliseconds[27]. The repeated electrical pulses cause damage to the cell membranes[26]. Initially the cell membrane damage is reversible, but it becomes irreversible after a period of time leading to apoptosis[26]. Because of the extremely short ablation time, care must be taken to ensure proper electrode positioning as mid treatment adjustment is not possible. Most IRE devices require simulation planning with the use of multiple probes placed in parallel to achieve the desired ablation zone.

IRE results in a well-defined ablation zone with sharp margins and relatively little damage to nearby tissues[27]. Because IRE does not utilize thermal methods for ablation, adjacent tissue architecture is well preserved[28]. The combination of fast ablation times

and minimal damage to nearby tissues makes IRE well suited for treatment of lesions in sensitive locations, including those adjacent to blood vessels and bile ducts. In addition, this eliminates the problems with heat sink seen in other thermal ablative techniques. However, the use of multiple parallel probes results in a significant increase in procedural cost and complexity[29]. One potential drawback to IRE is that imaging changes related to the ablation zone may take several minutes to manifest by ultrasound[30]. IRE also requires general anesthesia with paralytic agents as the electrical current generated during the procedure can cause muscle spasms and arrhythmias[31]. To lessen this risk, the IRE generator is connected to an ECG triggering device and pulses are delivered to the target/treatment zone during the cardiac refractory period[27].

Complications

A recent large systematic review investigated the safety and efficacy of IRE in several organs. The reported overall complication rate was 16% in 129 treated patients[26]. The most common complications included pneumothorax, portal vein thrombosis, biliary occlusion, pleural effusion, and cholangitis[26]. There was no periprocedural mortality reported in treated liver lesions, although 3 patients died after pancreatic IRE[26]. Self-reported post-procedural pain scores were similar between patients treated with IRE and RFA. Arrhythmias were reported in 4% of cases[26]. Ventricular arrhythmias were seen without synchronized pulse delivery while only atrial arrhythmias were seen in patients who received synchronized pulses[26]. No uncontrolled muscle spasms were reported in any of the reviewed studies in patients who received paralytic agents[26].

RESULTS OF INNOVATIVE ABLATION TECHNIQUES

HCC

Radiofrequency ablation: Numerous studies support the usage of RFA as a first line treatment for HCC in patients who are poor surgical candidates. One of the largest studies by Tateishi *et al*[32] evaluated RFA of 2140 nodules measuring less than 3 cm in 664 patients. Survival rates at 1-5 years post-treatment were similar for patients with first line RFA alone compared with those who underwent RFA as part of a combination therapy[32]. In addition, the rate of local progression of disease was similar for RFA alone when compared to ethanol treatment or hepatectomy[32]. A study by Lencioni *et al*[33] evaluated patients with early stage HCC (single lesion < 5 cm or up to 3 lesions < 3 cm each) who underwent RFA alone or palliative TACE or ethanol injection. Overall survival rates at 5 years were 48% with a median survival of 57 mo for the RFA group, which was not significantly different from the TACE or ethanol groups[33]. Histologic analysis of tumors which underwent RFA and subsequent transplantation found that 74% of ablated tumors were treated successfully by histologic criteria[34]. For tumors measuring less than 3 cm, the percentage successfully treated rose to 83%[34]. Another large study of 1502 HCC tumors in 1305 patients over 12 years by Kim *et al*[35] found survival rates of 59.7% and 32.3% at 5 and 10 years respectively. Additional studies have demonstrated similar overall recurrence and survival rates for patients who were poor surgical candidates using RFA as first line treatment[36].

Several recent studies have evaluated RFA as a first line treatment in tumors measuring more than 3 cm. A study by Lee *et al*[1] evaluated 162 patients who underwent RFA for up to three tumors with a maximum diameter of 5 cm. Overall 5 year survival and recurrence-free survival rates were 67.9% and 25.9% respectively[1]. The most significant predictors of poor survival were Child-Pugh class B, elevated serum α-fetoprotein level, and presence of portal-systemic collaterals[1]. The rate of local tumor progression at 5 years was 14.5% with tumor size being the only significant predictive factor[1]. Local tumor progression did have a significant negative effect on median recurrence free survival (28.0 mo *vs* 12.0 mo) and resulted in over two times more interventional procedures[1]. A study by Livraghi *et al*[37] evaluated RFA of 126 HCCs larger than 3 cm in 114 patients. Complete necrosis on follow up CT scan was observed in 47.6% of patients and near complete necrosis (90%-99%) was observed in 31.7% of patients. The observed complication rate was similar to other studies[37].

More recent studies have called into question the conclusion that RFA is equivalent to surgery in the treatment of HCC. A recent meta-analysis by Qi *et al*[38] evaluated 3 randomized control trials. Surgical resection was found to be superior to RFA with respect to overall survival (HR = 1.41) and recurrence free survival (HR = 1.41)[38]. However, surgical patients had a significantly higher incidence of complications and a significantly longer hospital stay than patients treated with RFA[38]. A more recent study by Miura *et al*[39] investigated 2804 patients who underwent ablation or surgical resection for a solitary HCC < 3 cm. Overall survival at 3 and 5 years was higher in the resection group (67%, 55%) than in the RFA group (52%, 36%)[39]. There were baseline differences between the two groups which somewhat limited the analysis. However, after propensity matching, the overall survival rate was still higher in the resection group (54%) *vs* RFA (37%)[39]. Surgical resection was also independently associated with improved survival (HR = 0.62)[39].

Cryoablation: Multiple studies have evaluated the utility of cryoablation in the treatment of HCC. Chen *et al*[40] performed percutaneous cryoablation in 76 lesions of unresectable HCC and 76 lesions of recurrent HCC. 1 and 3 year survival rates in the unresectable group were 81.4% and 60.3% while the disease-free survival rates were 67.6% and 20.8%[40]. Survival rates in the recurrent HCC group were 70.2% and 28.8% at 1 and 3 years respectively, while the disease-free survival

rates were 53.8% and 7.7%. There was a low overall complication rate (12.1%) and there were no peri-procedural deaths[40]. A similar study by Wang *et al*[41] evaluated cryoablation of 156 patients with HCC < 5 cm in diameter. The reported 1, 2 and 3 years overall survival rates were 92%, 82% and 64%[41]. Disease free survival rates were 72%, 56% and 43% at 1, 2 and 3 years[41].

One of the largest studies evaluating cryoablation and HCC was performed by Yang *et al*[19] and looked at 300 patients with unresectable HCC. A total of 223 tumors were incompletely ablated while 185 tumors were completely ablated[19]. The rate of local progression of disease at a median 36.7 mo follow up time was 31%[19]. The most significant risk factors for tumor recurrence were size and tumor location. The mean survival of patients after cryoablation was 45.7, 28.4 and 17.7 mo, in increasing order of tumor stage[19]. A study by Adam *et al*[17] looked at cryoablation *vs* RFA for unresectable HCC. Despite similar initial post-treatment results, they found a significantly higher rate of local progression of disease in patients treated with cryoablation *vs* RFA (53% *vs* 18%)[17].

Microwave: Many studies have demonstrated the safety and effectiveness of microwave ablation in the treatment of HCC. Dong *et al*[42] studied 234 patients who underwent microwave ablation, showing 1, 2, 3, 4 and 5 years survival rates of 92.7%, 81.6%, 72.9%, 66.4% and 56.7%. The reported local recurrence rate was 7%[42]. A more recent study from Ziemlewicz *et al*[43] of microwave ablation in 107 HCC lesions found an overall survival rate of 76.0% at median 14 mo follow up. The primary effectiveness was 93.7% for tumors 4 cm or smaller and 75.0% for tumors greater than 4 cm[43], with an overall primary effectiveness of 91.6%. This illustrates the ability of microwave to effectively treat larger tumors measuring more than 4 cm in diameter. No major complications or mortality were reported[43]. A study of microwave ablation in 182 patients with a single HCC was performed by Sun *et al*[44]. The complete ablation rate was 93%[44]. The overall survival rates were 89%, 74% and 60% at 1, 2 and 3 years respectively, while the recurrence-free survival rates were 51%, 36%, 27% at 1, 2 and 3 years respectively. Tumor recurrence was associated with increasing patient age and tumor size. The major complication rate was 2.7%[44].

Microwave ablation also compares favorably to treatment with RFA. A study of 102 patients with HCC found similar complete ablation rates of 94.9% for microwave and 93.1% for RFA[45]. The local recurrence rate was better with microwave ablation (11.8%) when compared to RFA (20.9%)[45]. A similar study by Shibata *et al*[46] reported complete ablation rates of 89% for microwave ablation compared to 96% for RFA. Overall complication rates were also similar.

Irreversible electroporation: There is less data on the efficacy of IRE in comparison with other ablative techniques because the procedure is relatively new. However, several studies have demonstrated the efficacy of IRE in treating hepatocellular carcinoma. Cheung *et al*[47] evaluated IRE of 18 HCC lesions in 11 patients with a size range of 1.0-6.1 cm and a mean follow up of 18 mo. In tumors measuring less than 3 cm, complete ablation was achieved in 93%, with an overall 73% complete ablation rate. Cannon *et al*[48] reported a primary efficacy of 97% in 14 HCC lesions ranging in size from 1.1-5.0 cm. Thomson *et al*[31] performed IRE in 18 patients with HCC, achieving complete tumor ablation in 15 patients.

Metastatic disease

Radiofrequency ablation: Percutaneous RFA is also increasingly used to treat hepatic metastases, including metastases from colorectal carcinoma (CRCLM), neuroendocrine tumors, and breast cancer[4]. The requirements for surgical resection of metastases are similar to HCC and therefore only 10%-20% of patients are surgical candidates at the time of presentation[49]. The ideal candidate for RFA has biopsy proven hepatic metastases without underlying liver disease. A study of patients with colorectal metastases who were not surgical candidates and underwent RFA found survival rates of 86%-99%, 46%-68%, and 24%-44% at 1, 3 and 5 years respectively[9]. A study by Oshowo *et al*[50] of patients with a solitary CRCLM reported a 3-year survival rate of 52% in patients who underwent RFA *vs* 55% in patients who underwent surgery. Kim *et al*[51] found similar overall and disease free survival rates in patients who underwent resection *vs* RFA for a solitary CRCLM < 3 cm. The disease free survival rate was significantly lower in patients with metastases > 3 cm[51]. There is additional data supporting the role of RFA as an adjunctive therapy in palliative treatment of CRCLM *vs* chemotherapy alone. Berber *et al*[52] evaluated RFA in 135 patients with colorectal metastases and found a median survival of 28.9 mo, compared to 11-14 mo in patients who underwent chemotherapy alone.

RFA has also been successfully used in treating hepatic metastases from neuroendocrine tumors. As with HCC and colorectal metastases, only 10% of patients with neuroendocrine metastases are surgical candidates at the time of presentation. Berber *et al*[53] evaluated the role of RFA in treating patients with carcinoid syndrome, as well as other neuroendocrine metastases. Two hundred and thirty-four tumors in 34 patients were treated with RFA[53]. Symptoms were improved in 95% of patients with significant or complete symptom control seen in 80% of patients[53]. This was compared to a response rate of 90% with surgery and 50%-88% with somatostatin analogues[53]. The rate of local progression of disease was 26% during the follow up period (1.6 years) while 41% of patients had no evidence of disease progression during the same period[53]. Another study by Elvin *et al*[54] of 109 RFA treatments of neuroendocrine metastases showed a local recurrence rate of 10% during follow up (mean 3.2

years) with CT evidence of successful treatment in 90% of patients.

Cryoablation: The data on using cryoablation for metastatic disease is limited compared to the data for RFA since few centers use cryoablation for treating liver lesions. An older study by Kerkar *et al*[55] in 2004 evaluated 56 patients who underwent cryoablation for colorectal metastases. The 3 and 5 years overall survival rates in the colorectal metastases group was 43% and 22% with a median survival of 30 mo[55]. A more recent and larger study by Ng *et al*[56] reported the results of cryoablation in 293 patients with unresectable colorectal metastases. 1-, 3-, 5- and 10-year survival rates were 87%, 41.8%, 24.2% and 13.3%[56]. Disease-free survival rates were 37.9%, 17.2%, 13.4% and 10.8% at 1, 3, 5 and 10 years[56]. "Recurrences" were reported elsewhere in the liver in 73%, at the cryoablation site in 23%, and at the edge of the ablation cavity in 14%[56].

Seifert *et al*[57] reported results of cryoablation in 13 patients with metastatic neuroendocrine tumors. Twelve patients (93%) had complete ablations without reported local progression of disease on follow up imaging. Of additional clinical importance, 7 patients who had preoperative hormone-related symptoms experienced helpful palliative results[57].

Zhang *et al*[58] reported recent results with cryoablation of breast cancer metastases. They performed cryoablation of 39 liver metastases in 17 patients. Tumor response was 92% in the immediate post-op period and 87.1% at 1 mo. Local progression was seen in 6 lesions (15.4%) at 3 mo. The 1 year survival rate was 70.6%.

Microwave ablation: One of the first studies to evaluate microwave ablation in the treatment of metastatic disease was by Shibata *et al*[59]. They compared microwave ablation to surgical resection in patients with metastatic colorectal cancer and found similar 1, 2 and 3 years survival rates (71%, 57% and 14% for microwave and 69%, 56% and 23% for resection), and mean survival rates (27 mo for microwave *vs* 25 mo for resection)[59]. A study by Tanaka *et al*[60] also found similar survival and recurrence rates in patients who underwent microwave alone compared to microwave and eventual resection for colorectal metastases. Another study reported identical five-year survival rates (24%) for patients with colorectal metastases treated with microwave ablation *vs* microwave and surgery[61].

Irreversible electroporation: Silk *et al*[62] reported results of IRE in 9 patients with a total of 19 metastatic colorectal cancer lesions ranging from 1.0-4.7 cm. They reported an efficacy of 55% with local tumor recurrence in 5 of 9 patients at 9 mo[62]. Thomson *et al*[31] reported a primary efficacy of 67% in a total of 45 metastatic lesions (including colorectal, breast, and neuroendocrine cancers) treated with IRE. Kingham *et al*[63] evaluated IRE of 28 metastatic lesions including metastatic colorectal and neuroendocrine cancers. They reported a total local failure rate of 7.5% with time to recurrence ranging from 66-230 d.

ABLATION MODALITY

The choice of ablation modality is important to potential treatment success. While each case is unique and modality choice is often driven by local expertise and operator experience, several general concepts prevail. RFA is very safe and effective in smaller hepatic tumors. However, RFA is less effective with larger tumors and tumors near blood vessels. In contrast, microwave ablation has been shown to be more effective with larger tumor sizes and is affected less by the heat sink effect. Although cryoablation has historically been avoided with hepatic tumors due to concerns about complications, it has been used very safely more recently following the development of smaller probes. Lastly, in limited studies, IRE has been shown to be safe and effective in the treatment of both HCC and metastatic disease especially near sensitive structures such as blood vessels and bile ducts, although continued research is needed to demonstrate long term efficacy.

CONCLUSION

Percutaneous ablation has become widely accepted as a curative technique in the treatment of HCC and hepatic metastatic disease. Specifically, ablation is useful in the treatment of patients who are not surgical candidates but in whom curative treatment is desired. Percutaneous ablation is safe and effective. Although additional studies are needed, percutaneous ablation continues to evolve as an option in the treatment of HCC and metastatic disease.

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ORIGINAL ARTICLE

Observational Study

Cirrhotic cardiomyopathy: Isn't stress evaluation always required for the diagnosis?

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Author contributions: All authors had made substantial contributions to the study; Barbosa M, Guardado J, Marinho C, Quelhas I and Cotter J participated in the study concept and design; Barbosa M, Guardado J, Rosa B and Quelhas I were involved in acquisition, analysis and interpretation of the data; Barbosa M, Guardado J and Rosa B performed statistical analysis; Barbosa M and Guardado J drafted the manuscript; Marinho C, Quelhas I, Lourenço A and Cotter J reviewed the manuscript; all authors read and approved the final manuscript.

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Data sharing statement: Technical appendix, statistical code, dataset is available from the corresponding at maraisabelbarbosa@net.sapo.pt. Consent was not obtained but the presented data are anonymized and risk of identification is low.

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Abstract

AIM: To describe the proportion of patients with cirrhotic cardiomyopathy (CCM) evaluated by stress echocardiography and investigating its association with the severity of liver disease.

METHODS: A cross-sectional study was conducted. Cirrhotic patients without risk factors for cardiovascular disease were included. Data regarding etiology and severity of liver disease (Child-Pugh score and model for end-stage liver disease), presence of ascites and gastroesophageal varices, pro-brain natriuretic peptide (pro-BNP) and corrected QT (QTc) interval were collected. Dobutamine stress echocardiography (conventional and tissue Doppler imaging) was performed. CCM was considered present when diastolic and/or systolic dysfunction was diagnosed at rest or after pharmacological stress. Therapy interfering with cardiovascular system was suspended 24 h before the examination.

RESULTS: Twenty-six patients were analyzed, 17 (65.4%) Child-Pugh A, mean model for end-stage liver disease (MELD) score of 8.7. The global proportion of patients with CCM was 61.5%. At rest, only 2 (7.7%) patients had diastolic dysfunction and none of the patients had systolic dysfunction. Dobutamine stress echocardiography revealed the presence of diastolic dysfunction in more 6 (23.1%) patients and of systolic

dysfunction in 10 (38.5%) patients. QTc interval prolongation was observed in 68.8% of the patients and increased pro-BNP levels in 31.2% of them. There was no association between the presence of CCM and liver impairment assessed by Child-Pugh score or MELD (P = 0.775, P = 0.532, respectively). Patients with QTc interval prolongation had a significant higher rate of gastroesophageal varices comparing with those without QTc interval prolongation (95.0% *vs* 50.0%, P = 0.028).

CONCLUSION: CCM is a frequent complication of cirrhosis that is independent of liver impairment. Stress evaluation should always be performed, otherwise it will remain an underdiagnosed condition.

Key words: Dobutamine stress echocardiography; Cirrhotic cardiomyopathy; Cirrhosis; Corrected QT interval prolongation; Liver impairment

Core tip: Our study demonstrates that cirrhotic cardiomyopathy (CCM) is a frequent condition that is independent of the severity of liver disease. Furthermore, it shows that CCM is currently underdiagnosed, even after a comprehensive evaluation at rest. Consequently, a stress test should always be considered in the diagnostic approach to CCM, as it is here. Moreover, an association between corrected QT (QTc) interval prolongation and the presence of gastroesophageal varices was revealed, irrespective of the diagnosis of CCM. As such, the clinical significance of QTc interval prolongation is emphasized and it can be regarded as a marker of severe liver disease.

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INTRODUCTION

The presence of cardiac dysfunction related to chronic liver disease was first hipothesized by Kowalski *et al*[1] in 1953. Subsequent studies revealed that cirrhosis is associated with a hyperdynamic circulation (increased cardiac output and diminished systemic vascular resistance)[2-6], which corresponds to a high-output heart failure under resting conditions[7,8]. This clinical entity, formally named cirrhotic cardiomyopathy (CCM), is unrelated to the etiology of cirrhosis[3-5,8-10] and is different from alcoholic disease[9]. It has been defined as a cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease[11]. Some decades ago, this cardiac dysfunction was attributed to the effects of alcohol toxicity on the heart; however, data from studies performed since 1980s showed that the dysfunction at rest and the blunted cardiac response to stress was associated with cirrhosis *per se* rather than being an adverse effect of alcohol, which justify the specific cardiac disease term - "CCM"[3,8,12].

The pathophysiology of diastolic dysfunction is an increased stiffness of the myocardial wall, most likely because of a combination of mild myocardial hypertrophy, fibrosis and subendotelial edema[3,9]. Systolic dysfunction relates to the inability of the heart to maintain an adequate arterial blood pressure and output[5-7]. QT interval prolongation is the main electrophysiological abnormality in cirrhosis[2-8,13,14]. Several mechanisms have been implicated in the impaired contractile function of the cardiomyocyte: Down-regulation of β-adrenergic receptors and impaired β-adrenergic signalling, altered cardiomyocyte plasma membrane biophysical characteristics, increased activity of the endocannabinoids, nitric oxide and cytokines systems, as well as abnormal myofilaments[3-5,7-9]. Though almost always clinically silent at rest, due to diminished preload and afterload that occurs in liver cirrhosis, CCM has been described as a blunted ventricular contractile response usually unmasked by physiological, pharmacological and/or surgical stress[3-9]. Consequently, overt congestive heart failure might result[4,5,9]. CCM has been linked to sodium and water retention, ascites formation and hepatorenal syndrome development[7,11,15-17], mainly following infections such as spontaneous bacterial peritonitis[2,3,17-19]. The prevalence and natural history of CCM is not accurately known and studies that better describe it are still needed[4,5,8]. It has also been proposed that this condition has prognosis significance[5-7]. There is no specific treatment for CCM, the goal being the management of congestive heart failure[2-5,7,8]. β-blockers therapy reduce the prolonged QT interval towards normal values[20,21] but its impact on survival is not clear[12,22]. Though liver transplantation may initially aggravate the CCM, it remains the ultimate therapy for cardiovascular complications of cirrhosis, being associated with normalization of cardiac function with improvements in cardiac hypertrophy, diastolic and systolic functions and QT interval, several months after transplantation[5,7,23-25].

The aims of our study were: (1) to describe the proportion of patients with CCM evaluated by stress echocardiography in a population of cirrhotic patients, defining blunted ventricular contractile response and/or impaired diastolic relaxation as predictors of CCM; and (2) to investigate whether CCM is related to severity of liver disease.

MATERIALS AND METHODS

Study population

A cross-sectional study was conducted during 2011 and 2012. Cirrhotic outpatients followed at our department

were included. The diagnosis of cirrhosis was based on clinical, biochemical, echographic, endoscopic, and, when available, histological criteria. Exclusion criteria were: Age under 18, known or suspected risk factors for cardiovascular disease (diabetes, systemic hypertension, smoking and obesity defined as body mass index > 30 kg/m^2), pulmonary major illness, severe anemia (Hg < 7 g/dL), severe systemic disease, hyperthyroidism and hypothyroidism, pregnancy and baseline electrocardiographic or echocardiographic evidence of structural heart disease, such as bundle branch block, regional wall motion abnormalities or valvular heart disease. Inclusion criteria were as strict as dictated by the definition discussed at the 2005 World Congress of Gastroenterology in Montreal and presented thereafter, so the cardiac dysfunction can be attributed to the CCM *per se;* moreover, they are similar to that described in other reports[26,27]. All the therapy interfering with cardiovascular system was suspended 24 h before the electrocardiographic and echocardiographic examinations not to alter the examinations' results. The study protocol was approved by the Ethics Committee of our hospital. Written informed consent was obtained from every patient included in the study.

Clinical and analytical data

Data regarding gender, age, etiology and severity of liver disease (Child-Pugh classification and model for end-stage liver disease (MELD), presence of ascites, gastroesophageal varices, history of overt encephalopathy, heart rate and systolic and diastolic blood pressure were retrospectively recorded. Compensated disease refers to Child-Pugh class A and decompensated disease includes Child-Pugh class B or C patients.

Laboratory parameters - hematological, biochemical [including pro-brain natriuretic peptide (pro-BNP)] and clotting profiles were measured in fasting venous blood samples.

Electrocardiographic examination

Patients were submitted to a 12-lead electrocardiogram and corrected QT interval (QTc interval), adjusted for heart rate, was calculated, according to Bazett's formula [QTc = QTmax/(RR)$^{1/2}$ interval]. A QTc interval > 440 ms was considered prolonged.

Echocardiographic examination

Transthoracic echocardiographic examination (standard 2D-echocardiographic imaging, pulsed Doppler interrogation of mitral inflow and tissue Doppler imaging (TDI) of the annular region of the left ventricle) using a General Electric™ Vivid 7, was performed by an experienced cardiologist in the echocardiography laboratory. Diastolic and systolic functions were evaluated at rest and after pharmacological stress with intravenous infusion of dobutamine. An initial dose of dobutamine at 5 μg/kg per minute was administered and increased to 10, 20, 30 and 40 μg/kg per minute every 3 min, in order to achieve, at least, 85% of maximum heart rate predicted for age (220-age). In case maximum heart rate was not reached, atropine (0.25 mg every minute up to maximum dose of 1 mg) was added to the 40 μg/kg per minute dobutamine infusion. After the examination, intravenous metoprolol (2.5 mg every 5 min up to a maximum dose of 10 mg) was administered until basal heart rate was achieved. Off-line analysis of echocardiographic images was performed by two investigators under blind conditions and agreement between the two observers was required.

Echocardiographic systolic left ventricular function parameters [left ventricular end-diastolic volume (LV EDV), LV end-systolic volume (LV ESV) and LV ejection fraction (LV EF) using Simpson's rule] were evaluated at rest and after a dobutamine inotropic dose perfusion (20 μg/kg per minute), as recommended to assess LV contractile reserve (LV CR). LV systolic dysfunction was considered present at rest when LV EF was below 50%. A reduced left ventricular contractile reserve was defined as an increase in LV EF < 10% after dobutamine infusion.

To assess left ventricular diastolic function, peak early filling (E-wave) velocity, late diastolic atrial filling (A-wave) velocity and E-wave deceleration time (DT) were measured by pulsed Doppler examination and E/A ratio (early diastolic/atrial filling ratio) was calculated. Average early diastolic myocardial velocity (e'-septal and lateral sides of the mitral annulus average) was obtained by TDI. E/e' average ratio was calculated combining the parameter E from the pulsed wave Doppler and the parameter e' from the TDI. Left ventricular diastolic dysfunction at rest was diagnosed if septal e' velocity was < 8 cm/s and lateral e' velocity was < 10 cm/s, according to the American Society of Echocardiography recommendations[28] (patients whose e' velocities values were within normal limits for age were considered as not having diastolic dysfunction). Three categories of increasing severity of diastolic dysfunction were defined according to the following parameters: Grade Ⅰ - E/e' average ratio ≤ 8, E/A ratio < 0.8 and DT > 200 ms; grade Ⅱ - E/e' average ratio between 9 and 12, E/A between 0.8 and 1.5 and DT between 160 and 200 ms; and grade Ⅲ - E/e' average ratio ≥ 13, E/A ≥ 1.5. E/e' average ratio at rest and after stress (using dobutamine maximum dose perfusion) has been applied in the diastolic stress test. If myocardial relaxation is normal, E and e' velocities increase proportionally, and the E/e' ratio remains unchanged or is reduced; in patients with impaired myocardial relaxation, the increase in e' with stress is much less than that of mitral E velocity and the E/e' ratio increases.

CCM

CCM was diagnosed when LV diastolic dysfunction and/or systolic dysfunction was present, irrespective of the presence of other supportive criteria [electrophysiological abnormalities as QTc interval prolongation or increased

Table 1 Baseline characteristics of cirrhotic patients

	n = 26 patients
Gender (male), *n* (%)	22 (84.6)
Age (yr)	54.6 ± 10.4
Cirrhosis etiology	
Alcoholic, *n* (%)	20 (77.0)
Viral, *n* (%)	3 (11.5)
Mixed, *n* (%)	3 (11.5)
Child-Pugh score (units)	6.2 ± 1.3
Child-Pugh class	
A, *n* (%)	17 (65.4)
B, *n* (%)	8 (30.8)
C, *n* (%)	1 (3.8)
MELD score (units)	8.7 ± 5.3
Medical therapy	
Propranolol, *n* (%)	15 (57.7)
Diuretics, *n* (%)	12 (46.1)
Ascites, *n* (%)	5 (19.2)
Mild/moderate (diuretic responsive) ascites, *n* (%)	3 (11.5)
Severe (diuretic refractory) ascites, *n* (%)	2 (7.7)
Gastroesophageal varices, *n* (%)	22 (84.6)
Decompensation by ascites, *n* (%)	12 (46.1)
Decompensation by variceal bleeding, *n* (%)	12 (46.2)
Decompensation by overt encephalopathy, *n* (%)	3 (11.5)
⩾ 2 Decompensations by ascites, *n* (%)	5 (19.2)
⩾ 2 Decompensations by variceal bleeding, *n* (%)	4 (15.4)
⩾ 2 Decompensations by overt encephalopathy, *n* (%)	2 (7.7)
Heart rate (bpm)	67.8 ± 13.0
Systolic pressure (mmHg)	115.7 ± 11.6
Diastolic pressure (mmHg)	62.1 ± 21.6
Sodium (mEq/L)	140.6 ± 2.7
Creatinine (mg/dL)	0.9 ± 0.2
Albumin (g/dL)	3.6 ± 0.4

MELD: Model for end-stage liver disease.

cardiac biomarkers (pro-BNP)].

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows (SPSS INC. Chicago, IL, United States). Variables with normal distribution were expressed as mean ± SD and variables with non-normal distribution as median and range. Student's *t*-test, Fisher's Exact Test and Pearson's correlation were used when appropriate. *P* values < 0.05 were considered as significant. The statistical review of the study was performed by the author Mara Barbosa.

RESULTS

Clinical and analytical data

Seventy-three cirrhotic patients were evaluated and only 26 fulfilled the inclusion criteria. The main reasons for exclusion were: Diabetes mellitus (51%), hypertension (26%), ischemic cardiac disease (9%) and arrhythmia (6%). Regarding included patients, 22 (85%) were men, with mean age 55 ± 10 years. The etiology of cirrhosis was predominantly alcoholic (77%). The majority of patients (65%) were compensated (Child-Pugh class A). Mean MELD score was 8.7 ± 5.3. Five (19.2%) patients had ascites and the vast majority (84.6%) had gastroesophageal varices. Baseline characteristics of patients are listed in Table 1. Non selective β-blockers (propranolol) were suspended in 15 patients and diuretics (furosemide and/or spironolactone) in 12 patients.

Pro-BNP levels were 110.8 ± 110.6 μg/mL and were elevated in 8 (30.8%) patients (normal levels < 125 μg/mL). Pro-BNP values were not significantly different between alcoholic and non-alcoholic patients (*P* = 0.757). Pro-BNP levels were similar in Child-Pugh class B/C and Child-Pugh class A patients (*P* = 0.651). There was no correlation between MELD score and pro-BNP values (*P* = 0.950). The presence of ascites, gastroesophageal varices and history of overt encephalopathy was not significantly associated with more elevated pro-BNP levels (*P* = 0.525; *P* = 0.615 and *P* = 0.186, respectively).

Electrocardiographic characteristics

QTc interval duration was 460 ± 23 ms. Prolongation of the QTc interval was found in 77% of the patients. The existence of a prolonged QTc interval or its duration was unrelated to the etiology of cirrhosis (alcoholic *vs* non-alcoholic) (*P* = 0.562 and *P* = 0.696, respectively). Regarding severity of disease, there was a significant correlation between QTc interval duration and MELD score (*P* = 0.453, *P* = 0.020) but not between QTc interval duration and Child-Pugh score (*P* = 0.322, *P* = 0.108); QTc interval prolongation was not more frequent in patients with decompensated (Child-Pugh class B/C) *vs* compensated cirrhotic patients (*P* = 0.380); however, patients with QTc interval prolongation tended to have higher MELD score (9.8 ± 3.7 *vs* 5.0 ± 8.2, *P* = 0.053). Patients with QTc interval prolongation had a statistically significant higher rate of gastroesophageal varices comparing with those without QTc interval prolongation (95.0% *vs* 50.0%, *P* = 0.028). However, the presence of QTc prolongation was not associated with the presence of ascites or history of overt encephalopathy (*P* = 0.678 and *P* = 0.438, respectively). Regarding QTc interval duration, there was no relation with ascites, gastroesophageal varices or history of encephalopathy. Although more elevated, pro-BNP levels were not significantly increased in patients with a prolonged QTc interval (*P* = 0.483). Furthermore, there was no correlation between pro-BNP levels and QTc interval duration (*P* = 0.125).

Echocardiographic characteristics

Echocardiographic examinations were performed as planned, maximum heart rate predicted for age was achieved in all patients and no adverse events were recorded.

Left ventricular systolic function: At rest, none of the patients had LV EF below 50% (69.1% ± 8.1%) and LV EDV and LV ESV were within normal limits (94.2 ± 29.7 mL and 28.4 ± 9.5 mL, respectively). A reduced LV CR was observed in 10 (38.5%) patients with LV EF mean increment of 0.4% ± 7.6% *vs* 20.7% ± 10.4% in the other 16 patients with normal LV CR. Comparing the two groups, at rest, LV EF and LV ESV were similar (73.0% ± 7.1% *vs* 66.7% ± 7.9%, *P* = 0.051 and 30.1 ± 12.3

Table 2 The characteristics of patients with and without cirrhotic cardiomyopathy

	CCM (n = 16)	Non-CCM (n = 10)	P value
Gender (male), n (%)	14 (87.5%)	8 (80.0%)	0.625
Age (yr)	52.8 ± 9.9	57.4 ± 11.2	0.284
Etiology (alcoholic/non-alcoholic), n (%)	14 (87.5)/2 (12.5)	9 (90.0)/1 (10.0)	0.677
Child-Pugh score (units)	6.3 ± 1.3	6.1 ± 1.2	0.775
Child-Pugh class (A/B + C), n (%)	9 (56.2)/7 (43.8)	8 (80.0)/2 (20.0)	0.399
MELD score (units)	8.1 ± 5.8	9.5 ± 4.6	0.532
Ascites, n (%)	4 (25.0)	1 (10.0)	0.617
Gastroesophageal varices, n (%)	14 (87.5)	8 (80.0)	0.625
History of overt encephalopathy, n (%)	1 (6.3)	2 (20.0)	0.538
Sodium (mEq/L)	140.7 ± 2.9	140.5 ± 2.3	0.865
Creatinine (mg/dL)	0.8 ± 0.2	0.9 ± 0.1	0.343
Albumin (g/dL)	3.6 ± 0.3	3.5 ± 0.4	0.446

CCM: Cirrhotic cardiomyopathy; MELD: Model for end-stage liver disease.

mL *vs* 27.3 ± 7.5 mL, P = 0.466, respectively), but LV EDV was significantly increased in the group of reduced LV CR (111.4 ± 32.8 mL *vs* 83.5 ± 22.4 mL, P = 0.016). After pharmacological stress, LV ESV mean reduction was significantly inferior in the group of reduced LV CR (6.1 ± 12.6 mL *vs* 44.0 ± 21.8 mL, P = 0.000) and LV EDV mean reduction was similar in the two groups (0.53 ± 11.6 mL *vs* 5.2 ± 18.5 mL, P = 0.481).

Left ventricular diastolic function: At rest, 2 patients were diagnosed with diastolic dysfunction (grade Ⅰ). In 8 (30.8%) patients, the E/e' average ratio increased from 6.9 ± 2.0 to 9.1 ± 2.7; in the others, the E/e' reduced from 8.9 ± 1.9 to 6.8 ± 2.3.

CCM

Sixteen (61.5%) patients were diagnosed with CCM: 10 patients had systolic dysfunction, 8 patients had diastolic dysfunction and 2 presented with both cardiac systolic and diastolic dysfunction. Among those patients with CCM, QTc interval prolongation was observed in 11 (68.8%) patients and increased pro-BNP levels were measured in 5 (31.2%) patients. QTc prolongation and elevation of pro-BNP were simultaneously present in 5 (31.2%) cases and none of the alterations was observed in 5 (31.2%) patients. The characteristics of patients with and without CCM are listed in Table 2. Of note, the presence of CCM was unrelated to the etiology and severity of cirrhosis and presence of ascites, gastroesophageal varices and history of overt encephalopathy. Moreover, sodium, creatinine and albumin values were similar between patients with and without CCM.

DISCUSSION

In order to describe CCM prevalence, natural history and prognosis accurately, an effort to define diagnostic criteria has been made. However, universal consensus is still lacking and important points remain to be elucidated, such as: The minimum number of criteria required to make the diagnosis, the need to always performing a stress test to unmask CCM and the most adequate stress test to use, as the disease is usually latent and revealed by stress. Moreover, recent studies have used tissue Doppler parameters to diagnose CCM[28,29], as they are more sensitive and less dependent on loading conditions[30,31], comparing with mitral inflow velocity variables. Consequently, there is a considerable heterogeneity in the results published in the literature.

Diastolic dysfunction relates to impaired myocardial relaxation[28] and elevated left ventricular filling pressures is the main physiological consequence of it[32]. During stress, left ventricular filling pressures change minimally in healthy subjects. However, if cardiac dysfunction is present, a rise in filling pressures is observed in order to maintain left ventricular filling and stroke volume[28]. The E/e' ratio was shown to relate significantly to left ventricular filling pressures during stress[33]. Diastolic dysfunction was present in only 2 cases at rest and in 8 after stress. In fact, stress dobutamine echocardiography could identify patients with diastolic dysfunction (average E/e' ratio increase) not recognized at rest. The observation of an impaired myocardial relaxation during stress provides a possible explanation for the frequent development of cardiovascular complications (such as pulmonary edema) after transjugular intrahepatic portosystemic shunt (TIPS) insertion and liver transplantation, as these interventions promote a sudden increase in the preload and, consequently, a rise in left ventricular filling pressures.

None of the patients was diagnosed with systolic dysfunction at rest, which is consistent with the data reported in the literature, when LV EF is used as diagnostic criteria. The pharmacological stimuli revealed the existence of a systolic dysfunction in a considerable number of patients (38.5%). Due to the hyperdinamic state, with central hypovolemia and diminished preload and afterload, it remains an underdiagnosed condition even after a careful echocardiographic evaluation at rest.

The proportion of patients with CCM in our population was 61.5%. A significant number of patients (68.8%) had concomitant QTc interval prolongation and a smaller fraction (31.2%) had increased levels of pro-BNP.

CCM was independent of the etiology of cirrhosis, as has already been described in previous studies[3-5,8-10]. Controversy exists regarding the relation between the CCM and the severity of the disease. Some studies suggest that CCM can be more severe in decompensated liver disease while others report CCM is not directly related to disease severity[7,26,29,33,34]. In our study, we did not find any relation between CCM and Child-Pugh classification or MELD. Furthermore, clinical markers of higher liver impairment (ascites, gastroesophageal varices and history of overt encephalopathy) did not predict the presence of CCM.

QTc interval prolongation was a very common finding in this population of cirrhotic patients. This result is in agreement with the data reported in the literature. It is already established that QTc interval prolongation is significantly related to the severity of the underlying liver disease[13,35,36]. In our study, QTc interval duration was positively correlated with the degree of liver dysfunction assessed by MELD score, but not by Child-Pugh classification, probably because of small sample size. Interestingly, patients who were diagnosed with QTc interval prolongation had more commonly gastroesophageal varices, the last being an established surrogate of more severe liver disease and increased risk. However, this could not be demonstrated in patients with ascites. Recently, Trevisani *et al*[37] reported further QTc interval prolongation in the setting of acute gastrointestinal bleeding in cirrhotic patients. Although the clinical significance of QTc interval prolongation is not completely clarified[2,4-7,13,22], it may increase the risk of cardiac events and be associated with a poorer survival[13]. Therefore, close monitoring during stressful events is advised[6].

In summary, our study demonstrated that CCM is a frequent condition that is independent of the severity of liver disease. Furthermore, it showed that CCM is currently underdiagnosed, even after a comprehensive evaluation at rest. Consequently, a stress test should always be considered in the diagnostic approach to CCM, as it is highlighted in the current study. Moreover, an association between QTc interval prolongation and the presence of gastroesophageal varices was revealed, irrespective of the diagnosis of CCM. As such, the clinical significance of QTc interval prolongation is emphasized and it can be regarded as a marker of severe liver disease. A limitation of our study is its small sample size.

Hepatologists should be aware of this silent entity and actively search for it because it is of major importance in the management of the cirrhotic patient as it contributes to the high cardiovascular morbidity and mortality related to TIPS insertion and liver transplantation. It remains of the utmost importance to better define CCM diagnostic criteria, to suggest specific stress test protocols and to update echocardiographic criteria for the diagnosis, probably including TDI parameters which have already been used in several studies besides ours, in order to achieve more reproducible results. Also, the performance of strain evaluation by speckle tracking analysis, a new sophisticated echocardiographic technique, might be a promising method to diagnose CCM in patients with advanced liver disease as it can detect subtle systo-diastolic dysfunction before left ventricular ejection fraction becomes impaired[12,29]. Data regarding the impact of CCM in the natural history of cirrhosis is also needed.

COMMENTS

Background

Cirrhotic cardiomyopathy (CCM) relates to a cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease. It is independent of the etiology of cirrhosis and is different from alcoholic disease. Athough almost always clinically silent at rest, CCM is usually unmasked by physiological, pharmacological and/or surgical stress, such as transjugular intrahepatic portosystemic shunt or liver transplant. In this study, the authors aimed at describing the proportion of patients with CCM evaluated by stress echocardiography in a population of cirrhotic patients, and at investigating whether CCM is related to severity of liver disease.

Research frontiers

Very few prior reports address the question of diagnostic evaluation of CCM in cirrhotic patients using accurate criteria and stress testing. The results of the authors' study contribute to the diagnostic approach to CCM in these patients.

Innovations and breakthroughs

This study demonstrated that CCM is a frequent condition that is independent of the severity of liver disease. Also, it revealed that CCM is currently underdiagnosed at rest, even after a comprehensive electrocardiographic and echocardiographic evaluation. A substantial number of patients were diagnosed has having CCM only after the stress echocardiographic evaluation. Furthermore, an association between corrected QT (QTc) interval prolongation and the presence of gastroesophageal varices was revealed, irrespective of the presence of CCM. As such, QTc interval prolongation can be regarded as a surrogate of severe liver disease.

Applications

This study suggests that a stress test should always be considered in the diagnostic approach to CCM, otherwise it will remain an underdiagnosed entity.

Terminology

This study demonstrated that stress echocardiography was useful at revealing CCM. As such, it can identify patients at risk of cardiac decompensation and can be used as diagnostic tool of CCM in cirrhotic patients in clinical practice.

Peer-review

This is an interesting manuscript, and especially interesting for the general gastroenterologists, hepatologists, cardiologists and the internist.

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