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REVIEW

# Anti-hepatitis C virus drugs and kidney

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## Abstract

Hepatitis C virus (HCV) mainly targets the liver but can also induce extrahepatic manifestations. The kidney may be impacted *via* an immune mediated mechanism or a cytopathic effect. HCV patients are clearly at a greater risk of chronic kidney disease (CKD) than uninfected patients are, and the presence of CKD increases mortality. Interferon-based therapies and ribavirin are difficult to manage and are poorly effective in end-stage renal disease and hemodialysis. These patients should be given priority treatment with new direct anti-viral agents (DAAs) while avoiding peginterferon and ribavirin. The first results were convincing. To aid in the correct use of these drugs in patients with renal insufficiency, their pharmacokinetic properties and potential renal toxicity must be known. The renal toxicity of these new drugs was not a safety signal in clinical trials, and the drugs are generally efficient in these frail populations. These drugs are usually well tolerated, but recent cohort studies have demonstrated that these new regimens may be associated with renal side effects, especially when using sofosbuvir combinations. HCV, renal diseases and comorbidities are intimately linked. The close monitoring of renal function is required, particularly for at-risk patients (transplanted, HIV-coinfected, CKD, hypertensive or diabetic patients). New DAA regimens, which will soon be approved, will probably change the landscape.

**Key words:** Nephrotoxicity; Hepatitis C; Direct anti-viral agents; Kidney; End-stage renal disease



**Core tip:** Hepatitis C patients are clearly at risk of chronic kidney disease (CKD). New direct anti-viral agents (DAAs) with different pharmacokinetic properties are generally efficient in such populations. However, renal toxicity has been described in frail patients such as patients with CKD, transplants and human immunodeficiency virus co-infections under real-life conditions, especially with sofosbuvir combinations. New DAAs, which will be soon approved, will probably change the

landscape favorably. Close monitoring of renal function is required for at-risk patients, but patients without comorbidities are probably at a very low risk of renal toxicity.

Carrier P, Essig M, Debette-Gratien M, Sautereau D, Rousseau A, Marquet P, Jacques J, Loustaud-Ratti V. Anti-hepatitis C virus drugs and kidney. *World J Hepatol* 2016; 8(32): 1343-1353 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i32/1343.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i32.1343

## INTRODUCTION

Hepatitis C virus (HCV) mainly targets the liver but also targets the kidney *via* either an immune mediated mechanism (cryoglobulinemic vasculitis) or a cytopathic effect[1-3].

Epidemiological studies show that the risk of chronic kidney disease (CKD) is 20% higher in HCV patients than in uninfected individuals[4]. HCV increases the risk of both end-stage renal disease (ESRD)[5] and renal mortality[6]. Moreover, patients who are infected with HCV exhibit an increased risk of developing diabetes, high blood pressure and secondary vascular renal diseases[7]. Finally, chronic hepatitis C is the most commonly seen viral infection in patients with renal insufficiency[8]; its treatment is warranted and remains a great challenge.

Historically, interferon-based therapy was considered nephrotoxic in a dose-dependent or idiosyncratic manner[9]. First-generation protease inhibitors (*i.e.*, telaprevir and boceprevir in association with peginterferon and ribavirin) have also been implicated[10], although their role remains controversial[11]. Although ribavirin is not nephrotoxic, it accumulates in patients with CKD, and its secondary effects (particularly anemia) are much more severe.

Although new direct anti-viral agents (DAAs) were very well tolerated in phase Ⅲ trials, recent real-life studies have demonstrated some nephrotoxicity in frail populations that were treated with sofosbuvir-based regimens[12,13].

After a brief review of the pharmacokinetics of anti-HCV drugs, we review their potential renal toxicity and clinical experiences related to the use of these drugs in populations at risk of renal disease.

## PHARMACOKINETICS OF HCV TREATMENTS

### *Treatments that are available in 2016*

The combination of pegylated interferon and ribavirin with or without first-generation protease inhibitors (boceprevir and telaprevir) is no longer used in many developed countries[14-16]. However, it may still be relevant in developing countries.

Standard care in countries where DAAs are available is based on the combinations of two or three DAAs from different families: Second-generation protease inhibitors, NS5B polymerase inhibitors, and NS5A inhibitors. Ribavirin may be added in cirrhotic patients to shorten treatment duration.

All but two DAA phase Ⅲ studies did not include patients with severe renal insufficiency (4-5 CKD stages)[17,18]. Sparse data are thus available, and guidelines recommend that these patients be referred to expert centers[14].

To justify the proper use of HCV treatments in renal insufficiency, the pharmacokinetic properties of these drugs should be remembered.

### *Pharmacokinetics of interferon, pegylated interferons and ribavirin*

Interferons are natural cytokines. Alpha interferon and its pegylated form are active against viral replication. Pegylation prolongs the half-life of interferon, thus necessitating fewer injections[19-21]. The kidney plays a central role in interferon clearance. Interferon is filtered through glomeruli and undergoes lysosomal proteolytic degradation during proximal tubular reabsorption[22,23].

Ribavirin is a guanosine analog that exhibits broad-spectrum activity against DNA and RNA viruses. Its mechanism of action is based on the erroneous incorporation of ribavirin triphosphate into replicating RNA strands, thereby inhibiting chain elongation[24]. When used with interferon, ribavirin acts synergistically, preventing relapses and breakthroughs, and remains relevant in the DAA era in special circumstances. The major side effects of ribavirin are hemolytic anemia and teratogenicity. The renal excretion of ribavirin and its metabolites accounts for 40% of its clearance; the remainder is eliminated through the spleen *via* its principal metabolite, ribavirin triphosphate, which is captured in erythrocytes. Based on the product characteristics, the ribavirin area under the concentration curve (AUC) is doubled when calculating estimated glomerular filtration rates (eGFRs) between 30 and 45 mL/min per 1.73 $m^2$ and is tripled when calculating eGFRs between 13 and 30 mL/min per 1.73 $m^2$[25].

### *Pharmacokinetics of DAAs*

**First-generation protease inhibitors:** Telaprevir and boceprevir are significantly high CYP3A4, P-glycoprotein (P-gp) inhibitors and are also OATP1B1/2 and OCT 1 and 2 inhibitors, respectively.

Thus, they interact significantly with calcineurin inhibitors in transplant patients and with some human immunodeficiency virus (HIV)-specific medications, thereby increasing the renal toxicity of these drugs by increasing their exposure[26,27]. These drugs are poorly eliminated by the kidney (1% for telaprevir[28], 9% for boceprevir[29]). Telaprevir is excreted by the tubular cells through organic cation transporter 2 (OCT2) and presents a risk of interaction with medications such as dolutegravir[30].

**New DAAs:** Most new DAAs are eliminated in the bile, with the exception of sofosbuvir, which is the keystone

of the main approved DAA regimens.

Sofosbuvir weakly inhibits CYP3A4, intestinal P-gp, and BCRP. Seventy-two percent of sofosbuvir is eliminated by the kidney, primarily as its main metabolite GS-331007[31]. The mechanism of clearance warrants study, even if it is reasonable to evoke tubular excretion by analogy with HIV or hepatitis B virus (HBV) analogs. GS-331007 AUC is greater than 55%, 88% and 451% in cases of mild, moderate and severe renal insufficiency, respectively. GS-331007 exposure is increased by at least 10 to 20 times in patients with ESRD[32].

**Several DAAs can be used in combination with sofosbuvir:** (1) NS3/4 protease inhibitor: Simeprevir moderately inhibits CYP3A and intestinal P-gp and potentially inhibits OATP1B1 and MRP2. Its urinary excretion is less than 1%[33]. On average, the simeprevir AUC is increased by 62% in subjects with severe renal impairment. The drug is not eliminated by dialysis; and (2) NS5A inhibitors: Daclatasvir is a substrate of CYP3A4 and P-gp and moderately inhibits OATP1B1/3 and P-gp. Its excretion in urine is < 1%. In case of severe renal insufficiency, AUC is increased by 27%, but no dose adjustment is needed[34]. Ledipasvir is a weak inhibitor of P-gp and BCRP. Its renal excretion is < 1%[35], and its pharmacokinetics are not altered by severe renal impairment[36]. Velpatasvir moderately interacts with CYP3A4, CYP2C8, OATP and P-gp[37] and is primarily eliminated in the feces (> 99%). The sofosbuvir/velpatasvir combination will be available soon. According to very preliminary data, this combination appears well tolerated in subjects with severe renal impairment. Velpatasvir AUC is approximately 50% higher in these subjects than in subjects with normal function[38].

**Other combinations exist:** (1) paritaprevir/ritonavir (anti-protease inhibitor), ombitasvir (anti-NS5A inhibitor) and dasabuvir (anti-polymerase inhibitor). Paritaprevir/ritonavir is a powerful CYP3A4 inhibitor. Ritonavir is a well-known inhibitor of many renal transporters including OAT1, OAT2, MRP2, MRP4 and MATE1[39]. The four-drug combination is a substrate of P-gp and CYP3A4 and is mainly eliminated in the bile[40,41]. In case of CKD 1, paritaprevir and dasa–buvir AUCs are increased by 20%, and ritonavir AUC is increased by 42%. In patients with CKD 2 and 3, paritaprevir and dasabuvir AUCs are increased by 37% and ritonavir AUC is increased by 80%. In patients with CKD 4, paritaprevir and dasabuvir AUCs are increased by 50%, and ritonavir AUC is increased by 114%. Ombitasvir AUC remains unchanged[42]; and (2) grazoprevir and elbasvir: This regimen will be available soon. Both molecules are substrates of CYP3A4, OATP and P-gp[43]. Less than 1% of grazoprevir and elbasvir are excreted by the kidney; the $AUC_{0\text{-}24H}$ values of grazoprevir and elbasvir are higher in subjects with severe renal insufficiency relative to controls [1.65- (1.09, 2.49) and 1.86-fold (1.38, 2.51) (90%CI), respectively]. Drug removal by hemodialysis is negligible[44]. Clinical experience shows that dose adjustment is not needed in the setting of non-dialysis-dependent stage 4-5 CKD and dialysis-dependent stage 5 CKD[17].

## SPECIFIC NEPHROTOXICITY OF HCV DRUGS

### *Interferon or pegylated interferon and ribavirin*

A dose-dependent or idiosyncratic renal toxicity of alpha interferon and pegylated interferon is well established although rare[45]. This nephrotoxicity is mostly reported in cases of malignancy[46,47]. However, no correlations were found among the occurrence of renal involvement, the type of interferon used, administration route, treatment dosage and duration, and the patient's profile. The histological features are nonspecific and various, mainly involving minimal forms of glomerular damage, including cellular hyperplasia and focal segmental glomerulosclerosis, which are often associated with nephrotic syndrome[45,48-51]. Interferon may worsen any pre-existing glomerular lesions[52]. Microangiopathic thrombosis has also been described[53,54]. More rarely, interstitial fibrosis (usually mild) as well as nonspecific interstitial inflammation and tubular atrophy, and interstitial nephritis associated with nephrotic syndrome[55] or acute tubulopathy[47,56,57] have been reported.

Proteinuria (usually a self-limited proteinuria that does not exceed 1 g/d) is observed in 15% to 20% of patients taking interferon[58,59]. Nevertheless, hepatitis C-associated glomerulonephritis may be cured with alpha interferon-based treatment, independent of SVR[60].

Renal failure generally occurs during the first weeks of treatment and rarely occurs after several months[61].

The involved physiopathological mechanisms are not clear. In a cellular model, Lechner *et al*[62] demonstrated that interferon directly affects tubular barrier function in renal epithelial cells in a reversible time- and dose-dependent manner. More recently, the same team showed that alpha interferon can activate caspase-3, -8 and -9, which favors the apoptosis cascade in renal proximal tubular epithelia. Gresser *et al*[63] showed that the daily administration of interferon to newborn mice can lead to severe glomerulopathy associated with glomerular sclerosis and IgG and C3 deposits[64].

Ribavirin renal toxicity has not been documented and is not probable in monotherapy[65,66]. Nevertheless, by analogy with the ribavirin apoptotic activity observed in K562 leukemia cells, potential tubular toxicity has been hypothesized[65,67].

### *New treatments and nephrotoxicity*

**Boceprevir and telaprevir:** The first-generation protease inhibitors boceprevir and telaprevir have been combined with pegylated interferon and ribavirin. No renal side effect was found in phase Ⅲ studies[68-74], which is consistent with the weak renal clearance of these drugs. Nevertheless, in a large cohort (1486 patients),

Mauss *et al*[10] showed a reversible decrease of eGFR in patients taking telaprevir or boceprevir. Similar reports involving telaprevir therapy confirmed this observation and suggested a link with anemia occurrence[75-78]. Recently, Kunze *et al*[30] described competition between telaprevir and OCT2, which interacts with creatinine tubular transport and is involved in proximal tubular secretion. Our team validated this hypothesis with a predictive model suggesting that the clinically observed creatinine increase is not due to renal toxicity of the drug[11]. Independent of this pharmacological effect, one of our patients experienced acute renal failure at week 20 of telaprevir treatment. In addition to extra-membranous glomerulonephritis, the renal biopsy showed particularly intense interstitial fibrosis that would exceptionally be described by pegylated interferon and probably implies telaprevir or a combination of telaprevir-pegylated interferon[3].

**New DAAs:** The renal toxicity of new DAA was not a safety signal in phase Ⅲ clinical trials[79-83]; however, most of the included patients presented with eGFR values of greater than 60 mL/min per 1.73 $m^2$ and few comorbidities. The prescription of sofosbuvir is not desirable for patients with an eGFR of less than 30 mL/min per 1.73 $m^2$. In practice, however, half of the daily dose[84] or a full dose taken every other day[85] was found safe. Various recommendations[14-16] specify that renal function should be monitored during treatment with sofosbuvir (grade B). Indeed, on the one hand, the drug is cleared by the kidney; on the other hand, a structural analogy with HBV nucleotide analogs is observed. Therefore, competitive risks with other drugs (antiviral or anti-calcineurins) that are eliminated by the tubule are awaited. In a prospective unselected HCV population, we were unable to find evidence for the induction of subclinical tubulopathy by the antiviral treatment when using tools for the early detection of proximal tubular injury (unpublished data). However, potential proximal tubular toxicity can be hypothesized.

DAAs are usually combined with sofosbuvir, *i.e.*, simeprevir, daclatasvir and ledipasvir do not appear to increase renal risk, although it is difficult to distinguish between the contributions of sofosbuvir and other drugs with which it is combined to the occurrence of renal failure: (1) simeprevir: Renal failure resulting from simeprevir therapy was not found in phase Ⅲ studies[86,87], except in association with sofosbuvir[13,88]; (2) daclatasvir: No renal warning was observed in phase Ⅲ studies[89], except when daclatasvir was associated with sofosbuvir in liver transplant patients[90]; and (3) ledipasvir: One case report suggested possible acute renal toxicity, but this occurred in association with sofosbuvir[91].

Concerning the combination ombitasvir, paritaprevir/ritonavir, dasabuvir, plasma creatinine increase was described in 2 of the 293 patients who had experienced previous interferon-based treatment[92]. Other phase Ⅲ studies did not describe any renal adverse event[93-96].

## EXPERIENCES ON ANTI-HCV THERAPIES IN POPULATIONS AT RENAL RISK

### *ESRD and hemodialysis*

HCV prevalence is high among patients on long-term dialysis (5% to 10% in Europe and in the United States and 10% to 70% in developing countries)[97]. HCV decreases global survival in this population[98].

HCV screening is recommended once yearly in hemodialysis patients. Patients generally present with normal transaminase levels[99], low viral load[100], and moderate fibrosis stage[8,101,102], although fibrosis appears to progress more rapidly in this population. For these reasons, anti-HCV treatment is warranted.

Three meta-analyses of historical treatment with pegylated alpha interferon and ribavirin showed a 40% SVR in ESRD[103-105]. The results obtained did not differ between alpha interferon and pegylated alpha interferon[106]. Ribavirin is generally contra-indicated in patients with eGFR values of less than 50 mL/min due to the high risk of ribavirin metabolite accumulation in erythrocytes, which increases the amplitude of hemolytic anemia[24,107]. However, at minimal doses, ribavirin was used after each dialysis session[108] or 5 d per week[109]. The erythropoietin doses were usually increased[109].

First-generation protease inhibitors in combination with pegylated interferon and ribavirin gave potentially interesting results[110-113], but the observed high antiviral efficacy was accompanied by numerous serious adverse effects[112].

ESRD and dialysis patients should be given priority treatment with new DAAs while avoiding peginterferon and ribavirin.

The currently available data on the approved DAAs are sparse. The adequate dose of sofosbuvir is unknown, and ribavirin should be avoided (see above).

Small preliminary studies, mainly based on the sofosbuvir/simeprevir combination[84,114,115], have shown a SVR rate of between 87% and 100% in ESRD genotype 1 patients. In a real-life TARGET cohort evaluating a sofosbuvir and simeprevir regimen, similar results were observed, with an increased benefit when adding ribavirin; however, anemia risk was increased[13]. In summary, the safety of sofosbuvir in ESRD is unclear, and larger trials are awaited.

Recently, preliminary results of the RUBY-1 trial including 20 patients with CKD 4 renal insufficiency receiving the approved regimen of ombitasvir, paritaprevir/ritonavir, and dasabuvir with (genotype 1a) or without (genotype 1b) ribavirin showed a SVR of 90%; however, ribavirin had to be stopped in 9 of the 13 G1a patients[18].

More recently, elbasvir and grazoprevir were administered together once daily in the largest trial to date (the Phase Ⅲ C-SURFER study); the trial included 224 ESRD patients, 179 of whom were hemodialysis dependent, and achieved a 99% SVR12 in genotype 1 patients[17]. Elbasvir and grazoprevir are expected to be approved shortly.

Thus, two regimens are or will be recommended in genotype 1 patients with severe renal insufficiency: Paritaprevir-ritonavir-ombitasvir-dasabuvir for patients with G1b and grazoprevir-elbasvir for patients with all G1 subtypes.

### *Patients with renal impairment*

In the TARGET cohort, the sofosbuvir/simeprevir combination (with or without ribavirin or pegylated interferon) was found to be efficacious and safe in HCV-infected patients of differing CKD stage. Compared with patients without renal insufficiency, these patients experienced a deterioration of their eGFR (25% with an initial eGFR < 30 mL/min per 1.73 $m^2$, 13% with an eGFR of between 31 and 45 mL/min per 1.73 $m^2$, and 1% to 2% with an eGFR > 45 mL/min per 1.73 $m^2$). These results suggest that sofosbuvir-based treatments used in kidney patients warrant close monitoring[13]. In the TARGET cohort, patients with a basal eGFR of less than 30 mL/min per 1.73 $m^2$ showed a high risk of acute renal insufficiency (25%)[13].

### *Kidney transplantation*

HCV prevalence among kidney transplant patients is approximately 10%, and most of the patients are viremic[116]. HCV decreases global survival in this population[117].

HCV also increases sepsis, diabetes, glomerulonephritis and rejection[102,117-120].

Anti-viral treatment is recommended for preventing fibrosis progression, risk of fibrosing cholestatic hepatitis and sepsis. Interferon is no longer recommended in this setting due to the strong risk of rejection[121], although this risk has been shown to be lower than expected[122,123]. Moreover, meta-analyses have demonstrated a weak SVR rate (18% to 26.9%) and a high rate of withdrawal: 21.1% to 35% with alpha interferon[124,125] and 40.6% with pegylated interferon[125]. No data with pegylated interferon and boceprevir or telaprevir-based triple therapy are available. However, the data obtained from liver transplant experience show that it is very difficult to manage drug interactions with calcineurin inhibitors, thus leading to serious adverse events[26].

A few published preliminary studies using sofosbuvir-based combinations showed a SVR > 95%; however, the immunosuppressing drug concentrations varied, a finding that should be studied and monitored[126-130]. Liver transplantation experience is more important, and treatment of such patients has shown good results in terms of efficacy, tolerance and medication interactions[90,131].

Recently, the concept of pre-transplant treatment has become preeminent, especially for patients of genotypes 1 and 4, due to the availability of regimens avoiding sofosbuvir[17]. However, patients with genotypes 2 and 3 for whom sofosbuvir-based regimens are recommended should be treated after kidney transplantation while awaiting new pangenotypic combinations[132].

### *Liver transplantation*

In the French CUPILT cohort of liver transplant patients who were treated with sofosbuvir and daclatasvir, 37.1% experienced a 25% decrease of GFR during or after treatment; however, in 10.9% of the cases, this GFR decrease was not reversible. The existence of prior kidney disease and fibrosing cholestatic hepatitis were both independent predictors of decreased GFR. The authors emphasized the importance of close renal function monitoring in this population[12]. These data were confirmed in an American multicenter study[133]. Moreover, patients with fibrosing cholestatic hepatitis who were treated with sofosbuvir, ribavirin and pegylated interferon ($n$ = 8) or daclatasvir, sofosbuvir and ribavirin ($n$ = 14) experienced high rates of renal failure (4/8 and 7/14, respectively), including 1 with creatinine clearance of less than 30 mL/min per 1.73 $m^2$[90].

### *Coinfected patients*

In coinfected patients of the ION-4 study, who were treated with sofosbuvir/ledipasvir, 4 of the 335 patients exhibited worsened renal function (a creatinine increase of 35 μmol/L or more); tenofovir $AUC_{tau}$ increased by 20% and 30% in two patients, one patient discontinued tenofovir, and the drug dose was reduced for one patient[134].

Renal function improved in all patients after treatment discontinuation.

### *Particular cases*

**Acute renal insufficiency:** Acute renal insufficiency has mainly been reported in cohorts with high renal risk. Recently, the first case of acute kidney injury, as documented by renal biopsy, was described in a patient receiving sofosbuvir and ledipasvir and suffering from hypertension and diabetes mellitus type 2: The biopsy showed an acute allergic interstitial nephritis with diabetic nephropathy. Corticosteroid therapy was introduced, and this stabilized the renal function[91].

**Adolescents and children:** The pharmacokinetics of new antiviral drugs are not known in this population. To our knowledge, only one study using ledipasvir/sofosbuvir (90/400 mg) in 100 adolescent patients (12 to 17 years old) with HCV genotype 1 for 12 wk resulted in an SVR12 rate of 97%, a similar result to that obtained in adults. Ledipasvir/sofosbuvir was well tolerated with no grade 3-4 adverse events, serious adverse events, or treatment discontinuations due to adverse events[135] in particular renal events. In the context of the universal use of new DAAs, a study in children aged 3 to < 12 years is ongoing (ClinicalTrials.gov Identifier: NCT02249182).

In summary, sofosbuvir-based combinations have exhibited renal toxicity in frail patients such as CKD, transplant and HIV co-infected patients under real-life conditions. Real-life studies suggest a risk of eGFR deterioration in patients with previous renal impairment, suggesting that these combinations be used cautiously in this setting including, in particular, diabetes mellitus and hypertension.

Physiopathologically, tubular toxicity can be suggested by structural analogy between this drug and antiretroviral analogs; however, this was not demonstrated in patients

with normal renal function. Nevrtheless, these new anti-HCV DAAs appear to act synergistically with drugs that are known to exert a toxic action on the tubule, such as anticalcineurins and tenofovir. Finally, a classic drug-induced renal tubulointerstitial disease of immunological origin has recently been described in at least one documented case with renal biopsy.

New combinations, such as paritaprevir-ritonavir-ombitasvir-dasbuvir for genotype 1b and grazoprevir-elbasvir for all genotype 1 subtypes, show promise in patients with severe renal impairment.

## CONCLUSION

HCV treatment should be offered to all patients with ESRD or kidney transplant candidates, regardless of liver fibrosis stage, due to the intimate link between HCV, renal diseases and comorbidities such as cardiovascular complications and diabetes and because of the impact of HCV on mortality.

There is no clear recommendation for the use of currently approved DAAs in cases of severe renal insufficiency; these drugs may be prescribed under certain conditions, preferably without ribavirin. However, expert opinions are needed.

New DAAs, which will soon be approved, will probably favorably change the landscape.

DAA regimens can present renal side effects, especially sofosbuvir combinations. Close monitoring of renal function is required in at-risk patients comprising patients with CKD, ESRD and hemodialysis, hypertension and diabetes, HIV coinfection, and transplant patients. Current recommendations require the universal monitoring of renal function in patients treated with DAAs. However, patients with none of the above described comorbidities are probably at very low risk of renal toxicity and will no longer require such close monitoring in future.

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REVIEW

# Toll-like receptors in pathophysiology of liver diseases

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## Abstract

Toll-like receptors (TLRs) are pattern recognition receptors that participate in host defense by recognizing pathogen-associated molecular patterns alongside inflammatory processes by recognizing damage associated molecular patterns. Given constant exposure to pathogens from gut, strict control of TLR-associated signaling pathways is essential in the liver, which otherwise may lead to inappropriate production of pro-inflammatory cytokines and interferons and may generate a predisposition to several autoimmune and chronic inflammatory diseases. The liver is considered to be a site of tolerance induction rather than immunity induction, with specificity in hepatic cell functions and distribution of TLR. Recent data emphasize significant contribution of TLR signaling in chronic liver diseases *via* complex immune responses mediating hepatocyte (*i.e.*, hepatocellular injury and regeneration) or hepatic stellate cell (*i.e.*, fibrosis and cirrhosis) inflammatory or immune pathologies. Herein, we review the available data on TLR signaling, hepatic expression of TLRs and associated ligands, as well as the contribution of TLRs to the pathophysiology of hepatic diseases.

**Key words:** Toll-like receptors; Innate immunity; Liver disease; Pathophysiology; Signaling



**Core tip:** Toll-like receptors (TLRs) are known to be pattern recognition receptors that recognize pathogen- and damage-associated molecular pattern molecules and thus participate in the activation of innate immune system. TLR signaling plays a significant role in liver diseases, whereas inflammatory or immune pathologies targeting distinct liver cells are based on complex immune responses. Herein, we review the current data on TLR signaling, hepatic expression of TLRs and associated ligands, as well as the contribution of TLRs to the pathophysiology of hepatic diseases.

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## INTRODUCTION

Liver, main filter organ acting as a first line of defense, is continuously exposed to massive gut-derived antigenic load *via* the portal vein, whereas inflammatory signs occur under normal conditions owing to highly specific immune properties leading to immune tolerance[1-7].

Pathogen-associated molecular patterns (PAMP) are specific signature molecules essential to entire categories of microorganisms[8-11]. Innate immune system recognizes PAMPs *via* pattern recognition receptors (PRRs)[7-9,12,13] and consequent downstream signaling cascades for proper host recognition and prevention of immune system hyperactivation[7-9,14].

Toll-like receptors (TLRs) are a family of PRRs that induce innate immune system by recognizing PAMPs and damage-associated molecular pattern molecules (DAMPs)[15-18]. Although the recognition of PAMPs enables a prompt and effective protection against invading pathogens[5,11,12], TLRs also contribute to the activation of adaptive immune responses, epithelial regeneration and carcinogenesis and regulation of sterile inflammation[5,19,20].

Consistent with their extensive hetapocellular expression[7,18,21,22], TLRs have recently been recognized as principal elements of the hepatic immune system that also play a crucial role in liver physiology and pathophysiology[11,15,23]. Despite being constantly exposed to gut-derived PAMPs, healthy liver is free of inflammation risk due to presence of "liver tolerance" in which modulation of TLR signals also plays a role[5,15,23-25]. A tight regulation of TLR activation occurs at many levels involving the receptor itself, the signaling cascade and a distinct compartmentalization of TLRs[24,26,27]. Acute and chronic liver diseases are highly associated with triggering TLR signaling by gut-derived microbiota in the breakdown of the tolerance and sterile insult-associated products of damaged cells[28].

Ligand mediated stimulation of TLRs activates downstream adaptor molecules, including myeloid differentiation primary response protein 88 (MyD88), myeloid toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). This triggers signaling cascades that converge on nuclear factor-κB (NF-κB), interferon (IFN) response factors (IRFs) and mitogen-activated protein (MAP) kinases[23,29-32]. As a result, transcription of certain proinflammatory agents including IL-6, IL-12, IL-23, and tumor necrosis factor α (TNF-α) is induced[23,29-32].

TLR-mediated inflammatory-signaling pathways are shown to be associated with entire spectrum of liver diseases, from hepatitis, liver fibrosis and cirrhosis to alcoholic and nonalcoholic liver disease, ischemia/reperfusion injury, liver regeneration and hepatocellular carcinoma[4,5,7,8,15,18,23,33].

Herein, we review the available literature on TLR signaling, hepatic expression of TLRs and associated ligands, as well as the contribution of TLRs to the pathophysiology of hepatic diseases.

## TLR FAMILY, DISTRIBUTION, LIGANDS

TLRs are a group of evolutionarily conserved type Ⅰ transmembrane proteins responsible for innate immune and inflammatory responses[34-38]. They comprise an extracellular domain with receptor specific leucine-rich repeat motifs and a highly conserved cytosolic domain alike to the IL-1 receptor called TIR[13,29,36,37].

Of 13 TLRs exist in mammals, only TLRs 1-10 exist in humans[9,26,39-41]. The presence of multiple widely expressed TLRs enables recognition of different pathogens and thus initiation of appropriate immunologic response by the innate immunity system[30,42,43]. PAMPs include microbial molecular structures such as Gram-negative related lipopolysaccharide (LPS); Gram-positive bacteria related lipoteichoic acid and peptidoglycan (PGN); lipoglycans, lipoarabinomannan, lipopeptides and lipomannans from mycobacteria; zymosan from yeast; and DNA from viruses and bacteria[34,44].

DAMP include extracellular matrix and plasma membrane components, nuclear and cytosolic proteins and elements of damaged organelles[9,34,45,46].

Each TLR is able to recognize a particular molecular pattern[29]. TLR1, TLR2, TLR4, TLR5 and TLR6 bind to molecules associated with bacterial membrane such as LPS, lipoprotein and PGN, whereas TLR3, TLR7, TLR8 and TLR9 detect viral and bacterial or endogenous nucleic acids, including ssRNA, dsRNA, and unmethylated cytosine phosphate guanine (CpG)-containing DNA[29]. TLR4 along with TLR2 can recognize antigens from bacteria, fungi, parasites, viruses and DAMPs[47,48]. TLR10 is the only family member among humans with no definite ligand, function or localization[9,13].

Given their ability to detect wide range of non-microbial host-derived stimuli and their extensive expression in various cell types, TLRs are considered to participate in development, progression and resolution of several noninfectious inflammatory and immune diseases[37,49].

## TLR SIGNALING PATHWAYS

Healthy liver contains low mRNA levels of TLRs and shows no activation of TLR-signaling pathways[5,50,51]. However, in the case of a breakdown in TLR tolerance against endogenous ligands under pathologic conditions, the TLR-related immune response induces TLR-ligand complex activated expression of proinflammatory/anti-inflammatory cytokines and interferons[7,9,27,52].

The differential host cell response after TLR ligand stimulation is associated with the fact that TLRs selectively use four main adaptor molecules, including MyD88, TIR domain-containing adaptor protein (TIRAP, or MyD88 adaptor-like), TIR domain-containing adaptor protein inducing interferon-b (TRIF) and TRAM[7,9,27,30,52].

Signal transduction pathways following ligand-induced receptor dimerization involve one or more TIR-containing adaptor molecules, such as IL-1 receptor-associated

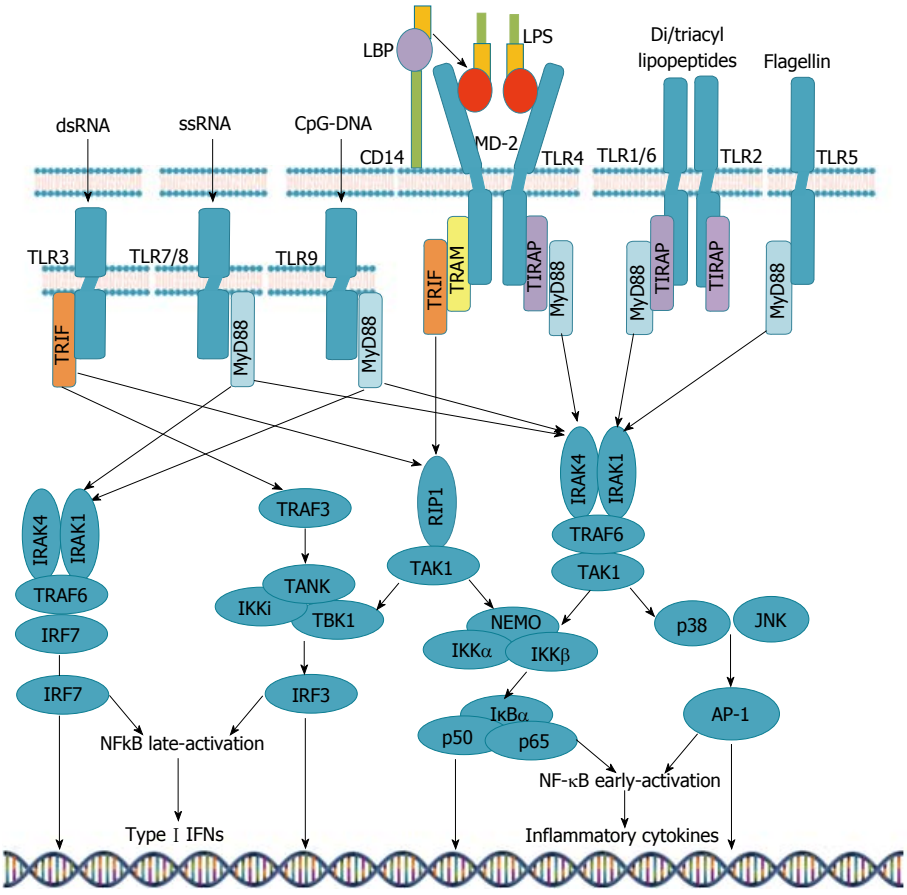


**Figure 1 Toll-like receptors signaling pathways.** TLR: Toll-like receptors; LPS: Lipopolysaccharide; NF-κB: Nuclear factor; IFNs: Interferons; LBP: LPS-binding protein; TIRF: Toll/interleukin-1 receptor-domain-containing adaptor-inducing interferon-β; MyD88: Myeloid differentiation primary response protein 88; TRAM: TRIF-related adaptor molecule; TIRAP: TIR domain-containing adaptor protein; IRAK: IL-1 receptor-associated kinase; TRAF: Tumor necrosis factor receptor-associated factor; TBK1: TANK binding kinase-1; IKK: IκB kinase; AP: Activator protein; JNK: c-Jun N-terminal kinase.

kinase (IRAK)-1, IRAK-4, TNF receptor-associated factor (TRAF)-6 and TANK binding kinase (TBK)-1, MAP kinases and IκB kinase (IKK). This leads to activation of the nuclear transcriptional factor kappa-B (NF-κB), interferon (IFN) regulatory factor 3 (IRF-3) and activator protein (AP)-1[37,53].

Upon binding with their ligand, all superfamily receptors except TLR3 use MyD88 to initiate signaling which may also act along with other adaptors, such as TIRAP, in the response induced by TLR4, TLR1/2, and TLR2/6. Activation of TLRs 5, 7, 8 and 9 also leads to NF-κb and AP-1 production, with no need for TIRAP to stimulate MyD88. TLRs 7 and 9 act through IRAK-1, 4 and TRAF-6, phosphorylate IRF-7 and lead to type 1 interferon mRNA expression. TLR3-mediated signaling uses only the TRIF adaptor molecule, which is also recruited by TLR4 in concert with another adaptor called TRAM[9,12,23,32,39,54] (Figure 1).

Hence, while intracellular signaling is similar, the final outcome of TLR activation differs depending on the nature of PAMPs, concomitantly activated TLRs and PRRs, the level of cytokines, and the cell stimulated[13,27,55-57]. Moreover, chronically activated signaling pathways is likely to induce transcription of oncogenic factors, which adds a further level of complexity to the intracellular signaling for these receptors[13,27,58].

## TLR EXPRESSION AND SIGNALING IN HEPATIC CELL POPULATIONS

Under constant exposure to gut-derived microbiota, strict regulation of TLR signaling pathways is crucial in the liver, which otherwise may lead to inappropriate production of proinflammatory cytokines and interferons creating a predisposition to several autoimmune and chronic inflammatory diseases[9].

Liver cells are classified as parenchymal or non-parenchymal cells. Hepatocytes comprise 60%-80% of the parenchymal cells, whereas the remaining population of non-parenchymal cells include Kupffer cells (KCs), sinusoidal endothelial cells (SECs), hepatic stellate cells (HSCs), dendritic cells (DCs), biliary epithelial cells (BECs) and intrahepatic lymphocytes[1,9,33].

Besides distinct function of liver cells with a highly specific distribution of TLR[1,33], liver comprises many populations of cells with immune competence that may respond to TLR signals, indicating the complexity of immune responses underlying inflammatory or immune pathologies associated with the liver cells[10].

mRNA levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8,

**Table 1 Toll-like receptor expression and their signaling in the liver**[5,9,11,15,23,33,49]

| TLR subfamily | Members | Expression of cell population in the liver (protein level) | Location | Ligand (origin) | Signaling | Final product-effect |
|---|---|---|---|---|---|---|
| TLR2 subfamily | TLR1/2 | NK cells, DCs (h) | Plasma membrane | Bacterial lipoproteins Triacylated lipopeptides | TIRAP-MyD88-NF-κB/AP-1/IRF5 pathway | Pro- and anti-inflammatory cytokines excluding type 1 IFNs; the apoptotic cascade *via* recruiting FADD leading to caspase-8 activation |
| | TLR2/6 | Hepatocytes, Kupffer cells, NK cells, B cells, activated T cells, DCs (m), biliary epithelial cells | | Diacylated lipopeptides LPS of Gram-positive bacteria Fungal zymosan Mycoplasma lipopeptides | TIRAP-MyD88-NF-κB/AP-1 pathway | |
| | TLR10 | Unknown | | ND | | |
| TLR3 subfamily | TLR3 | Hepatocytes, LSECs, Kupffer cells, NK cells, NKT cells, activated T cells, cDCs (m), biliary epithelial cells | Endosome | Double-stranded RNA (viruses) | PI3K/TRIF-IRF3 pathway TRAM-TRIF-NF-κB pathway PI3K/TRIF-RIP1-NF-κB pathway | Production of type 1 IFNs; the apoptotic cascade *via* recruiting FADD leading to caspase 8 activation; DC maturation |
| TLR4 subfamily | TLR4[1] | Hepatocytes, LSECs, Kupffer cells, NK cells, B cells, activated T cells, DCs (m), biliary epithelial cells, HSCs | Plasma membrane | LPS of Gram-negative bacteria; fusion protein (respiratory syncytial virus), envelope protein (mouse mammary-tumor virus); HMGB1, hyaluronan, HSP60, free fatty acids (endogenous ligands); HSP72 (cells during stress and injury) surfactant protein A; fibrinogen; fibronectin extra domain A | TIRAP-MyD88-NF-κB/AP-1 pathway TRAM-TRIF-NF-κB/IRF3 pathway | Pro- and anti-inflammatory cytokines excluding type 1 IFNs; the apoptotic cascade *via* recruiting FADD leading to caspase 8 activation; DC maturation; activating caspase-1 through adaptor molecule apoptosis associated speck-like protein[2] |
| TLR5 subfamily | TLR5 | Biliary epithelial cells | Plasma membrane | Flagellin protein (bacteria) | MyD88-NF-κB/IRF5 pathway | Pro- and anti-inflammatory cytokines excluding type 1 IFNs |
| TLR9 subfamily | TLR7/8 | NK cells, B cells, DCs (h), DCs (m) | Endosome | Single-stranded RNA (viruses), double-stranded, shortinterfering RNA (siRNA) | MyD88 and endosomal acidification (maturation)-IRF7 pathway; MyD88-NF-κB pathway | High levels of type 1 IFN production in pDCs; proinflammatory cytokine production |
| | TLR9 | LSECs, Kupffer cells, NK cells, B in mDCs and macrophages | | Imidazoquinoline CpG-containing viral or bacterial DNA Endogenous host-DNA | | |

[1]TLR4 requires LPS-binding protein (LBP), CD14 and MD2 to recognize LPS; [2]Containing a caspase recruitment domain (ASC)[33]. RIP1: Receptor-interacting protein 1; FADD: Fas-associated death domain; TLR: Toll-like receptors; LPS: Lipopolysaccharide; DCs: Dendritic cells; HSCs: Hepatic stellate cells; LSECs: Liver sinusoidal endothelial cells; IFNs: Interferons; DC: Dendritic cell; MyD88: Myeloid differentiation primary response protein 88.

TLR9, TLR10 and signaling molecules such as MD-2 and MyD88 are lower in liver as compared with the levels observed in other organs[50,51,59]. This discrepancy indicates the high tolerance to TLR ligands from the intestinal microbiota in liver[11], whereas no specific liver cell population is considered central in TLR-mediated pathologies, with the different effects of TLR ligation varying from cell to cell[10] (Table 1).

### *Hepatocytes*

Constituting 60% of liver cells, hepatocytes are the principal site for PRR production[5,33]. They express mRNA for all TLRs and are responsive to multiple PAMPs, while respond fairly weakly to TLR2 and TLR4 ligands[5,9,33]. While TLR4 expression in hepatocytes is not upregulated by proinflammatory mediators, hepatocytes show increased responsiveness to TLR2 ligands under inflammatory conditions leading to up-regulation of TLR2 expression by LPS, TNF-alpha, bacterial lipoprotein, and IL-1β in an NF-κB-dependent manner[5,11,33,60,61].

### *Kupffer cells*

Accounting for approximately 20% of non-parenchymal cells, KCs play a significant role in host defense by orchestrating the inflammatory response *via* functional properties, including phagocytosis, antigen processing and presentation, and secretion of proinflammatory mediators such as cytokines, prostanoids, nitric oxide, and reactive oxygen intermediates[5,9,11,33,62].

KCs express TLRs 2, 3, 4 and 9 and have a higher threshold for activation when compared with other immune cells given their milieu[5,9,33,63].

KCs are less responsive to "LPS tolerance" in the physiological environment, whereas upon activation, they produce several pro-inflammatory (IL-6, IL-12, IL-18 and TNFα) and anti-inflammatory (IL-10) mediators[33,64-66]. Additionally, KCs produce IFN-β, upregulate the expression of MHC-Ⅱ/costimulatory molecules and promote T cell proliferation and IFN-γ production; when stimulated with TLR3/TLR4 ligands; TLR1/TLR8 ligands and TLR1/2/4/6 ligands, respectively[22,33].

### Hepatic stellate cells

Constituting < 1% of non-parenchymal cells, HSCs undergo an activation process after liver injury and become the main liver cell type that produce extracellular matrix, contributing onset of liver fibrosis[67-70].

HSCs express TLRs 4 and 9, whereas expression of TLR2 is induced by TLR4 stimulation in HSCs[68-70]. Activated HSCs express TLR4 and CD14 and respond to LPS upon the activation of IKK/NF-κB and c-Jun N-terminal kinase (JNK) as well as the secretion of proinflammatory cytokines such as transforming growth factor (TGF)-β, IL-6, IL-8 and several chemokines such as MCP-1, MIP-2, intercellular cell adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin[9,33,70]. TLR4 enhances TGF-β signaling, and stellate cell activation was shown to promote hepatic fibrosis[71]. In chimeric C3H/HeJ mice with TLR4 mutation in HSC or KCs, amelioration of hepatic fibrosis by LPS indicated a cardinal role for KCs and HSC in hepatic inflammation and fibrosis[9,72]. LPS was shown to downregulate the TGF-β pseudoreceptor BAMBI in quiescent HSCs to induce TGF-β signaling and stellate cell activation[71]. Additionally, TLR9 signaling activated *via* DNA from apoptotic hepatocytes was shown to modulate liver fibrosis *via* its effects on HSC differentiation through increased collagen production and inhibited HSC migration[73]. Hence, LPS and other TLR ligands are suggested to facilitate fibrogenic responses in the liver *via* their direct effects on HSCs[9,11,33].

### Biliary epithelial cells

Accounting for approximately 5% of non-parenchymal cell population in the liver, BECs are commonly exposed to several gut-derived microbes[74,75]. BECs mainly express TLRs 2, 3, 4 and 5, which are upregulated by IFN-γ stimulation[74,75]. TLR2 and TLR4 activation results in increased IRAK-M expression and provide negative feedback in human intrahepatic BECs[76].

Under normal conditions, increased IRAK-M expression is critical in preventing undesired induction of the TLR signaling cascade, while in case of inflammatory conditions, upregulation of BEC-associated TLRs leads to IFN-c and TNF-α exposure, participating in biliary pathogenic responses[9,75].

### Sinusoidal endothelial cells

Making up 50% of the non-parenchymal cells, SECs function in hepatic perfusion and nutrient supply[66,77-79]. They express TLR3, 4 and 9 and show increased NF-κB activation and CD54 expression alongside a limited ability to trigger leukocyte adhesion after LPS stimulation[66,77-79]. Although these effects indicate a scavenging role and thus the likelihood of SECs acting as antigen presenting cells, the exact role of the TLR signaling in inflammatory process in SEC remains inconclusive[9,11,33,66,77-79].

Isolated SECs from WT mice were shown to respond to TLR1, 2, 6 and 9 ligands *via* producing TNF-α; to TLR3 ligands by producing TNF-α, IL-6 and IFN-β; and to TLR4 ligands *via* production of TNF-α and IL-6[22,33]. Upon TLR8 ligand binding, SECs leads to TNF-α production alongside upregulation of major histocompatibility complex (MHC)-Ⅱ and co-stimulatory molecules. Stimulation of SECs by TLR1, 2 or 6 ligands is suggested to be associated with activation of allogeneic T cells, as evaluated by the mixed lymphocyte reaction[22,33]. The SEC immune response is also modulated by LPS tolerance, which appears to be based on prostanoid expression rather than regulation at the level of TLR4 surface expression[78]. Although SECs have been suggested to be involved in the hepatic uptake of LPS in some studies, several studies have not confirmed such a role[33,80,81].

### Hepatic dendritic cells

Comprising < 1% of non-parenchymal cells, hepatic DCs are recruited into the liver sinusoids during inflammation and then they may migrate to periportal and pericentral areas[5,33,82,83]. Plasmacytoid DCs (pDCs), myeloid DCs, lymphoid DCs, mixed lymphoid + myeloid DCs and natural killer DCs are amongst the DC subsets, whereas lymphoid and myeloid DCs are considered conventional DCs[33,82,83].

Each DC subset show distinct TLR expression pattern in humans with TLR1, 7 and 9 expression *via* pDCs, while expression of all TLRs excluding TLR9 by other DC subsets[20,33,84]. Cytokines TNF-α, IL-6 and IL-12 TLR7 are produced by hepatic pDCs upon TLR7 and TLR9 activation, whereas TNF-α and IL-6 in response to TLR2, TLR3 and TLR4 activation[50,85].

## TLRs IN THE PATHOPHYSIOLOGY OF LIVER DISEASES

Increasing evidence suggests that TLRs have significant contribution to the pathogenesis and progression of several liver diseases, *i.e.*, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), viral hepatitis, autoimmune liver disease and hepatic inflammation-fibrosis-carcinoma (IFC) sequence including hepatic fibrosis and/or cirrhosis and hepatocarcinoma[9,11,13,15,23,33].

LPS/TLR4 and TLR2 signaling have been suggested to be principal actors in the human hepatic IFC sequence associated with viral chronic hepatitis[86], while the participation of TLR3 in the pathophysiology of several liver diseases has also been suggested in the recent studies[11,15,23,87] (Figure 2).

### NAFLD and steatohepatitis

NAFLD and steatohepatitis is characterized by a pathologic spectrum that ranges from fatty liver (hepatic steatosis) to cirrhosis with intervening non-alcoholic steatohepatitis (NASH) and usually occurs in association with obesity and insulin resistance[13,72,88-90].

Increased serum PAMP levels were observed in both experimental models and in NAFLD patients[9,18,91-96]. A shift in microbial populations to adopt an "obese" phenotype in NAFLD is referred to as "metabolic endotoxaemia", in which a high-fat diet is associated with

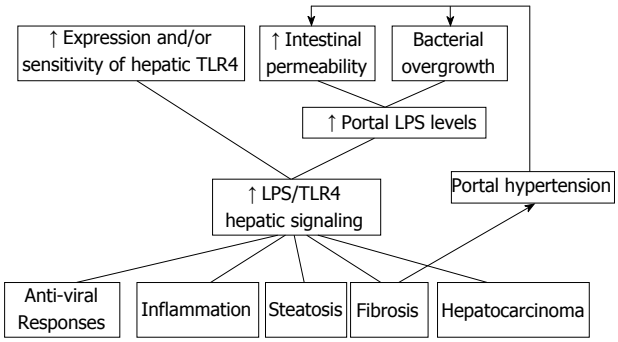


**Figure 2 Enhanced lipopolysaccharide/toll-like receptors 4 signaling in chronic liver diseases.** Induction of anti-viral responses, inflammation, steatosis, fibrosis, and hepatocarcinoma *via* LPS/TLR4 signaling alongside hepatic fibrosis mediated portal hypertension which further increases bacterial overgrowth and intestinal permeability, creating a positive feedback process. TLR4: Toll-like receptors 4; LPS: Lipopolysaccharide.

elevated levels of LPS translocation[27,90,97].

While TLR2, TLR4 and TLR9 participate in the development of NASH and NAFLD, LPS-TLR4 is considered to be the main pathway for the progression of NAFLD[98-100]. The role of bacterial overgrowth has also been associated with development of NASH, emphasizing the interaction between bacterial overgrowth, gut permeability and liver injury[90,101,102].

While the role of adipose tissue macrophages in the development of NAFLD is not yet clear, KCs are known to play a pivotal role in the development of NAFLD alongside accompanying hepatic inflammation and related complications[18,98].

When inflammation occurs in NAFLD, NF-κB and transcriptional factor AP1 are activated, stimulating the production of TNF-α and IL-10, in particular, by KCs[23,103]. Studies in animal models indicated the likelihood of TLRs 2, 4 and 9 to participate in NAFLD onset or progression[9,18,91,104]. LPS/TLR4 and TLR9 signaling in KCs have been associated with both onset and progression of NAFLD by inducing reactive oxygen species (ROS)-dependent activation of X-box binding protein-1 and IL-1b, respectively, whereas induction of hepatic steatosis occurs independent of TLR2 signaling in KCs[18,104-106] (Figure 2).

While free fatty acids and denatured host DNA are considered to be potential candidates to activate TLR2, TLR4 and TLR9 signals, no clear-cut evidence exists to confirm their capacity to activate TLRs in NAFLD[18]. TLR4 signaling has been considered to play a major role in the pathogenesis of NAFLD that operates *via* KCs stimulation and increased ROS and TNF-α production[13].

### ALD

ALD is described along a disease spectrum ranging from steatosis and steatohepatitis to fibrosis and cirrhosis and potential development of hepatocellular carcinoma (HCC)[90,107].

Despite a strong association between alcohol and hepatotoxicity, the exact pathogenesis has not yet been elucidated[90]. Involvement of the gut microbiota *via* a "leaky" gut has been indicated in the development of ALD[18], whereas the role of alcohol has also been suggested in increasing gut permeability by disrupting tight junctions[108,109]. Increased plasma LPS levels and hepatic endotoxin levels, which leads to increased TLR4 signaling on KCs, HSC, LSECs and hepatocytes and thus the release of pro-inflammatory cytokines have been associated with inflammation and liver damage[9,107,108,110].

Recent studies indicate significant contribution of TLR4 signaling and thus the crucial role of both KCs and HSCs in development of gut-derived endotoxin related effects in ALD[18]. Chronic alcohol consumption is also associated with the increased expression of TLR1, TLR2, TLR4 and TLR6-TLR9, which further potentiates the secretion of the pro-inflammatory TNF-α in response to LPS[111].

KCs produce pro-inflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8, chemokines) and profibrogenic factors (TGF-β) under post-LPS mediated TLR4-dependent stimulation, and consequent liver inflammation and stellate cell activation induce liver fibrosis[9,15,112,113]. The TLR4-dependent downstream signaling cascade in ALD was shown to proceed *via* the MyD88-independent pathway, possibly *via* adapter molecule TRIF[114]. Nonetheless, increased expression of not only TLR4 but also other TLRs such as TLR1, 2, 6, 7, 8 and 9 was shown in an experimental chronic alcohol model[115].

Although activation of KCs *via* TLR4 signaling is a key event in the pathogenesis of alcohol-induced liver injury[18], recent data emphasize the activation of TLR4 signaling in HSCs as well, indicating the their contribution to alcohol-induced hepatocyte injury, steatosis, inflammation, and fibrogenesis[18,116]. In HSCs, activated TLR4 signaling downregulates TGF-β pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI), resulting in enhancement of TGF-β signaling, whereas BAMBI downregulation is dependent on MyD88 but not TRIF[18,110]. The TLR4-TRIF-IRF3-dependent pathway associated with bone marrow-derived cells including KCs is considered to be more important than the TLR4-MyD88-dependent pathway in the development of alcoholic steatohepatitis[18,110,114].

Acting through upregulation of TLR4 and MD-2 and induction of a Th1-type immune response, bacterial DNA recognition by TLR9 was also shown to be associated with LPS induced liver injury[117], indicating the likelihood of TLR9 signaling to contribute to pathogenesis of ALD[18].

### Hepatic fibrosis and cirrhosis

The development of hepatic fibrosis and consequent cirrhosis upon continued liver insults may occur in any type of chronic hepatic injury, including viral hepatitis, alcohol, autoimmune and metabolic disease[9,67].

Prolonged or repeated liver injury leads to a maladaptive interplay of hepatocytes, HSCs and KCs in association with TLR expression, eventually resulting in abnormal extracellular matrix protein deposition in the

liver[35,67,118].

LPS-TLR4 activation is considered essential for hepatic fibrogenesis, whereas TLR4 is expressed on KCs and HSCs, the key mediators of hepatic fibrogenesis[27,75,80,81].

KCs express the highest levels of TLR4 and act as the principal target of LPS leading to release of several pro-inflammatory and pro-fibrogenic mediators[5,27,71,114,119]. However, HSCs are crucial in the pathogenesis of fibrosis and cirrhosis given their myofibroblastic phenotype and ability to produce collagen, the principal component of fibrotic tissue[9,120].

Activation of HSC occurs either *via* pro-inflammatory cytokines and growth factors secreted by LPS-TLR4-stimulated KCs, or directly *via* LPS-TLR4-dependent HSC stimulation[9,71]. LPS/TLR4 signaling in HSCs is essential for development of liver fibrosis and acts *via* stimulating production of chemokines that recruit KCs alongside enabling unrestricted activation of HSCs by KCs-derived profibrogenic cytokine TGF-β[11,13,103,121] (Figure 2).

TLR4 activation in HSCs is considered to be the main step for collagen production and the main mediator of fibrosis and cirrhosis[9,11,67,70,71].

KCs induce fibrogenesis by means of proinflammatory and profibrogenic cytokine secretion, whereas HSCs are the leading source of extracellular matrix production in the fibrotic liver[11,67].

TLR9 signaling-associated metabolic pathways are also considered important in the genesis of hepatic fibrosis *in vivo*, leading to activation of pathways such as IL-1 production and thus HSCs by upregulating pro-fibrogenic genes, such as procollagen type Ⅰ and tissue inhibitor metalloproteinase-1[16,69,103,104].

Moreover, a deficiency of TLR3-mediated NK cell-dependent apoptosis of HSCs has been linked to the progression of alcohol-induced liver fibrosis[122,123]. Upregulation of TLR2 was shown to promote liver inflammation and fibrogenesis in NASH[106] and HSCs activation and inflammation response during carbon tetrachloride-induced liver fibrosis mediated *via* MAPK and NF-jB signaling pathways[124], whereas TLR5 was also shown to be directly involved in the progression of fibrosis *via* activation of the NF-κB and MAPK signaling pathways[52].

### Hepatitis B

Hepatitis B virus (HBV) is a DNA virus responsible for acute hepatitis, which is self-limiting in 80%-90% of adults and chronic in 10%-20% of cases[5,125]. Hepatitis B is associated with an increased risk of developing cirrhosis, hepatic decompensation and HCC, but prognosis shows interpersonal variation depending on the viral susceptibility and induction of antiviral immune response[126,127].

Indicating the role of TLRs in HBV infection, the activation of TLR3, TLR7 and TLR9 as well as TLR4 and TLR5, has been associated with blockage of viral replication *via* IFN-dependent inhibition of HBV[76,128,129]. Moreover, HBV leads to TLR downregulation alongside restriction of receptor activity, increasing the likelihood of persistent infection[27].

*In vitro* HBV studies on TLR expression in HepG2 cells revealed elevated expression of TLRs 2, 3, 4, 5, 6, 7 and 9 mRNA upon ligand binding along with an induced IFN response and abolished HBV DNA replication and RNA transcription, whereas no or very limited expression of TLRs 1, 8 and 10[9,130]. Furthermore, transfection of HBV-positive cell lines with TLR adaptor molecules was shown to be associated with elevated TLR activity and a consequent reduction in HBV DNA and mRNA levels[131], whereas HBV replication was completely abolished after injection of TLR3, TLR4, TLR5, TLR7 and TLR9 ligands into HBV transgenic mice[129].

TLR1, TLR2, TLR4 and TLR6 were shown to be down-regulated in HBV-infected peripheral blood monocytes along with a decreased cytokine response to TLR2 and TLR4 ligands[132]. Downregulation of TLR2 on hepatocytes and hepatic KCs was demonstrated in HBeAg-positive CHB-infected patients, whereas upregulation of TLR2 and cytokine expression was observed in HBeAg-negative CHB patients[133]. Hence, HBeAg-induced downregulation of TLR2 *via* precore protein has been accused for the accelerated progression of disease in HBeAg-positive patients[9,133].

Although HBV is able to downregulate TLRs and thus avoid anti-viral pathways, prolonged infection and loss of HBeAg is considered likely to upregulate TLR signaling pathways such as TLR2 that are not primarily involved in anti-HBV responses while trigger hepatic inflammation and disease progression[11].

*In vitro* analysis of HBV-Met cells revealed that TLR-treated KCs and SECs to have a modulatory effect on HBV replication[134]. TLR3- and TLR4-stimulated KCs and TLR3-activated SECs were shown to affect HBV replication *via* MyD88-independent pathway[66]. HBV-suppressing effect was mediated by IFN-β in case of TLR3 ligand activation, whereas by cytokines of an undefined nature in case of TLR4-activated KCs[66].

HBV is a stealth virus and thus does not induce an IFN response during the early phase of infection, whereas its recognition by liver resident cells is considered likely to activate innate immune responses without IFN induction[107,135]. Notably, HBV was shown to be recognized by hepatic NPCs, mainly by KCs, leading to NF-κB-dependent induction of the release of the inflammatory cytokines IL-6, IL-8, TNF-α and IL-1β as well as reduced expression of transcription factors essential for HBV gene expression and replication including hepatocyte nuclear factor (HNF) 1α and HNF4α[136].

### Hepatitis C

Hepatitis C virus (HCV) is a hepatotropic virus responsible for development of chronic hepatitis and related complications such as liver cirrhosis, liver failure or HCC[137,138].

Similarly to HBV, current evidence indicates that HCV selectively impairs activation of TLR signaling controlling HCV replication, while it concomitantly stimulates TLR pathways that generate a chronic inflammatory state

leading to persistent liver injury[11,27,139,140].

HCV-induced inhibition of TLR signaling contributes to its chronicity related to virus dissemination, inflammation and eventual progression to fibrosis and cirrhosis[9,11].

Regulation of HCV replication by non-parenchymal liver cells occurs through the production of IFN-β upon their stimulation by TLR3 and TLR4[141]. The inhibitory effect of HCV proteins on TLR7 and TLR9, is also likely to prevent virus clearance[27]. Furthermore, activation of TLR2 along with TLR1 and TLR6 and possibly TLR4 by HCV core protein and NS3 promotes hepatic inflammation and injury[142-145].

In the presence of HCV, significantly decreased TLR7 expression along with TLR7-independent activation of IRF-7 pathway was demonstrated both *in vitro* and *in vivo*[146].

The NS3/4A serine protease of HCV, HCV NS3 protein and HCV NS5A act *via* three signaling pathways including the TLR3-TRIF-TBK1-IRF-3, TLRMyD88, and RIG-I/MDA5-IPS-1 pathways to enable HCV to evade innate immune signaling[33]. Moreover, LPS, the HCV core protein and IFN-γ have been suggested to amplify inflammatory monocyte/macrophage activation *via* formation of MyD88-IRAK complexes, increased NF-κB activation and increased production of TNF-α, leading to the loss of TLR tolerance[147].

Based on these findings, both host- and virus derived factors have been considered likely to act on macrophages to induce persistent inflammation during chronic HCV infection[53,107].

### *Hepatocarcinoma*

Diseases associated with uncontrolled innate immunity related to TLR ligand exposure in the liver (fibrosis, hepatitis B and C infection, ALD and NASH) are also among the etiologies for HCC. Therefore, it appears likely that TLRs play a role in the development of inflammation-associated liver cancer and are involved in the progression of HCC[18,107]. Hence, chronic hepatic inflammation and fibrosis, as regulated by TLR activation, promotes HCC formation in approximately 10% of cases of cirrhosis[9,54].

TLRs, TLR4 in particular, are considered to play a significant role in associating hepatic chronic inflammation and hepatocarcinoma[13]. A significant regression in liver tumors in TLR4 and MyD88 deficient mice indicates a prominent contribution of TLR signaling to hepatocarcinogenesis[23,148].

HCC has been indicated to be promoted *via* gut microbiota and TLR4 in association with increased production of proinflammatory cytokines (TNF-α, IL-6), hepatomitogen epiregulin expression and prevention of apoptosis, whereas a reduction in the development of HCC was shown *via* gut sterilization, germ-free status or TLR4 inactivation[18,149,150].

Activation of KCs *via* TLRs is considered to be involved in the process of tumorigenesis[18] by inducing proinflammatory cytokines and hepatomitogens responsible for enhanced development of HCC[150,151], whereas TLR4 expression on non-marrow-derived resident liver cells is considered to be required for the promotion of HCC[149].

TLR4 contributes significantly to hepatic inflammation and fibrosis, whereas upregulation of inflammatory factors such as COX-2 and NF-κB by TLR4 as well as the TLR adaptor protein Myd-88 is also important in hepatocarcinogenesis[148,152-155]. TLR3 expression is suggested to contribute to hepatocarcinoma *via* pro-apoptotic activity, while activation of TLR9 *via* CpG DNA of HBV has been associated with malignant transformation in liver cells[27,156,157].

Although, TLR2 binding with ligands such as HMGB1 and HSPA1A is associated with tumor enhancement, the effect of TLR2 activation is considered likely to differ according to the phase of HCC carcinogenesis, with anti-oncogenic potential slowing down the onset and development of HCC in earlier phases, whereas pro-oncogenic potential during later stages that promotes the progression of inflammation and fibrosis[158].

Activation of the NF-κB and JNK pathways and higher expression levels of IKKα and IKKβ are considered critical in the production of the cytokines related to TLR-induced liver damage and HCC progression[107].

Recently, spontaneous HCC development was demonstrated in hepatocyte-specific TAK1 deleted (TAK1DHEP) mice along with a resistance for HCC development that occurs *via* deletion of MyD88, TLR4 or TLR9 signaling[159].

Alcohol and HCV are suggested to interact in causing progression of liver disease and malignancy, whereas TLR4, TLR4 downstream gene Nanog and activated LPS-TLR4 are also considered to contribute to this synergy *via* triggering proliferative and anti-apoptotic signals to non-marrow-derived resident liver cells and thus HCC progression[9,149,150,160].

### *Ischemic/reperfusion injury and liver allograft rejection*

Ischemia-reperfusion (I/R) injury in partial hepatectomy and liver transplantation is associated with the release of various endogenous ligands for hepatic tissue TLRs and thus the activation of complex signaling pathways that induce neutrophilic and T-lymphocytic tissue inflammation and injury[53,161,162].

Among the most studied TLRs in hepatic I/R, TLR4 was shown to participate in certain acute sterile injury models, including liver I/R, by mobilizing the immune system upon detection of endogenous ligands, whereas limited data are available on TLR2 and TLR9[163,164].

MyD88-independent activation of TLR4 by DAMPs is considered central to the inflammatory process observed in I/R lesions[165-167], whereas HSP, heparan sulfate, fibronectin, fibrinogen, hyaluronan and HMGB1 are known to act as endogenous ligands for TLR4 activation in hepatic I/R injury[5,163].

Release of HMGB1 activates the cell surface TLR4 on KCs and leads to a subsequent release of cytotoxic mediators (TNF-α, IL-6 and chemokine IP-10), alongside an inappropriate activation of the pro-apoptotic protein kinase JNK and stress-responsive NF-κB, all of which are mediators of cell injury[5,163,168,169]. Cellular expression

of TLR4 is further upregulated *via* newly synthesized mediators such as TNF-α, leading to formation of a vicious cycle of proinflammatory cytokine production[61,163,170].

Downstream TLR4 signaling pathways in I/R injury seems to be independent of MyD88 signaling, whereas TRIF-dependent activation of the interferon response and IRF1 expression is considered critical for mediating I/R injury in hepatocytes in terms of releasing the danger signal HMGB1[164,171,172]. Hence, TLR4, IRF1 and HMGB1 are considered three important and interacting mediators of I/R injury[164].

Albeit not consistent, available data suggest that besides lack of TLR4, downregulation of TLR2 expression in the donor organ also suppress I/R injury[27,165,173]. Accordingly, given the amelioration of liver injury in I/R *via* non-selective inhibition of TLR2 and TLR4 activation by certain molecules such as bicyclol or N-acetylcysteine, role of TLRs in I/R lesion has been emphasized[27,174,175].

TLR9, which shows affinity toward both pathogen-derived and endogenous host DNA, is considered to play a crucial role in non-pathogen-induced hepatic I/R injury by causing neutrophil activation, liver necrosis, and inflammatory cytokine release[163,176,177].

Although TLR signaling dependent early activation of the innate immune system is consistently reported in the setting of I/R injury, additional studies are required to fully explore the roles of other TLRs and TLR signaling pathways in I/R injury[163,164].

### *Liver regeneration after partial hepatectomy*

Recognizing the mechanism of liver regeneration is important not only for managing acute liver failure and post-transplant hepatic dysfunction but also for disturbed liver regeneration in NASH or NAFLD and advanced liver fibrosis[178]. The deposition of excessive amounts of extracellular matrix, the presence of persistent inflammation, the transformation of SECs and HSCs, portal blood flow reduction and increased JNK activity are considered among the factors associated with the regenerative ability of fibrotic livers[178,179].

TLR/MyD88-mediated pathways are associated with onset of liver regeneration after partial hepatectomy (PH) *via* activation of NF-κB, release of TNF-α and IL-6 and the expression of the immediate early genes for cell replication in hepatocytes, whereas distinct TLR ligands responsible for the priming process have not yet been clarified[33,178]. No contribution of TLR2, TLR4 or TLR9 to MyD88-mediated pathways and no influence of TLR2 or TLR4 on proinflammatory cytokine production or gene replication have been reported for liver regeneration after PH[33,180,181].

In fact, given the inhibition of regenerative process *via* excessive TLR signaling produced by LPS injection after PH, the magnitude of TLR signaling is considered critical for intact liver regeneration[178,182].

TLR3 signaling, which utilizes a distinct adaptor protein, TRIF, is considered to attenuate the initiation of liver regeneration *via* TLR3-dependent NF-κB activation in hepatocytes and TLR3-induced IFN-γ through STAT1 and consequent induction of the IRF-1 and p21 pathways[178,183,184].

In addition, although a non-TLR MyD88-dependent pathway with IL-1 and IL-18 has been suggested to play a role in allograft rejection initially, findings on the existence of normal liver regeneration after PH in caspase 1-deficient mice indicate unremarkable participation of IL-1β and IL-18 in liver regeneration[178].

### *Hepatic autoimmune disorders*

Although antibody formation against self-antigens is key to the development of autoimmune hepatic diseases, including autoimmune hepatitis, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)[185], recently the influence of gut microbiota on the propagation of these diseases has been indicated[90].

Given that the liver is considered a classical immunoprivileged site, TLR signals may act as an important promoter for overcoming this immunoprivilege and inducing hepatic autoimmune disease[11,13,186].

Previous studies have suggested regulator role of gut-derived products on T cell function within the liver[90], based on the connection between TLR4 signaling and the trapping of CD8+ T cells in the murine liver[187], as well as contribution of TLR9 to the homing and stimulation of hepatic NKT cells *via* a KC and IL-12 dependent process[188]. The role of LPS/TLR4 signaling has been indicated in the pathogenesis of PBC and PSC[13]. Monocytes from PBC patients have been suggested to show increased sensitivity to activation of selective TLRs (TLR2, TLR4, TLR3, TLR5 and TLR9), while the subsequent release of proinflammatory cytokines has been associated with development of self-tolerance and autoimmune progression[189] (Figure 2).

LPS was shown to accumulate in significant amounts in the biliary epithelia of PBC patients, whereas positivity for IgM antibodies against lipid A, an immunogenic and toxic component of LPS, is confirmed in 64% of PBC sera[190,191]. TLR4 expression is significantly elevated in BECs, periportal hepatocytes and blood monocytes of PBC patients[192,193], whereas LPS/TLR4 signaling has been associated with an increased release of proinflammatory cytokines such as IL-1b, IL-6, IL-8 and TNF-α[189]. TLR4 ligand-stimulated NK cells have been suggested to be associated with BEC damage in the presence of TLR3 ligand-activated monocytes among PBC patients[194]. Despite similar levels of TLRs in BECs isolated from livers from patients and controls, stimulation *via* TLR3 agonist poly I:C and co-culture with liver-infiltrating mononuclear cells resulted in elevated chemokine levels in livers from patients[195]. Moreover, when compared to patients with autoimmune hepatitis and Hepatitis C, patients with PBC showed higher levels of TLR3 and IFN-α/β in portal tracts and liver parenchyma[196]. Furthermore, TLR9 ligand (CpG) stimulation of peripheral blood monocytes from PBC patients was demonstrated to activate IgM-producing B cells and to increase TLR9 expression on these cells[197,198]. These findings emphasize the role of innate immunity not only in the pathogenesis and pro-

gression of PBC but also in the regulation of adaptive immune responses[9].

The role of TLRs in PSC has not been extensively studied[11]. Abnormal LPS accumulation was demonstrated in BECs in PSC[190]. Stimulating isolated BECs with anti-BEC antibodies from patients with PSC leads to increased expression of TLR4 along with higher levels of inflammatory cytokines in the presence of LPS[199].

Accordingly, increased LPS accumulation and TLR4 expression in BECs has been suggested to induce breakdown of self-tolerance and onset of bile duct damage in PBC and PSC thorough their stimulatory effects on selective pro-inflammatory cytokines with a critical role[13]. Given the signs of inflammatory bowel disease to exist in most patients with PSC and the likelihood of gut factors to induce response onset per se with no preceding immune cell dysfunction, future investigations are needed addressing the role of gut microbiota in conjunction with PSC and PBC to provide a better understanding of the mechanisms and treatment of these complex diseases[90].

## CONCLUSION

TLRs have been recognized as key regulators of innate and adaptive immune responses in the liver, although growing evidence suggests the critical role of TLR dysregulation in the pathogenesis and progression of many liver diseases[9,107]. TLRs, mainly TLR4 and TLR2, play a fundamental role in the inflammation and fibrosis of the liver and promote the progression of chronic liver diseases[27,35,86]. Indeed, LPS/TLR4 signaling is enhanced and essential in liver diseases such as ALD, NAFLD, PSC, CBP and fibrosis, and inhibition of TLR4 has been associated with amelioration of liver injury, emphasizing the contribution of LPS/TLR4 signaling to the pathogenesis of liver diseases[13].

The local innate immune system represented by liver cells participates in tolerance induction or inflammation alongside its interaction with the adaptive immune system, whereas suppression of the TLR system in the liver by pathogens enhance chronicity of infection[107]. Therefore, targeting TLR signaling at different levels of cascade appears to offer therapeutic potential in the management of chronic liver disease[11].

LPS/TLR4 signaling pathway has been recognized as an important pharmacological target in chronic liver diseases. Suppression of TLR4 signaling *via* modulation of LPS production, TLR and co-receptor expression and downstream signaling molecules has been shown to ameliorate liver injury, indicating the contribution of LPS/TLR4 signaling to the pathogenesis of chronic liver diseases. Given the likelihood of systemic suppression of TLR4 to disable responding pattern of TLR4 to invading pathogens, modulation of intestinal microbiota *via* probiotics and symbiotics become a preferred therapeutic strategy for liver diseases, associated with favorable tolerability and safety[13,23]. Besides, certain synthetic ligands of TLRs have been considered to act as target molecules for drug development given their effects on regulation of innate and adaptive immune responses, including TLR activators (for infections and certain cancers), TLR inhibitors (for inflammatory diseases and sepsis) as well as TLR neutralizing antibodies[34,37]. Further investigation of the role of TLR pathways in liver diseases addressing the downstream mediators and regulation of TLR signaling, the specific cell populations involved, the role of TLR polymorphisms and the mechanisms underlying liver tumorigenesis is needed to transfer knowledge on TLR pathophysiology into clinical practice in treating human liver diseases[5,23].

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ORIGINAL ARTICLE

Basic Study

# Changes in cellular proliferation and plasma products are associated with liver failure

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Author contributions: All authors have seen and approved the content of the manuscript and contributed meaningfully to the work. In summary, Melgaço JG conceived the study, performed the experiments, analyzed the data and wrote the manuscript; Pinheiro LA, Vieira YR, de Oliveira JM and Vitral CL acquired the data for cytokine quantification, serological data and the virological marker assay; de Oliveira JM reviewed the analysis and wrote the manuscript; Lewis-Ximenez LL collected the clinical data and the samples from the self-limited acute hepatitis patients and healthy subjects; Araújo CCV and Pacheco-Moreira LF collected the clinical data and the samples from the acute liver failure patients; Soriani FM, Sucupira PHF and Menezes GB performed the DNA extraction and quantified the mitochondrial DNA using molecular biology assays; Cruz OG performed the statistical analysis; Vitral CL and Pinto MA participated in the study design and coordination; Pinto MA participated in the analysis of data and the preparation of the manuscript.

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## Abstract

***AIM***

To study the differences in immune response and cytokine profile between acute liver failure and self-limited acute hepatitis.

***METHODS***

Forty-six patients with self-limited acute hepatitis (AH), sixteen patients with acute liver failure (ALF), and twenty-two healthy subjects were involved in this study. The inflammatory and anti-inflammatory products in plasma samples were quantified using commercial enzyme-linked immunoassays and quantitative real-time PCR. The cellular immune responses were measured by proliferation assay using flow cytometry. The groups were divided into viral- and non-viral-induced self-limited AH and ALF. Thus, we worked with five groups: Hepatitis A virus (HAV)-induced self-limited acute hepatitis (HAV-AH), HAV-induced ALF (HAV-ALF), non-viral-induced self-limited acute hepatitis (non-viral AH), non-viral-induced acute liver failure (non-viral ALF), and healthy subjects (HC). Comparisons among HAV and non-viral-induced AH and ALF were performed.

***RESULTS***

The levels of mitochondrial DNA (mtDNA) and the cytokines investigated [interleukin (IL)-6, IL-8, IL-10, interferon gamma, and tumor necrosis factor] were significantly increased in ALF patients, independently of etiology ($P < 0.05$). High plasma mtDNA and IL-10 were the best markers associated with ALF [mtDNA: OR = 320.5 (95%CI: 14.42-7123.33), $P < 0.0001$; and IL-10: OR = 18.8 (95%CI: 1.38-257.94), $P = 0.028$] and death [mtDNA: OR = 12.1 (95%CI: 2.57-57.07), $P = 0.002$; and IL-10: OR = 8.01 (95%CI: 1.26-50.97), $P = 0.027$]. In the cellular proliferation assay, $NK^{bright}$, NKT and regulatory T cells (TReg) predominated in virus-specific stimulation in HAV-induced ALF patients with an anergic behavior in the cellular response to mitotic stimulation. Therefore, in non-viral-induced ALF, anergic behavior of activated T cells was not observed after mitotic stimulation, as expected and as described by the literature.

***CONCLUSION***

mtDNA and IL-10 may be predictors of ALF and death. TReg cells are involved in immunological disturbance in HAV-induced ALF.

**Key words:** Acute liver failure; Cytokines; Mitochondrial DNA; Cellular immune response; Hepatitis A virus



**Core tip:** Acute liver diseases induced by viral infections are considered major causes of liver failure and death in Brazil. To better understand this pathogenesis, we investigated in a pioneering way the cellular immune response, inflammatory mediators and mitochondrial products in patients with hepatitis A virus (HAV)-induced acute liver failure (ALF) in comparison to patients with non-virus-induced ALF in a cross-sectional study. The results showed that non-invasive samples could be helpful to assay early prognostic markers that would indicate the necessity for liver transplantation. The contribution of *in vitro* immune response involved in ALF can be helpful to show the necessity of mass vaccination against HAV.

Melgaço JG, Soriani FM, Sucupira PHF, Pinheiro LA, Vieira YR, de Oliveira JM, Lewis-Ximenez LL, Araújo CCV, Pacheco-Moreira LF, Menezes GB, Cruz OG, Vitral CL, Pinto MA. Changes in cellular proliferation and plasma products are associated with liver failure. *World J Hepatol* 2016; 8(32): 1370-1383 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i32/1370.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i32.1370

## INTRODUCTION

Acute liver failure (ALF) is a rare (0.5%-1% of the acute hepatitis cases) and devastating clinical syndrome resulting from an acute insult that occurs when a high percentage of liver cells are rapidly lost. Liver transplantation is the only effective therapy[1-4]. Non-invasive methods have been proposed to evaluate the liver damage[5-7] and predict the worst outcome (death)[8-10], with little success. Nevertheless, there are few studies on the systemically released inflammatory products that indicate liver failure or regeneration before liver transplantation, such as cytokine profile or mitochondrial DNA[11-15]. Additional early prognostic markers are urgently requested to evaluate the necessity of liver transplantation therapy.

The causes of ALF involve a variety of toxic, viral, metabolic, and vascular liver injuries. The etiology of ALF varies with geography[16], and the hepatitis A virus (HAV) is the major cause of acute hepatitis in Brazil[17,18] due to absence of an effective hepatitis A vaccination program. Recent studies have shown high counts of natural killer (NK) cells ($NK^{bright}$ and $NK^{dim}$) during self-limited hepatitis A[19]. Functionally, NK cells are important components of liver immunology, mediating pro-inflammatory functions, such as IFN$\gamma$ secretion by $NK^{bright}$ ($CD3^{-}CD56^{+}CD16^{-}$) cells, as well as the lysis of target cells by a subset of $NK^{dim}$ ($CD3^{-}CD56^{low}CD16^{+}$) cells[7,19-22].

Perrella *et al*[23] (2008) showed that regulatory T cells (TReg) ($CD4^+CD25^+$) are important factors in acute hepatitis A resolution. Trujillo-Ochoa *et al*[14] showed that serum IL-17 is elevated in children with acute hepatitis A infection; however, the involvement of TReg and helper T cells in ALF caused by hepatitis A is unknown.

The goal of our study was to evaluate plasma levels of inflammatory and anti-inflammatory cytokines and mtDNA in a pilot study with a case series of liver injury patients and their association with ALF and occurrence of death. We quantified the mechanism of viral (HAV) and non-viral liver dysfunction by phenotypically characterizing cytotoxic, helper, and TReg and analyzed the cytokine secretion in a peripheral blood mononuclear cell (PBMC) clonal proliferation assay.

## MATERIALS AND METHODS

### *Patients and samples*

Eighty-four subjects agreed in participate in this study in Rio de Janeiro, Brazil, from 2009 to 2012: 46 (54.76%) were consecutive outpatients with self-limited acute hepatitis (AH) that were referred to the Viral Hepatitis Clinic of Oswaldo Cruz Institute - Fiocruz; 16 (19.05%) inpatients were admitted to the Bonsucesso Federal Hospital, a referral hospital for patients with ALF requiring transplantation; and 22 (26.19%) were healthy donors.

All samples were assayed for HAV, hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV) serological markers using commercially available enzyme-linked immunoassays (ELISAs): Anti-HAV IgM (Abbott, United States), Vikia HBsAg (Biomerieux, France), Murex anti-HCV version 4.0 (Diasorin, South Africa), and bioELISA HEV IgM version 3.0 (Biokit, Spain). Blood samples were also assayed using rapid tests for syphilis (DPP®, Bio-manguinhos, Brazil), HIV-1/2 (DPP®, Bio-manguinhos, Brazil), dengue (SD BIOLINE, Standard Diagnostics, South Korea), and leptospirosis (SD BIOLINE, Standard Diagnostics, South Korea). Other current infections and autoimmune diseases were analyzed with a chemiluminescent ELISA for Epstein-Barr, cytomegalovirus and antinuclear antibodies (ANA). The respective reference levels of $\geqslant$ 20 U/mL, $\geqslant$ 30 UA/mL, and $\geqslant$ 1.5 UI/mL were considered positive. Herpes virus type 1 (HSV-1) and herpes virus type 2 (HSV-2) were investigated using a TaqMan-based multiplex assay as previously described[24]. Metabolic disorders were also investigated whether the routine exams (biochemical, hematological, *etc*.) presented alterations or whether the patient had a family history of metabolic disorders.

AH cases were defined by aminotransferase levels of at least 10 × the upper normal limit and the onset of jaundice in a previously healthy individual[25]. The cases were further categorized according to international normalized ratio (INR) and hepatic encephalopathy grade (HE). Cases with INR < 1.5 and no HE were classified as self-limited AH and those with INR $\geqslant$ 1.5 and an HE score above Ⅱ as ALF[4].

The timing of sample collection was based on the onset of jaundice and liver enzyme levels for self-limited AH patients. In ALF patients, the timing of sample collection was based on ALF diagnosis and hospital admission. In healthy subjects, the sample collection was based on the lack of infection found in their routine exams.

The study population was divided into five groups according to etiology and clinical condition: Group 1: Virus-induced self-limited hepatitis, of which all cases were caused by HAV-AH; group 2: Non-viral-induced self-limited hepatitis, which included drug and indeterminate causes (non-viral AH); group 3: Virus-induced ALF, of which all cases were caused by HAV-ALF; group 4: Non-viral-induced ALF, which included drug and indeterminate causes (non-viral ALF); and group 5: Healthy subjects, as the control group (HC).

To assess the PBMCs, blood samples were collected in the anticoagulant citrate-dextrose solution-A (Greiner Bio-one, Kremsmünster, Austria) and stored at -70 ℃ (plasma) or in liquid nitrogen (peripheral blood mononuclear cells, PBMCs) until assay. Plasma and PBMC samples used were thawed only once for the different assays.

The study protocol was approved by the National Commission on Ethics in Research (CONEP), and by the institutional review board of the Oswaldo Cruz Foundation, FIOCRUZ (222/03). Signed informed consent was obtained from all participants. The study was performed in compliance with the relevant laws and institutional guidelines and in accord with the ethical standards of the Declaration of Helsinki.

### *Quantitative detection of cytokines and mitochondrial-derived DNA in ALF, AH and healthy control subjects*

To assess the liver inflammatory/anti-inflammatory status, plasma levels of the cytokines IL-6, IL-8, IL-10, IFNγ and tumor necrosis factor alpha (TNFα) were quantified using commercially available Standard ELISA Development kits (Peprotech, United States). To assess hepatocellular damage, the total DNA was purified from the plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, United States) according to the manufacturer's instructions[26]. The mitochondrial DNA (mtDNA) was quantified by real-time PCR as previously reported[26] using 3 pairs of primers specific for human cytochrome B (sense 5'atgaccccaatacgcaaaat-3' and antisense 5'cgaagtttcatcatgcggag3'), human cytochrome C oxidase subunit Ⅲ (sense 5'atgacccaccaatcacatgc3' and antisense 5'atcacatggctaggccggag3'), and human NADH dehydrogenase (sense 5'atacccatggccaacctcct3' and antisense 5'gggcctttgcgtagttgtat3'). The total mtDNA value corresponds to the sum of the individual values from each test. Colorimetric commercial kits were used to assess the levels of liver enzymes and total bilirubin.

### *Quantitative evaluation of the clonal proliferation response and cell phenotypes of proliferated PBMCs from ALF and AH patients*

Twenty-nine PBMC samples from 62 patients were evaluated for the proliferative cellular immune response: 16

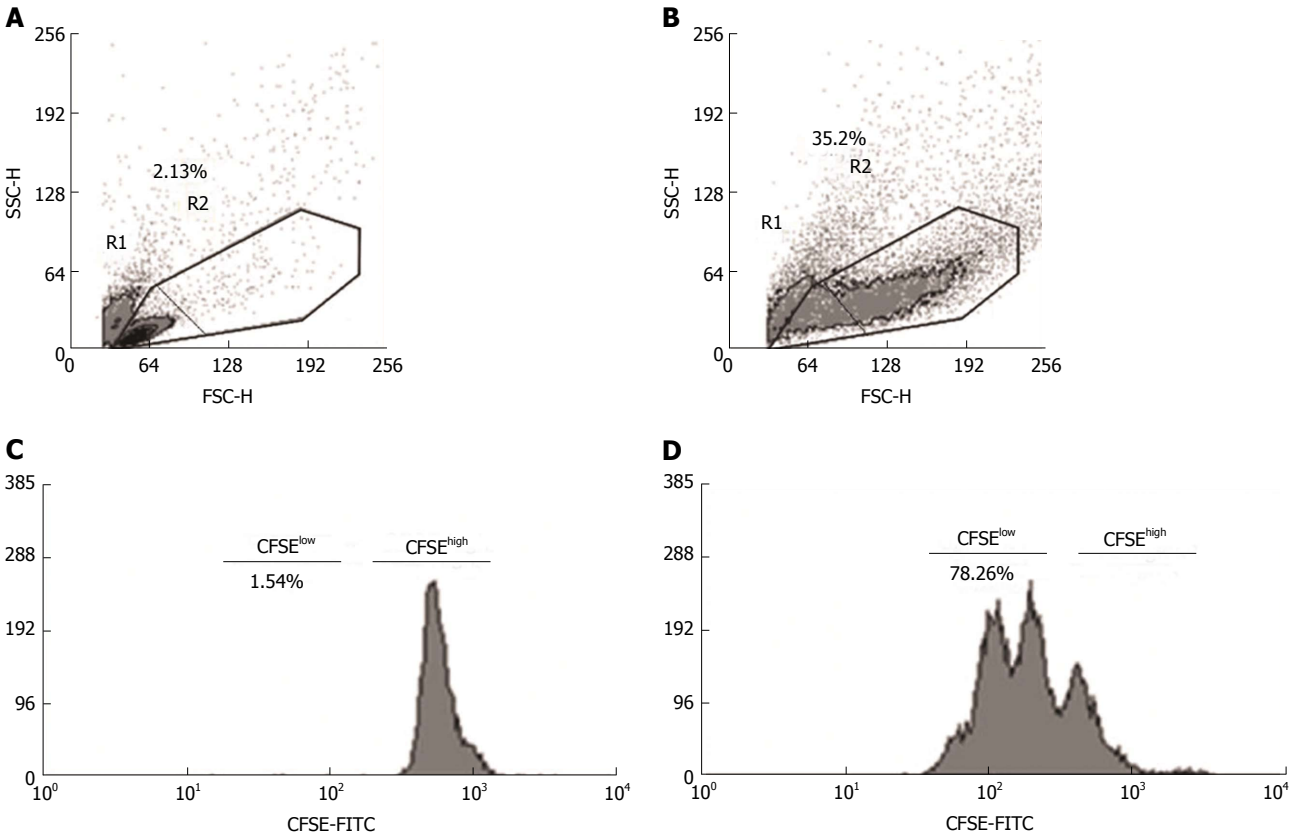


**Figure 1 Flow cytometry analysis of proliferating mononuclear cells in antigenic stimulation.** Mononuclear cell populations were gated using forward (FSC) and side (SSC) scatter, and the dot plot identifies the total cells (R1 + R2), resting cells (R1) and blasts (R2). Peripheral blood mononuclear cells, either unstimulated (A and C) or stimulated with antigens (PHA, LPS or HAV Ag) (B and D), were labeled with CFSE. The histograms show the proportion of total ($CFSE^{low}$ + $CFSE^{high}$), resting ($CFSE^{high}$) and proliferating cells ($CFSE^{low}$) observed using the Cyan flow cytometer and analyzed using the off-line software Summit version 6.0. CFSE: Carboxyfluorescein succinimidyl ester; PHA: Phytohemagglutinin; LPS: Lipopolysaccharide.

samples from patients with self-limited AH (8 patients diagnosed with HAV-induced hepatitis and 8 with non-viral hepatitis) and 13 samples from patients with ALF (8 patients diagnosed with HAV-induced hepatitis and 5 with non-viral hepatitis). Ten of twenty-two healthy subject samples were included in the cellular response assay.

The PBMCs from each patient were separated on a Ficoll density gradient by centrifugation (30 min at 400 g at 18 ℃). The concentration of viable cells was determined by trypan blue exclusion. Samples with less than 80% of viable cells at this stage were excluded. In the proliferation assay, the PBMCs were suspended in RPMI 1640 (Sigma Aldrich, United States) medium at a concentration of 5 × $10^6$ cells/mL and mixed with an equal volume of 10 mmol/L carboxyfluorescein succinimidyl ester working solution (CFSE-FITC) (Molecular Probes, Invitrogen, United States) that was diluted 1/1000 for all analyses. Cells that were not labeled with CFSE were used as a negative control for the flow cytometry analysis. The mitogen inducers phytohemagglutinin (PHA) and lipopolysaccharide (LPS) (Sigma Aldrich, United States) were used at final concentrations of 10 μg/mL and 1 ng/mL, respectively, for non-viral proliferation. The HAF-203 strain of HAV was propagated in FRhK-4 cells[27] and was used for viral-antigen-specific (HAV Ag) proliferation (viral titer of $10^6$ HAV-RNA/mL). Duplicate proliferation cultures were performed with 5 × $10^5$ cells/well in 96-well flat bottom culture plates. The plates were incubated at 37 ℃ in a 5% $CO_2$ incubator for 72 h with PHA, 24 h with LPS and 96 h with HAV Ag. After incubation, the cells were harvested for the flow cytometry assay.

To assess the cell phenotypes and proliferative response, 20000 live cells were collected from each sample using a Cyan flow cytometer (BD Biosciences, United States) and analyzed using the off-line software Summit version 6.0 (Dako Cytomation, United States) (Figures 1 and 2). PBMCs were labeled and quantified with αCD8-PerCP (clone DK25), αCD25-PE (clone ACT1), αCD56-PE (clone CM55B), αCD16-FITC (clone DJ130c) (all from Dako Cytomation, United States), αCD3-APC (clone OKT3), αCD29-FITC (clone MEM101a), αCD44-PECy7 (clone IM7), αFoxP3-FITC (clone PCH101) and isotypes (eBiosciences, San Diego, CA, United States). The intracellular staining for FoxP3 expression was performed with a Cytofix/Cytoperm® kit (BD Biosciences, United States). Total mononuclear cells were electronically gated in R1 *plus* R2 using forward (FSC) and side (SSC) properties; cellular debris and granular cells were excluded (Figure 1A and B). The proliferating cells (R1 + R2) were defined based on their FSC and SSC properties[28]. The proliferation index (PI)

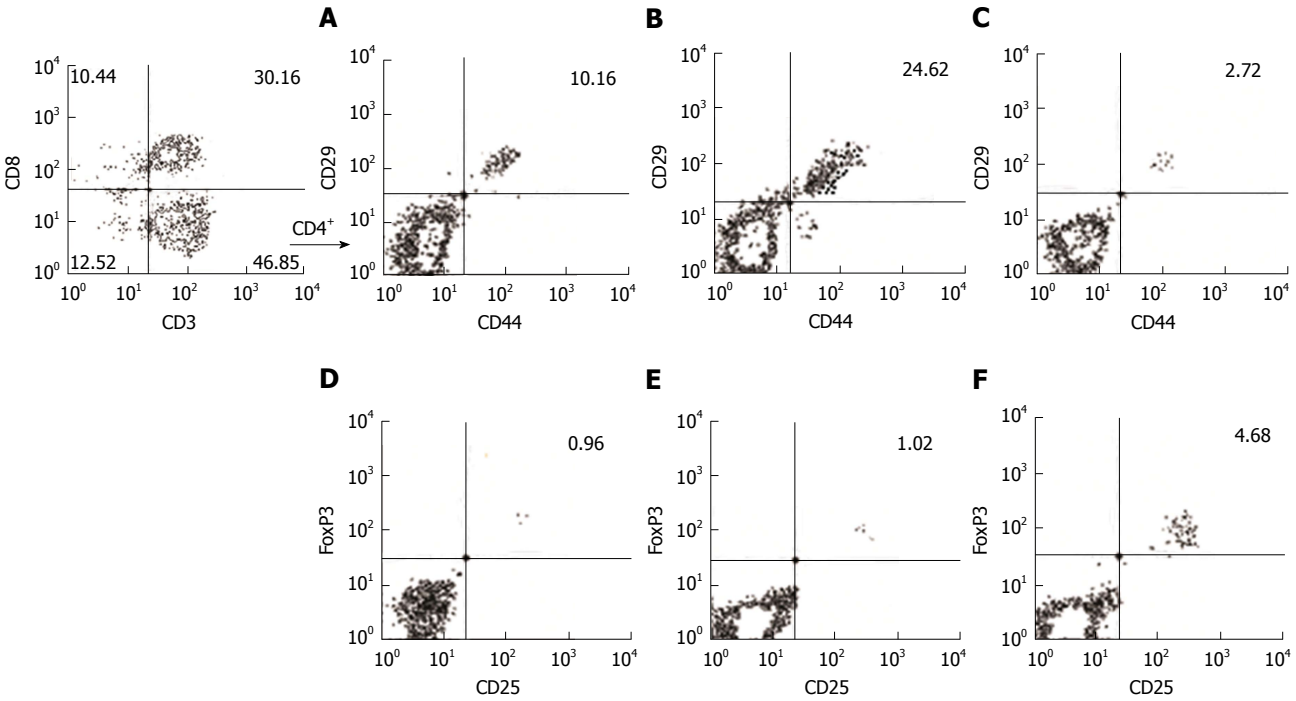


**Figure 2 Hepatitis A virus Ag-activated CD4$^+$ T cells in acute liver disease caused by hepatitis A virus.** The data from three subjects selected from our study groups were used to represent the gating strategy to select CD29$^+$CD44$^+$ and CD25$^+$FoxP3$^+$ on CD4$^+$ cells (CD3$^+$CD8$^-$). Representative contour plots of the frequency of migratory T helper cells (%) in HAV Ag-activated mononuclear cells from healthy subjects (A), patients with acute hepatitis A (B), patients with acute liver failure with HAV infection (C) and in HAV Ag-activated regulatory T cells from healthy subjects (D), patients with acute hepatitis (E), and patients with acute liver failure (F). HAV: Hepatitis A virus.

was determined by the software program; this index is a measure of the frequency of cells that have gone through more than three divisions (positive proliferation, CFSE$^{low}$) (Figure 1C and D)[28-30]. The final PI was determined by calculating the ratio of the average PI for mitogen- or antigen-stimulated cells divided by the average PI of unstimulated cells (Figure 1). The highly expressed surface markers on the T, NK and NKT cell subsets that were activated by antigenic stimulation (R1 + R2) were considered in the off-line software analysis (*e.g.*, Figure 1A and B, and Figure 2). The cell culture supernatants were assayed to quantify IL-6, IL-8, IL-10, IFNγ and TNFα using commercially available Standard ELISA Development kits (Peprotech, United States). Human cytokine IL-17/17A was quantified with the commercially available Mini ELISA Development kit (Peprotech, United States).

### *Statistical analysis*

The data are expressed as the mean ± standard deviation (SD) at a 95%CI. The distribution of the data in the groups was initially evaluated by the Kolmogorov-Smirnov test. The correlations were evaluated using the Spearman rank correlation test (R project for Statistical Computing (http://www.r-project.org/). The differences between self-limited AH, ALF, and healthy subjects were evaluated by intergroup comparisons using the Kruskal-Wallis test. If a significant difference was found, a pair of variables in the three groups was assessed with the Mann-Whitney *U*-test. For the plasma samples, receiver operating characteristic (ROC) curve analysis was used to compare the predictive strength of markers with chance. The area under the curve was used as a measure of the ability of the test to distinguish between the two groups. The software GraphPad Prism 5 for Windows, version 5.01 (San Diego, CA, United States), was used to perform statistical ROC curve analysis. Multivariate logistic regression was applied to select the independent predictors in plasma samples associated with ALF based on cut-off points (90% specificity and with the highest likelihood ratio value) obtained from ROC curve analysis. In the initial logistic model, all variables were tested for predictive strength. The variables showing statistically significant differences were kept in the final model. The logistic regression analyses were performed using SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL, United States). The significance for all statistical analyses was defined as $P < 0.05$.

## RESULTS

### *Characterization of the AH and ALF patients*

Non-viral ALF cases were caused by α-methyldopa (1 patient), rifampicin (1), and cryptogenic disease (3). The self-limited AH were caused by NSAIDs (2) and cryptogenic disease (6). HAV infection was the viral etiology found in self-limited AH (38) and ALF (11). The mean ± SD of viral load for the HAV was $1.4 \times 10^6 \pm 8.6 \times 10^5$ HAV-RNA/mL in plasma samples from ALF patients and $3.6 \times 10^3 \pm 1.8 \times 10^3$ HAV-RNA/mL in

**Table 1 Clinical characteristics of the studied population *n* (%)**

| | Acute liver failure (*n* = 16) | Acute hepatitis (*n* = 46) | Healthy control (*n* = 22) |
|---|---|---|---|
| Age (yr) | | | |
| Mean ± SD | 24.88 ± 21.52 | 21.21 ± 10.32 | 24.64 ± 8.79 |
| 25%, 75% | 9.25, 49 | 9.1, 29.75 | 15.2, 47 |
| Gender | | | |
| Male | 6 (37.50) | 25 (54.34) | 9 (40.9) |
| Diagnosis | | | |
| Hepatitis A | 11 (68.75) | 38 (82.60) | 0 |
| Drug toxicity | 2 (12.50) | 2 (4.34) | 0 |
| Indeterminate | 3 (18.75) | 6 (13.04) | 0 |
| Liver enzymes | | | |
| AST (UI/L) | 1095.5 ± 1460 | 344.5 ± 444.9 | 21.68 ± 4.87 |
| ALT (UI/L) | 806.12 ± 639.11 | 517.90 ± 884.30 | 14.36 ± 4.50 |
| Total bilirubin (mg/dL) | 21.47 ± 10.48 | 10.01 ± 6.88 | 0.85 ± 0.09 |
| Coma grade | | | |
| 0- I | 3 (18.75) | 0 | 0 |
| II -IV | 13 (81.25) | 0 | 0 |
| Coagulopathy | | | |
| INR (mean ± SD) | 4.88 ± 0.99 | 1.16 ± 0.04 | 0.98 ± 0.06 |
| Outcome | | | |
| Survived | 6 (46.15) | 46 (100.00) | 22 (100) |
| Died | 10 (53.84) | 0 | 0 |

INR: International normalized ratio; SD: Standard deviation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

samples from AH self-limited patients (AH).

The time of blood collection in self-limited AH was 1-4 wk in the HAV-AH group and 2-6 wk in the non-viral AH group. In the ALF patient group it was 1-3 wk for HAV-ALF and non-viral ALF. Three patients with acute HAV infection, INR < 1.5 and no coma grade (HE < I) had their samples collected before the evolution to liver failure. They progressed to death before transplant, according to medical records, so they were included in the ALF group. Table 1 shows more information about the study population, including age, gender, coma grade, coagulopathy, liver enzymes, total bilirubin, and outcome.

### *Elevated plasma cytokines and mtDNA are seen in AH and ALF patients compared to healthy controls*

The intensity of the inflammatory status was not associated with etiology ($P > 0.05$). Table 2 compares the systemic inflammatory parameters between clinical conditions. The cytokines IL-6, IL-8, IL-10 and IFNγ were significantly raised in the AH and ALF patients compared to the healthy subjects. TNFα was also elevated in the ALF patients compared to the healthy subjects (Table 2). Similarly, total mtDNA was significantly higher in both the AH and ALF groups than in the healthy controls. ALF patients showed a significant elevation in IL-6, IL-10, IFNγ and TNFα as well as high levels of mtDNA compared to the AH patients (Table 2).

### *Elevated plasma cytokines and mtDNA are positively correlated with the degree of liver damage, as represented by the presence of HE or coagulopathy*

When we evaluated the correlations between INR and HE and the plasma cytokine and mtDNA levels, the HE grade showed significant positive correlations with IL-6 ($P < 0.0001$), IL-10 ($P < 0.0001$), TNFα ($P = 0.0001$), and IL-8 ($P = 0.0034$) (Supplementary Figure 1A, C, E and G). The elevated INR values showed significant positive correlations with IL-6 ($P < 0.0001$), IL-10 ($P = 0.0002$), TNFα ($P = 0.0004$) and IFNγ ($P = 0.0057$) (Supplementary Figure 1B, D, F and H). A positive correlation was observed between mtDNA and HE ($P = 0.0002$; Supplementary Figure 1I) as well as INR ($P = 0.0043$; Supplementary Figure 1J).

### *Elevated cytokines and mtDNA are correlated with outcome in ALF*

To determine whether the plasma concentrations of the inflammatory or anti-inflammatory cytokines could be used as indicators of liver dysfunction, we used ROC curve analysis, which showed that IL-6 ($P < 0.0001$), IL-10 ($P < 0.0001$), TNFα ($P < 0.0001$), and IFNγ ($P < 0.00104$) had the highest diagnostic accuracy for ALF. When we evaluated hepatocyte damage, the ROC curve showed that mtDNA ($P = 0.0046$) had the highest diagnostic accuracy for ALF.

Among the cytokines, elevated IL-10 was the best indicator of ALF ($P = 0.028$). Although the IL-6, IL-8, IFNγ and TNFα levels had a positive correlation with hepatic encephalopathy, the association with ALF was not significant (Table 3). Elevated mtDNA ($P < 0.0001$) was associated with ALF diagnosis.

Subsequently, the indicators that were associated with death were investigated in all 62 acute liver disease patients: 52 survived (AH and ALF patients) and 10 died (ALF patients). Figure 3 shows that the mtDNA (*P*

**Table 2 Systemic inflammatory products in the plasma samples from patients with acute hepatitis or acute liver failure and healthy subjects**

| Plasma variables | HC ($n$ = 22) | AH ($n$ = 49) | ALF ($n$ = 13) | HC *vs* AH[a] | HC *vs* ALF | AH *vs* ALF |
|---|---|---|---|---|---|---|
| IL-6 (pg/mL) | 15.07 ± 25.92 (3.58-26.57)[1] | 68.93 ± 109.7 (38.39-99.46) | 509.30 ± 678.70 (147.6-870.9) | 0.0009 | < 0.0001 | < 0.0001 |
| IL-8 (pg/mL)[2] | ND | 10.50 ± 20.05 (4.92-16.09) | 144.70 ± 437.6 (-88.45-377.9) | < 0.001 | < 0.0001 | ns |
| IL-10 (pg/mL) | 1.81 ± 5.58 (-0.66-4.28) | 17.28 ± 51.97 (2.81-31.75) | 249.60 ± 379.60 (47.35-451.9) | 0.0006 | < 0.0001 | < 0.0001 |
| IFNγ (pg/mL) | 4.80 ± 18.00 (-3.18-12.79) | 113.0 ± 265.33 (39.1-186.8) | 229.70 ± 342.20 (47.37-412.1) | 0.0075 | < 0.0001 | 0.0016 |
| TNFα (pg/mL) | 1.08 ± 2.38 (0.02-2.13) | 27.25 ± 64.05 (9.42-45.08) | 179.40 ± 161.40 (93.42-265.4) | ns | < 0.0001 | < 0.0001 |
| mtDNA (ng/100 μL plasma) | 81.79 ± 121.6 (27.88-135.7) | 159.6 ± 202.2 (64.99-254.3) | 4228.00 ± 4286.0 (1944-6512) | 0.0131 | < 0.0001 | 0.0008 |

[1]Mean ± standard deviation (95%CI); [2]IL-8 levels in the plasma samples were evaluated only by the Kruskal-Wallis test. [a]$P < 0.05$. The differences between the acute liver failure patients, the self-limited acute hepatitis patients and the healthy controls were evaluated by intergroup comparisons using the Mann-Whitney *U*-test. IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; mtDNA: Total mitochondrial DNA; ND: Not detectable; ns: Not significant; HC: Healthy control; AH: Acute hepatitis (viral plus non-viral etiologies); ALF: Acute liver failure (viral plus non-viral etiologies).

**Table 3 Potential clinical and inflammatory parameters as indicators of acute liver failure syndrome and death**

| | Cut-off | Adjusted OR | 95%CI | *P* value |
|---|---|---|---|---|
| Plasma variables[1] | | | | |
| IL-6 (pg/mL) | > 197.6 | 1.36 | 0.04-40.27 | 0.856 |
| IL-10 (pg/mL) | > 55.77 | 18.86 | 1.38-257.94 | 0.028 |
| TNFα (pg/mL) | > 122.6 | 4.42 | 0.185-105.93 | 0.359 |
| mtDNA (ng/100 μL plasma) | > 174 | 320.54 | 14.42-7123.33 | 0.000 |
| Plasma variables[2] | | | | |
| IL-6 (pg/mL) | > 473.2 | 2.27 | 0.19-26.92 | 0.515 |
| IL-8 (pg/mL) | > 66.30 | 10.42 | 1.54-70.45 | 0.016 |
| IL-10 (pg/mL) | > 95.71 | 8.01 | 1.26-50.97 | 0.027 |
| TNFα (pg/mL) | > 313.7 | 0.27 | 0.03-2.17 | 0.220 |
| mtDNA (ng/100 μL plasma) | > 405.3 | 12.11 | 2.57-57.07 | 0.002 |
| INR | > 2.12 | 29.88 | 5.44-164.19 | 0.000 |

[1]Multivariate analysis from clinical and inflammatory parameters associated with ALF; [2]Multivariate analysis from clinical and inflammatory parameters associated with death. OR: Odds ratio; IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; mtDNA: Total mitochondrial DNA; ALF: Acute liver failure; INR: International normalized ratio.

< 0.01) and all investigated cytokines were significantly elevated in the non-surviving patients ($P < 0.01$). The ROC curve analysis showed that elevated INR, IL-6, IL-8, IL-10, TNFα, IFNγ and mtDNA in the plasma samples were able to discriminate survivors from non-survivors with a sensitivity and specificity above 70%. The high plasma levels of mtDNA, IL-8, IL-10 and INR were considered predictive factors for poor outcome (death) in patients with acute liver disease (Table 3). Despite the high levels of IL-6, and TNFα, these factors did not predict death (Figure 3 and Table 3).

### *Changes in the frequency of mononuclear cell phenotypes and cytokine secretion after the clonal proliferation assay are associated with virus (HAV)-induced AH and ALF syndrome*

The panel of phenotypic analyses for PBMC clonal proliferation was composed of activated and migratory T helper cells ($CD4^+CD29^+CD44^+$), activated and migratory cytotoxic T cells ($CD8^+CD29^+CD44^+$), activated NK cells [$CD3^-CD56^{low}CD16^+$ ($NK^{dim}$), $CD3^-CD56^+CD16^-$ ($NK^{bright}$)], and NKT cells ($CD3^+CD16^+CD56^+$). Mitogens (PHA and LPS) and virus particles (HAV Ag) were used for non-specific and specific PBMC proliferation, respectively. The mitogen stimulation showed a reduced frequency (anergic behavior) in all investigated phenotypes from HAV-induced hepatitis (ALF and AH patients) (Table 4). The same patients, when stimulated with HAV Ag, exhibited positive proliferation of the regulatory ($CD4^+CD25^+FoxP3^+$), NKT ($CD3^+CD16^+CD56^+$), and $NK^{bright}$ ($CD3^-CD56^+CD16^-$) phenotypes, and only the helper phenotype ($CD4^+CD29^+CD44^+$) frequency was reduced in HAV-induced ALF patients (Figure 2). In general, the PBMCs from HAV-induced AH showed a tendency toward negative proliferation after mitogen stimulation in all analyzed phenotypes. A significant decrease was detected in in the T helper and NKT cells (AH *vs* HC) (Table 4). The PBMCs showed a significant positive proliferation of the T helper, cytotoxic ($CD8^+CD29^+CD44^+$), and NKT cells with HAV-specific stimulation.

The secreted cytokines, IL-6, TNFα, IL-8 and IL-17, were reduced in the supernatant of HAV-induced hepatitis PBMCs from ALF patients compared to AH patients during mitogen stimulation. Additionally, IL-10 and IFNγ were reduced in ALF patients *vs* the HC subjects. In patients with AH A, we observed a significant reduction in IL-6 secretion and a general tendency toward a reduced

**Table 4 Variables from the mitogen-stimulated peripheral blood mononuclear cell phenotypes from patients with acute hepatitis A infection and healthy subjects**

| Phenotypes/cytokines (PHA/LPS) | HC ($n$ = 10) | AH ($n$ = 8) | ALF ($n$ = 8) | HC *vs* AH | HC *vs* ALF | AH *vs* ALF |
|---|---|---|---|---|---|---|
| PI of $CD3^+$ | 133.1 ± 71.12 (95.19-171.0) | 44.4 ± 25.83 (22.80-65.99) | 17.48 ± 5.94 (11.24-23.72) | 0.0155 | 0.0021 | 0.0426 |
| $CD4^+CD25^+FoxP3^+$ (%) | 17.23 ± 9.74 (12.03-22.42) | 17.08 ± 5.37 (12.59-21.57) | 5.99 ± 2.80 (3.65-8.33) | ns | 0.0009 | 0.0003 |
| $CD4^+CD29^+CD44^+$ (%) | 38.63 ± 18.37 (28.84-48.42) | 20.75 ± 7.82 (14.21-27.29) | 10.22 ± 4.74 (6.25-14.18) | 0.0062 | < 0.0001 | 0.0047 |
| $CD8^+CD29^+CD44^+$ (%) | 39.76 ± 19.91 (29.15-50.36) | 37.56 ± 25.01 (16.74-58.57) | 9.03 ± 4.59 (5.18-12.88) | ns | 0.0002 | 0.0104 |
| $CD3^-CD56^+CD16^-$ (%) | 8.31 ± 6.75 (2.07-14.56) | 4.19 ± 2.28 (2.08-6.30) | 0.50 ± 0.37 (0.15-0.84) | ns | 0.0006 | 0.0009 |
| $CD3^-CD56^{low}CD16^+$ (%) | 12.70 ± 8.93 (4.44-20.96) | 8.52 ± 5.68 (3.26-13.78) | 1.11 ± 0.66 (0.50-1.72) | ns | 0.0012 | 0.0018 |
| $CD3^+CD56^+CD16^+$ (%) | 13.66 ± 3.54 (11.77-15.55) | 7.20 ± 5.28 (2.79-11.63) | 1.83 ± 1.06 (0.94-2.72) | 0.0117 | < 0.0001 | 0.0070 |
| IL-6 (pg/mL) | 2625.33 ± 3320 (856.5-4394) | 565.7 ± 313.3 (303.8-827.6) | 156.8 ± 173.9 (11.40-302.2) | 0.0155 | < 0.0001 | 0.0070 |
| TNFα (pg/mL) | 1675.20 ± 623.4 (1343-2007) | 1405.0 ± 324.3 (1134-1676) | 145.3 ± 107.9 (55.09-235.5) | ns | < 0.0001 | 0.0002 |
| IL-10 (pg/mL) | 528.86 ± 755.1 (126.5-931.2) | 269.5 ± 145.8 (147.6-391.5) | 188.4 ± 267.1 (-16.88-393.7) | ns | 0.0454 | ns |
| IFNγ (pg/mL) | 3379.1 ± 1869 (2383-4375) | 2467.0 ± 2787 (137.1-4798) | 1249.0 ± 2067 (-479.2-2977) | ns | 0.0205 | ns |
| IL-8 (pg/mL) | 273.9 ± 116.3 (211.9-335.9) | 274.2 ± 148.5 (150.0-398.3) | 151.0 ± 156.4 (20.21-281.8) | ns | 0.0205 | 0.0379 |
| IL-17 (pg/mL) | 73.81 ± 107.0 (16.81-130.8) | 31.55 ± 35.58 (1.80-61.30) | 4.16 ± 3.20 (1.49-6.84) | ns | 0.0029 | 0.0116 |

The differences between the hepatitis A-induced acute liver failure (ALF) patients, the self-limited acute hepatitis A (AH) patients, and the healthy control (HC) subjects were evaluated by intergroup comparisons using the Mann-Whitney *U*-test. The significance cutoff for all statistical analyses was defined as $P < 0.05$. IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; ns: Not significant; PI: Proliferation index.

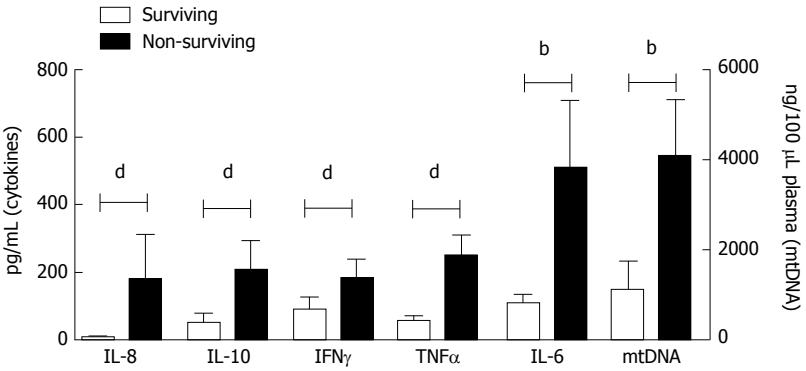


**Figure 3 Differences in inflammatory cytokines (interleukin 6, 8 and 10, interferon gamma, and tumor necrosis factor α) and hepatocyte damage (mitochondrial DNA) parameters between the surviving and non-surviving patients.** $^bP < 0.01$; $^dP < 0.001$. IL-6: Interleukin 6; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; mtDNA: Mitochondrial DNA.

secretion of all cytokines, but there were no significant differences (Table 4).

The analysis of the secreted cytokines after HAV Ag stimulation of PBMCs from HAV-induced hepatitis patients showed reduced levels of TNFα and IL-17 when comparing the ALF and AH patients. Reduced levels of secreted TNFα were also observed in the ALF patients compared to the HC subjects. Additionally, we observed elevated levels of secreted IL-10 and IFNγ. The ALF patients presented elevated levels of secreted IL-8 compared to the HC subjects. The levels of secreted IL-10, IFNγ, IL-8 and IL-17 were elevated in cultures from the AH patients (Table 5).

### *Changes in the frequency of mononuclear cell phenotypes and cytokine secretion after the clonal proliferation assay in non-viral-induced AH and ALF*

We observed a tendency toward positive proliferation for the migratory T helper ($CD4^+CD29^+CD44^+$) and cytotoxic T ($CD8^+CD29^+CD44^+$) cells for IL-6 and IL-17 release in the ALF patients compared to the AH patients. Significant elevations of $NK^{dim}$ ($CD3^-CD56^{low}CD16^+$) and $NK^{bright}$ ($CD3^-CD56^+CD16^-$) cell frequencies were associated with high levels of TNFα in the non-viral

**Table 5 Variables from hepatitis A virus Ag-stimulated peripheral blood mononuclear cell phenotypes from patients with acute hepatitis A infection and healthy subjects**

| Phenotypes/cytokines (HAVAg) | HC ($n$ = 10) | AH ($n$ = 8) | ALF ($n$ = 8) | HC *vs* AH | HC *vs* ALF | AH *vs* ALF |
|---|---|---|---|---|---|---|
| PI of $CD3^+$ | 1.09 ± 0.85 (0.64-1.55) | 3.15 ± 1.92 (1.54-4.76) | 3.34 ± 2.29 (1.42-5.25) | 0.0053 | 0.0044 | ns |
| $CD4^+CD25^+FoxP3^+$ (%) | 0.85 ± 0.96 (0.34-1.37) | 1.0 ± 0.97 (0.18-1.81) | 3.19 ± 1.49 (1.95-4.44) | ns | 0.0011 | 0.0070 |
| $CD4^+CD29^+CD44^+$ (%) | 11.99 ± 6.43 (8.55-15.42) | 27.93 ± 8.16 (21.1-34.76) | 5.46 ± 5.92 (0.50-10.42) | 0.0008 | 0.0077 | 0.0006 |
| $CD8^+CD29^+CD44^+$ (%) | 12.65 ± 4.31 (10.35-14.95) | 30.13 ± 6.74 (24.49-35.77) | 36.05 ± 10.59 (27.19-44.90) | 0.0001 | 0.0001 | ns |
| $CD3^-CD56^+CD16^-$ (%) | 0.19 ± 0.20 (0.05-0.34) | 0.30 ± 0.19 (0.13-0.46) | 1.33 ± 0.85 (0.62-2.04) | ns | 0.0009 | 0.0005 |
| $CD3^-CD56^{low}CD16^+$ (%) | 4.28 ± 2.22 (2.70-5.87) | 10.09 ± 8.94 (2.61-17.5) | 14.24 ± 11.81 (4.36-24.12) | ns | ns | ns |
| $CD3^+CD56^+CD16^+$ (%) | 1.67 ± 2.71 (0.22-3.12) | 4.25 ± 4.06 (0.85-7.65) | 15.06 ± 7.74 (8.58-21.53) | 0.0110 | 0.0003 | 0.0019 |
| IL-6 (pg/mL) | 50.49 ± 76.14 (9.92-91.06) | 76.41 ± 93.18 (-1.46-154.3) | 139.7 ± 165.9 (0.98-278.4) | ns | ns | ns |
| TNFα (pg/mL) | 92.49 ± 133.4 (21.42-163.6) | 23.96 ± 28.92 (-0.21-48.14) | 1.63 ± 1.01 (0.78-2.48) | ns | 0.0089 | 0.0098 |
| IL-10 (pg/mL) | 10.39 ± 13.97 (2.94-17.84) | 52.78 ± 62.06 (0.89-104.7) | 164.3 ± 75.56 (101.1-227.5) | 0.0297 | 0.0001 | 0.0148 |
| IFNγ (pg/mL) | 0.88 ± 1.08 (0.30-1.46) | 106.6 ± 183.9 (-47.14-260.4) | 1095 ± 1962 (-546-2735) | 0.0035 | 0.0001 | 0.0499 |
| IL-8 (pg/mL) | 88.64 ± 45.40 (64.44-112.8) | 148.9 ± 54.77 (103.1-194.7) | 150.2 ± 72.19 (89.84-210.5) | 0.0131 | 0.0110 | ns |
| IL-17 (pg/mL) | 3.36 ± 3.75 (1.36-5.36) | 32.61 ± 38.30 (0.59-64.64) | 7.58 ± 5.43 (3.05-12.13) | 0.0008 | ns | 0.0499 |

The differences between the hepatitis A-induced acute liver failure (ALF) patients, the self-limited acute hepatitis A (AH) patients and the healthy control (HC) subjects were evaluated by intergroup comparisons using the Mann-Whitney *U*-test. The significance cutoff for all statistical analyses was defined as $P < 0.05$. IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; ns: Not significant; PI: Proliferation index.

ALF patients compared to the AH patients and the HC subjects. The IL-8 levels were also significantly elevated in the ALF patients compared to the HC subjects (Table 5). In general, the comparison between the PBMCs from non-viral AH patients and the HC subjects showed a tendency toward a negative proliferation of all phenotypes investigated and the secreted cytokines IL-6, IL-10, IFNγ and IL-17 (Table 6).

### *Evidence of effects on the TReg and migratory T helper cells obtained from viral and non-viral AH and ALF patients*

To understand the influence of the TReg in AH and ALF, we evaluated the balance between the frequency of TReg with the innate and adaptive immune cells studied. Tables 5 and 6 reveal the change in the frequencies of TReg ($CD4^+CD25^+FoxP3^+$) and migratory T helper frequencies ($CD4^+CD29^+CD44^+$) in viral ALF after HAV stimulation and in non-viral ALF after mitogen stimulation. Figure 4A shows an elevated TReg-to-Thelper ratio in the HAV-induced ALF patients after HAV stimulation compared to the AH patients and the HC subjects. No changes in the ratios between the TReg and the other phenotypes were observed. After mitogen stimulation, the imbalance between the TReg-to-T helper ratio was significantly reduced in the non-viral ALF patients compared to the AH patients (Figure 4B). For the other investigated phenotypes, the alterations in this ratio were not significant.

## DISCUSSION

Acute viral hepatitis was represented by hepatitis A cases in our study. There are a large number of outbreaks of hepatitis A in Brazil; HAV infection is the major etiology of AH and ALF[17,31,32]. Here, we introduced plasma mtDNA level as a new predictor for HAV-induced ALF syndrome. In our opinion, the gross elevation of mtDNA in the ALF patients resulted from massive liver necrosis, as expected. mtDNA, inflammatory and anti-inflammatory cytokines and effector cells are involved in drug-induced liver failure in murine models and in patients[5,8,33].

Increased levels of cytokines and chemokines have been observed in all ALF and non-surviving patients, as described by other authors investigating both drug- and viral-induced ALF[5,34-36]. In our study, the high levels of IL-8 and IL-10 were predictive markers of death in acute liver disease.

Additionally, the imbalance between IL-10 and IL-12 levels has been noted in HBV-induced ALF[37], indicating an ineffective attempt to activate the anti-inflammatory pathway[38-40]. The elevated plasma levels of IL-8 that were detected in all cases of ALF are also described in patients with drug-induced ALF and are correlated with granulocyte migration into the liver parenchyma[5,41]. The elevated levels of circulating IL-6 and TNFα, also described by others[42,43], have been related to attempts

**Table 6 Variables from mitogen-stimulated peripheral blood mononuclear cells from non-viral acute hepatitis patients and healthy control subjects**

| Phenotypes/cytokines (PHA/LPS) | HC ($n$ = 10) | AH ($n$ = 8) | ALF ($n$ = 5) | HC *vs* AH | HC *vs* ALF | AH *vs* ALF |
|---|---|---|---|---|---|---|
| PI of $CD3^+$ | 133.1 ± 71.12 (95.19-171.0) | 173.3 ± 91.84 (96.51-250.1) | 268.4 ± 101.6 (142.3-394.5) | ns | ns | ns |
| $CD4^+CD25^+FoxP3^+$ (%) | 17.23 ± 9.74 (12.03-22.42) | 15.82 ± 8.13 (9.02-22.62) | 6.84 ± 5.12 (0.48-13.2) | ns | ns | ns |
| $CD4^+CD29^+CD44^+$ (%) | 38.63 ± 18.37 (28.84-48.42) | 21.84 ± 7.50 (15.56-28.11) | 36.67 ± 14.54 (18.61-54.73) | ns | ns | ns |
| $CD8^+CD29^+CD44^+$(%) | 39.76 ± 19.91 (29.15-50.36) | 22.26 ± 11.16 (12.92-31.59) | 29.59 ± 15.21 (10.71-48.47) | ns | ns | ns |
| $CD3^-CD56^+CD16^-$ (%) | 8.31 ± 6.75 (2.07-14.56) | 6.33 ± 4.13 (2.88-9.79) | 17.22 ± 4.94 (13.09-21.35) | ns | 0.0289 | 0.0030 |
| $CD3^-CD56^{low}CD16^+$ (%) | 12.70 ± 8.93 (4.44-20.96) | 11.38 ± 4.67 (7.05-15.71) | 27.66 ± 3.49 (22.11-33.21) | ns | 0.0061 | 0.007 |
| $CD3^+CD56^+CD16^+$ (%) | 13.66 ± 3.54 (11.77-15.55) | 11.63 ± 3.01 (9.11-14.15) | 8.52 ± 4.97 (2.35-14.70) | ns | ns | ns |
| IL-6 (pg/mL) | 2625.33 ± 3320 (856.5-4394) | 966 ± 622.6 (445.5-1486.0) | 1309 ± 851.6 (251.60-2366) | ns | ns | ns |
| TNFα (pg/mL) | 1675.20 ± 623.4 (1343-2007) | 1497 ± 219.8 (1313-1681) | 3217 ± 991.5 (1986-4448) | ns | 0.0044 | 0.0016 |
| IL-10 (pg/mL) | 528.86 ± 755.1 (126.5-931.2) | 217.1 ± 159.1 (84.12-350.2) | 152.1 ± 126.4 (-4.89-309.0) | ns | ns | ns |
| IFNγ (pg/mL) | 3379.1 ± 1869 (2383-4375) | 2257 ± 2872 (-143.3-4658) | 1378 ± 2533 (-1767-4524) | ns | ns | ns |
| IL-8 (pg/mL) | 273.9 ± 116.3 (211.9-335.9) | 293.9 ± 120.2 (193.4-394.4) | 733.1 ± 404.8 (230.4-1236) | ns | 0.0267 | ns |
| IL-17 (pg/mL) | 73.81 ± 107.0 (16.81-130.8) | 36.31 ± 34.62 (7.36-65.25) | 62.73 ± 5.78 (55.55-69.91) | ns | ns | ns |

The differences between the non-viral acute liver failure (ALF) patients, the self-limited acute hepatitis (AH) patients, and the healthy control (HC) subjects were evaluated by intergroup comparisons using the Mann-Whitney *U*-test. The significance cutoff for all statistical analyses was defined as $P < 0.05$. IL: Interleukin; IFNγ: Interferon gamma; TNFα: Tumor necrosis factor alpha; PI: Proliferation index; HC: Healthy control; ns: Not significant.

at liver regeneration[44,45] and liver injury[46], respectively. Therapeutic approaches targeting the clearance of inflammatory/toxic products (plasmapheresis, hemodiafiltration, and bioartificial livers) from the liver or anti-cytokine therapy are currently being considered[42,47-49] despite contradictory clinical results[50,51].

Even though the profile of monocytes was not explored here, several studies showed the important role of these cells in association with their activation, migration to the liver, and differentiation into hepatic macrophages induced by grow-factor β and IL-10 in humans[52,53] and experimental animal models[54]. Production of the inflammatory cytokines TNF, IL1-β, IL-6, IL-8 and MCP-1 by hepatic macrophages has been associated with cytokine storm in liver injury[52,53]. These findings could explain the biological relevance of high levels of circulating IL-6, IL-8 and IL-10 in ALF patients with the worst outcomes, which were produced by activated monocytes/macrophages, by antigen presentation, and by T cell proliferation.

When we evaluated the linear correlation between coagulopathy/encephalopathy and the plasma variables studied, we observed that the INR and HE scores increased in ALF cases. mtDNA, IL-6, IL-10, IFNγ, TNFα and IL-8 were also significantly elevated and were positively correlated with the elevated INR and/or HE scores observed in severe liver disease. Thus, this study also showed that elevated mtDNA and IL-10 are positively associated with the risk of ALF and mortality. Other authors described IL-10 as an important immunosuppressive cytokine that is released by TReg and is strongly expressed in HBV-induced acute-on-chronic liver failure[38,55,56].

Indeed, the most puzzling fact revealed here was the anergic behavior of the PBMCs from HAV-induced AH and ALF after *in vitro* mitotic stimulation. This fact may be explained by PBMC clonal exhaustion[57-59] or may suggest that the- TReg influence HAV Ag-primed PBMCs *in vivo* during AH and ALF syndrome[23]. In addition, when the TReg cells have been previously primed by a specific antigen (*e.g*., viral antigen), they may develop a non-specific suppressor activity, as described by others[60].

Here, the impairment of the PBMC response was associated with liver dysfunction in patients with AH A. The high TReg cell frequencies in HAV-induced ALF and the increase in IL-10 after HAV Ag stimulation were consistent with the reduced frequency found for the Th17 migratory phenotype ($CD4^+CD29^+CD44^+$ and IL-17 secretion) and the modulation of the T lymphocyte ($CD3^+$) and cytotoxic T cell ($CD8^+CD29^+CD44^+$) phenotypes.

Our results suggest that the negative regulation of the TReg cells attempts to control liver inflammation and disease progression by reducing the Th17 migration to the liver tissue in patients with HAV-induced ALF. A similar profile of antigen-specific and unspecific stimulation was

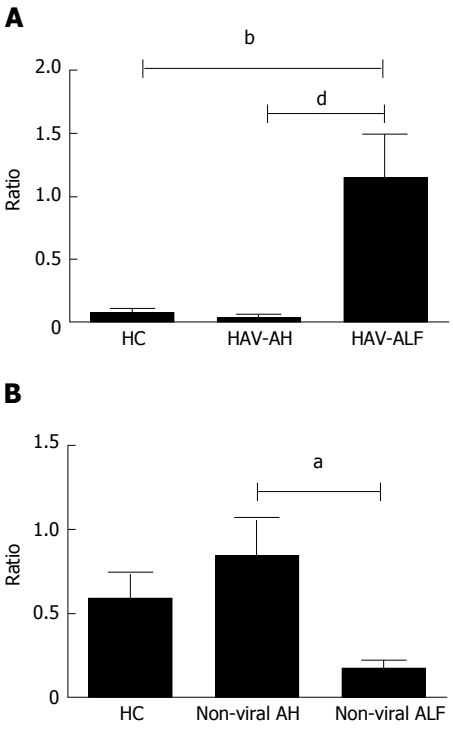


**Figure 4 Imbalance between peripheral CD4$^+$ regulatory T cells and migratory T helper cells in viral and non-viral acute hepatitis cases.** A: Comparison of the ratio of CD4$^+$ TReg-to-Thelper in HAV-induced acute liver disease (ALF and AH) and healthy controls (HC); B: Comparison of the ratio of CD4$^+$ TReg-to-Thelper cells in non-viral-induced acute liver disease (ALF and AH). $^aP < 0.05$, $^bP < 0.01$; $^dP < 0.001$. HAV: Hepatitis A virus; AH: Acute hepatitis; ALF: Acute liver failure.

observed in patients with HEV-induced AH and ALF[59] and in chronic hepatitis B infection after anti-CD3/CD28 (unspecific) stimulation[56]. Our results did not confirm the TReg influence in non-viral ALF patients, which corroborates other results[61-63]. The expansion of T helper cells (Th17) and the suppression TReg cell production are involved in the mechanisms of liver damage in drug-induced liver disease[61,63].

In HAV-AH, T helper cell proliferation was increased after HAV Ag stimulation and was reduced after mitogen stimulation. The scarce literature available describes defects in cell signaling in CD4$^+$ T cells that are secondary to ALF[59]. Other authors reported that an increase in TReg cells and a decrease in Th17 cells are associated with the survival of HBV-related acute-on-chronic liver failure patients[56], although contradictory opinions have been reported[38,64]. In our study, a similar profile was exhibited by migratory cytotoxic T cells (CD8$^+$CD29$^+$CD44$^+$) for both antigens (viral and mitogen) in HAV-induced AH and ALF. Impaired proliferation was also demonstrated with HEV Ag (pORF3), which was dependent on ERK activation (a member of mitogen-activated protein kinase) and involved in cell proliferation through the TCR/CD3 complex[65].

A linear reduction of NK$^{bright}$, NK$^{dim}$ and NKT cell reactivity occurred after mitogen stimulation in patients with HAV-induced AH and ALF, which was reversed by HAV Ag-stimulation. The loss of NK$^{dim}$ reactivity in our ALF patients corroborated the suppressor function of the TReg cells, as described above, which appears to modulate the NK-mediated liver injury. A marked elevation in the frequency of NK$^{bright}$ and NKT cells in patients with HAV-induced ALF reinforces the importance of these cells in liver injury[19,66-69].

The significant reduction in the secreted TNFα levels following the HAV Ag-stimulation of patients with HAV-induced AH and ALF shown here was also observed by other authors in HEV-induced AH and ALF[59]. However, TNFα, IL-17 and T helper cell reactivity are positively correlated with the progression to chronic liver disease and acute-on-chronic liver failure in hepatitis B infection[39,64]. In addition, Zhou *et al*[70] (2012) observed a reduced frequency of the CD4$^+$IL-2$^+$IFNγ$^+$TNF$^+$ population after resolution of hepatitis A, suggesting an increased risk of hepatitis relapse. We observed that the frequency of T cells was not reduced in mitogen stimulated non-viral-induced AH and ALF. The NK$^{bright}$ and NK$^{dim}$ cells with TNFα and IL-8 secretion were significantly elevated in patients with ALF compared to patients with AH and the HC subjects, as expected. The literature describes that the NK cells have an important role in liver damage during non-viral-induced liver diseases and contribute to ALF progression[7,33].

The relative weaknesses of our study included the variance in the plasma cytokine levels, the age of the patients, and the timing of sampling during the evolution of the disease. To minimize the effect of time on our analysis, the blood collection was performed considering the clinical manifestations in self-limited AH and the time of liver failure diagnosis and hospital admission for ALF patients. The sample size was small because the participants who were in the acute symptomatic phase (including pain and malaise) had to agree to the collection of additional samples for cellular immune response investigation; many patients did not return to the ambulatory clinic after resolution of their infection, hindering longitudinal assessment.

In conclusion, The increase of systemically released inflammatory and anti-inflammatory products is associated with AH and ALF. mtDNA and IL-10 may be useful clinical markers as part of a panel to indicate viral (HAV) and non-viral liver disease outcome. These markers, along with IL-8, may be useful to predict death. The anergic behavior of mononuclear cells in fulminant hepatitis A may, in part, be a consequence of the predominant TReg influence that is exclusively detected in HAV infection. Taken together, our results provide additional information to understand the complex immunological disturbances presented during ALF syndrome. Additional efforts are necessary to clarify the anergy mechanism in HAV infection.

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## COMMENTS

### Background

The immune response can induce gross inflammation and consequently liver damage in acute liver diseases, independently of etiology. The role of immune cells in inducing acute liver failure (ALF) in hepatitis A infection is still unknown. High levels of systemic inflammatory products and *in vitro* immune response can be helpful markers to evaluate the necessity for liver transplantation, mainly in hepatitis A patients. Additionally, to minimize the effects of liver failure caused by hepatitis A, universal vaccination should be improved in developing countries such as Brazil.

### Research frontiers

Circulating cytokines have been associated with liver failure. Imbalance between peripheral regulatory T cells and helper T cells has been correlated with the worst outcome in hepatitis B-induced liver failure, a disease preventable by vaccination.

### Innovations and breakthroughs

This is the first study evaluating biological markers to show the necessity of liver transplantation, particularly in hepatitis A patients. The role of antigen-specific T cells during ALF caused by hepatitis A virus was investigated in a pioneering way in comparison to non-viral etiologies.

### Applications

Non-invasive samples as early prognostic markers are urgently needed to determine the necessity of liver transplantation. These findings can be helpful to highlight the development of facilities for laboratory diagnostics in acute liver diseases progression. This study supports the mass vaccination against hepatitis A in developing countries.

### Peer-review

The authors describe interesting findings in the circulating cytokines, mitochondrial damage and cell proliferation when comparing different clinical statuses in acute liver diseases (self-limited acute hepatitis and ALF) and healthy controls. The correlation of these factors with the severity of liver disease and outcome is also interesting. This study evaluated accurate markers to predict the necessity for liver transplantation, which is very important for guiding clinical work. Data from T cells in the hepatitis A cohort with liver failure, as the authors note, have not been reported.

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ORIGINAL ARTICLE

Retrospective Study

# Systemic-to-pulmonary artery pressure ratio as a predictor of patient outcome following liver transplantation

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Author contributions: Rebel A and Bauer B collected and analyzed the data, and drafted the manuscript; Nguyen D, Sloan PA, DiLorenzo A and Hassan ZU provided analytical oversight and supervised the study; all authors contributed and revised the manuscript for importance; all authors have read and approved the final version to be published.

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Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at arebe2@email.uky.edu.



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## Abstract

### *AIM*

To assess the value of the mean systemic-to-pulmonary artery pressure (MAP/mPAP) ratio for predicting outcomes following orthotopic liver transplant (OLT).

### *METHODS*

A retrospective data analysis was performed and data (mean arterial blood pressure, mean pulmonary artery pressure and Cardiac Index) were collected at several points during OLT. Outcomes evaluated were duration of postoperative endotracheal intubation [ET; minutes after intensive care unit (ICU) arrival], length of ICU stay, total hospitalization and frequency of immediate postoperative complications. A total of 91 patients were included in the data analysis. Based on the intraoperative course of the MAP/mPAP ratio, 2 hemodynamic responses were identified: Group 1 (MAP/mPAP ratio increase during anhepatic period with postreperfusion recovery, $n$ = 66); and Group 2 (MAP/mPAP ratio with no change during anhepatic period or decreased without recovery, $n$ = 25).

### *RESULTS*

The main finding was that the lack of increased MAP/mPAP ratio in the anhepatic period was associated with: (1) longer intubation times; and (2) prolonged ICU

stays and total hospitalization time, when compared to patients with an increase in MAP/mPAP ratio during the anhepatic period.

***CONCLUSION***

The data from this retrospective study should raise awareness to the mean systemic to pulmonary artery pressure ratio as a potential indicator for poor outcome after OLT. Further prospective studies are needed for validation.

**Key words:** Anesthesiology; Liver transplantation; Right heart function; Outcome; Morbidity



**Core tip:** The aim of this study was to assess the value of the mean systemic-to-pulmonary artery pressure (MAP/mPAP) ratio for predicting outcomes following orthotopic liver transplant. The intraoperative pattern of this ratio has not been previously described. Performing a retrospective analysis we identified 2 different MAP/mPAP patterns: Group 1 (MAP/mPAP ratio increase during anhepatic period with postreperfusion recovery, $n$ = 66); and Group 2 (MAP/mPAP ratio with no change during anhepatic period or decreased without recovery, $n$ = 25). The main finding was that the lack of increased MAP/mPAP ratio in the anhepatic period was associated with longer intubation times, and prolonged hospitalization time.

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## INTRODUCTION

The mean systemic-to-pulmonary artery pressure ratio (MAP/mPAP) has been shown to be a valuable predictor of outcomes following cardiac surgery. Previous studies documented that the MAP/mPAP ratio was easy to obtain during cardiac surgery, and correlated with the development of pulmonary hypertension and diastolic dysfunction[1-4]. Outcomes following orthotopic liver transplant (OLT) are dependent on the ability of the patient's cardiovascular system to compensate for the physiological stress related to OLT. While detailed and expensive cardiac evaluation is routinely performed on patients before OLT, the extent of cirrhotic cardiomyopathy and biventricular dysfunction is often underestimated[5,6]. Intraoperatively, due to circumstances related to advanced multi-organ disease, limited cardiac reserve and procedural related stressors including blood loss, fluid shifts, acidosis, or hypothermia, the patient may present more hemodynamic challenges than anticipated[7].

The MAP/mPAP ratio as a predictor of patient outcome following OLT has not been investigated. We hypothesized that the pattern of the MAP/mPAP ratio during the different stages of OLT may predict the ability of the circulatory system to compensate for the surgery related stress. If the MAP/mPAP pattern indicates sufficient cardiac reserve, the patient should have a better outcome than patients with a MAP/mPAP ratio that is less favorable. In order to more reliably risk stratify these patients undergoing OLT, we performed a retrospective data analysis to explore the feasibility of obtaining useable data during OLT. With desirable outcomes defined as less morbidity/mortality, decreased need for mechanical ventilation and shorter length of stay, the aim of this study was to assess the value of the MAP/mPAP ratio for predicting desirable outcomes following OLT.

## MATERIALS AND METHODS

The Institutional Review Board (IRB) reviewed the study protocol and gave approval access to an institutional database to retrieve patient information. The IRB waived the need for informed consent since the de-identified data review demonstrated minimal risk to patient population.

The retrospective data analysis was performed on patients undergoing OLT for end-stage liver disease in the time period from October 2011 through October 2014 at a single University Hospital. All patients undergoing OLT during this time period regardless of underlying liver disease, model for end-stage liver disease (MELD) score and age were included. Exclusion criteria were patients undergoing OLT as a combined procedure with kidney transplant, redo-OLT or OLT as a treatment for acute liver failure. Patient records with incomplete intraoperative information were excluded from the data analysis.

The selected time period was based on the absence of changes in surgical approach or staff (transplant surgeon/anesthesiology). All patients underwent cardiac evaluation with transthoracic echocardiography prior to listing for liver transplant. None of the included patients were diagnosed with or had signs of pulmonary hypertension prior to OLT.

All OLT were performed using the end-to-end inferior vena cava (IVC) anastomosis technique requiring total IVC cross-clamp during the anhepatic period. Using this technique, the anhepatic period was less than 60 min for all OLTs in this study period. Intraoperative anesthesia care for all patients followed a standardized protocol for anesthesia induction, intravenous access, monitoring and vasoactive medications. All patients remained intubated at the conclusion of their surgical procedure and were transported to a dedicated intensive care unit (ICU) for anesthesia emergence and recovery.

Patient demographic data were collected including preoperative creatinine level, comorbidities, MELD score,

**Table 1 Patient demographics and surgical characteristics**

| | Age (yr) | Gender (F:M ratio) | MELD (score) | OR duration (min) | Crystalloid (mL) | Colloid (mL) | PRBC (units) | FFP (units) |
|---|---|---|---|---|---|---|---|---|
| Group 1 ($n$ = 66) | 55.6 ± 8.6 | 24:42 (36%) | 18.7 ± 8.4 | 400.9 ± 43.5 | 5804 ± 2824 | 1440 ± 972 | 3.3 ± 3.7 | 2.8 ± 3.0 |
| Group 2 ($n$ = 25) | 58.9 ± 5.4 | 7:18 (28%) | 16.3 ± 6.9 | 427.9 ± 63.3 | 6086 ± 3424 | 1607 ± 626 | 3.9 ± 3.1 | 4.0 ± 4.6 |
| $P$-value | 0.081 | 0.189 | 0.204 | 0.081 | 0.690 | 0.428 | 0.473 | 0.148 |

Data are shown as mean ± SD. $P$ value was obtained using paired $t$-test for all parameters except gender. The 1-tail exact binomial calculation with probability value of 0.5. Group 1 ($n$ = 66): Patients with MAP/mPAP ratio increase during anhepatic period; Group 2 ($n$ = 25): Patients with MAP/mPAP ratio decrease or no change during anhepatic period. MELD: Model For End-Stage Liver Disease score; OR duration: Anesthesia time from induction to ICU transfer; Crystalloid: Amount of intraoperative normal saline; Colloid: Amount of intraoperative 5% Albumin; PRBC: Packed red blood concentrate; FFP: Fresh frozen plasma; MAP/mPAP: Mean systemic-to-pulmonary artery pressure.

age and gender. Basic intraoperative information such as procedure time, intraoperative intravenous fluids and blood component therapy were extracted from the surgical records. During the retrospective chart review, the following intraoperative hemodynamic data were gathered: Mean arterial blood pressure, mean pulmonary artery pressure and Cardiac Index (CI). These hemodynamic parameters were collected at several time points during the surgical procedure: Baseline (30 min after incision), pre-anhepatic (1 h before IVC cross-clamp), anhepatic (15 min before reperfusion), neo-hepatic (15 min after reperfusion), and 1 h neo-hepatic (1 h after reperfusion).

### MAP/mPAP patterns

Based on pilot observations, the MAP/mPAP ratio was expected to increase during the anhepatic phase. During the data analysis, patients indicating an increase in MAP/mPAP ratio by $\geqslant$ 1 from baseline to anhepatic phase were categorized into Group 1. Patients showing a decrease in MAP/mPAP ratio of $\geqslant$ -1 or no change (< 1 to > -1) from baseline to anhepatic phase were categorized into Group 2. We chose the anhepatic period as comparison to the baseline value because this surgical stage is characterized by a single hemodynamic alteration (preload reduction) and for a prolonged duration. In our institution, the anhepatic period is approximately 45-55 min. Therefore, all patients received a similar type of cardiac stress. To account for fluctuations in this anhepatic period we chose a measurement point at 15 min before reperfusion (IVC cross-clamp release) to allow sufficient equilibration time for reduction in cardiac preload caused by the IVC flow interruption.

### Vasopressor use

Further chart review was performed regarding the use of vasoactive agents in the anhepatic phase of the operation. The following medications are available intraoperatively: Norepinephrine (NE), Epinephrine (EPI), Vasopressin (V), Dopamine (DOP) and Phenylephrine (PHE). Intraoperative documentation allowed the investigators to identify of the frequency and dosing of vasoactive medication at 30 to 15 min before reperfusion (anhepatic measurement of MAP/mPAP ratio). The patients were sorted into three categories: No vasoactive medication use, low dose vasoactive medication use (NE < 0.05 μg/kg per minute, EPI < 0.03 μg/kg per minute, V < 0.03 units/min, or PHE < 0.1 μg/kg per minute) and high dose vasoactive medication use (NE $\geqslant$ 0.05 μg/kg per minute, EPI $\geqslant$ 0.03 μg/kg per minute, V $\geqslant$ 0.03 units/min or any vasopressor combination).

### Outcomes

The patient outcomes evaluated were duration of post-operative endotracheal intubation (ET, minutes after ICU arrival), length of ICU stay (LOS ICU, days post OLT) and length of hospital stay (LOS Total, days post OLT to hospital discharge). Postoperative complications were recorded if they occurred in the first 14 postoperative days after OLT. The frequency of reintubation within 48 h post extubation, need for renal replacement therapy and the need for ICU readmission were recorded. Mortality < 1 mo post OLT was also recorded.

Data is reported as mean ± SD. Statistical analysis was performed by the paired $t$-test or $\chi^2$ test. A $P$ value of < 0.05 was used to identify statistical significance.

## RESULTS

A total of 100 patients were included in the study. Due to incomplete data recordings, 9 patients were excluded from data analysis. Thus, data from a total of 91 patients was included in this analysis. The demographic patient characteristics and characterization of intraoperative course are shown in Table 1. Age, gender and MELD scores were equally distributed in both groups. The most common causes for end-stage liver disease in our patient collective were Hepatitis C related liver cirrhosis (48 patients, Group 1: 35 patients; Group 2: 13 patients), NASH related cirrhosis (21 patients, Group 1: 16 patients; Group 2: 5 patients) and alcohol induced liver cirrhosis (25 patients, Group 1: 16 patients; Group 2: 9 patients). Primary sclerosing cholangitis related liver cirrhosis was the leading diagnosis in 5 patients (all in Group 1). Other rare OLT indications were autoimmune hepatitis or alpha-trypsin 2 deficiency (one patient each, all Group 1). Two patients had hepato-pulmonary syndrome prior to OLT (both in Group 1).

Pre-OLT Creatinine was not different between the two groups (Group 1: 1.26 ± 0.67 mg/dL, Group 2: 1.30 ± 0.82 mg/dL). There were no significant differences in surgical duration, fluid requirements or use of blood

**Table 2 Mean systemic-to-pulmonary artery pressure ratio and cardiac index, measured at several times during the liver transplant: Baseline (30 min after incision), preanhepatic (1 h before inferior vena cava cross-clamp), anhepatic (15 min before reperfusion), neohepatic (15 min after reperfusion), and 1 h neohepatic (1 h after reperfusion)**

| | Group 1 MAP/mPAP increase during anhepatic period ($n$ = 66) | Group 2 MAP/mPAP no change or decrease during anhepatic period ($n$ = 25) | $P$-value |
|---|---|---|---|
| MAP/mPAP baseline | 3.32 ± 0.734 | 4.13 ± 1.191 | 0.185 |
| % change preanhepatic | 0.278 ± 1.276 | 0.184 ± 1.268 | 0.754 |
| % change anhepatic | 2.892 ± 1.827 | -0.088 ± 1.137 | < 0.01[a] |
| % change neohepatic | 0.206 ± 1.411 | -0.688 ± 1.325 | < 0.01[a] |
| % change 1 h neohepatic | 0.098 ± 0.828 | -0.611 ± 1.373 | < 0.01[a] |
| CI baseline | 4.111 ± 1.229 | 4.214 ± 0.961 | 0.879 |
| % change preanhepatic | 0.039 ± 0.790 | -0.204 ± 0.832 | 0.200 |
| % change anhepatic | -1.606 ± 1.018 | -1.188 ± 1.082 | 0.089 |
| % change neohepatic | -0.327 ± 1.316 | -0.058 ± 1.231 | 0.378 |
| % change 1 h neohepatic | 0.335 ± 1.106 | 0.454 ± 1.038 | 0.643 |
| ET | | | |
| min | 967 ± 1361 | 1719 ± 1933 | 0.040[a] |
| h | 16.1 ± 22.7 | 28.7 ± 32.2 | |
| Median (min) | 540 | 1045 | |
| LOS ICU (d) | 3.9 ± 4.4 | 12.1 ± 19.2 | < 0.01[a] |
| Median (d) | 2 | 6 | |
| LOS hospital (d) | 12.0 ± 12.5 | 26.3 ± 33.2 | < 0.01[a] |
| Median (d) | 8 | 11 | |

% change is calculated as difference from value to baseline value; data are shown as mean ± SD, and median value for ET and LOS. $P$ value was obtained using paired $t$-test. [a]$P < 0.05$. ET (duration of postoperative endotracheal intubation): Minutes from ICU arrival to extubation to supplemental oxygen; LOS ICU: Length of stay in intensive care unit; LOS total: Time from ICU arrival to hospital discharge; MAP/mPAP: Mean systemic-to-pulmonary artery pressure ratio; CI: Cardiac index.

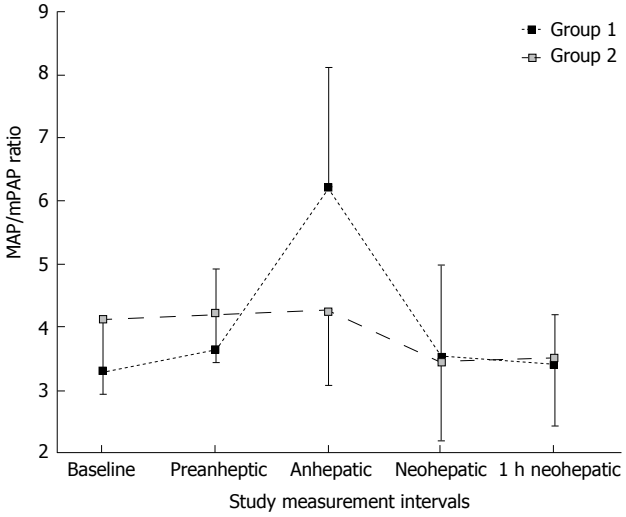


**Figure 1 Mean systemic-to-pulmonary artery pressure ration.** MAP/mPAP = Mean systemic-to-pulmonary artery pressure ratio, measured at several times during the liver transplant: Baseline (30 min after incision), preanhepatic (1 h before IVC cross-clamp), anhepatic (15 min before reperfusion), neohepatic (15 min after reperfusion), and 1 h neohepatic (1 h after reperfusion). Group 1 ($n$ = 66): Patients with MAP/mPAP ratio increase during anhepatic period; Group 2 ($n$ = 25): Patients with MAP/mPAP ratio decrease or no change during anhepatic period. Data are shown as mean ± SD. $P$ value was obtained using paired $t$-test. IVC: Inferior vena cava; MAP/mPAP: Mean systemic-to-pulmonary artery pressure.

component therapy between the patient collectives (Table 1).

The intraoperative MAP/mPAP values for each group at the different measurement points are shown in Figure 1. MAP/mPAP values at baseline and at the preanhepatic period were slightly higher in Group 2 than in Group 1 (Table 2). However, the difference was not statistically significant. Patients in Group 1 demonstrated a significant increase in MAP/mPAP during the anhepatic period. The MAP/mPAP ratio pattern in Group 2 showed less variability throughout the surgical procedure (Figure 1). The analysis of the absolute MAP/mPAP values indicated that MAP/mPAP recovered to baseline range after reperfusion in both groups. However, the percent change of MAP/mPAP ratio at baseline to the different measurement points indicated that MAP/mPAP recovered to baseline after reperfusion in Group 1. The percent change of MAP/mPAP ratio was significantly decreased in Group 2 after reperfusion, lasting up to 1 h post reperfusion (Table 2). In Group 1 the MAP/mPAP ratio increased by 2.9 ± 1.8 during the anhepatic period, while MAP/mPAP ratio in Group 2 only changed by 0.1 ± 1.1 at this observation point. CI measurements did not parallel the hemodynamic patterns shown by the MAP/mPAP ratio (Table 2). There were no significant changes in CI between both groups at any observation points during the surgical procedure.

The majority of patients required vasoactive medication during the anhepatic phase and the presence of these medications may have influenced the hemodynamic measurements at the anhepatic measurement point (Table 3). Approximately 80% of patients in both groups received some vasopressor assistance during the anhepatic phase. Phenylephrine, Vasopressin and Norepinephrine were the most commonly used vasopressors. While approximately half of patients required some low-dose vasoactive medication, the frequency

**Table 3 Vasopressor use**

| Vasopressor use frequency | Group 1 MAP/mPAP increase during anhepatic period ($n$ = 66) | Group 2 MAP/mPAP no change or decrease during anhepatic period ($n$ = 25) | *P*-value |
|---|---|---|---|
| No vasopressor use | 14 | 5 | 0.624 |
| Low dose vasopressor use | 39 | 12 | 0.341 |
| High dose vasopressor use | 13 | 8 | 0.214 |

Following medications are intraoperatively available: Norepinephrine (NE), Epinephrine (EPI), Vasopressin (V), Dopamine or Phenylephrine (PHE). Based on intraoperative documentation at 15 min before reperfusion (anhepatic measurement of MAP/mPAP ratio). The patients were sorted into three different categories: No vasoactive medication use; low dose vasoactive medication use (NE < 0.05 mcg/kg per minute or EPI < 0.03 mcg/kg per minute or V < 0.03 units/min or PHE < 0.1 μg/kg per minute) and high dose vasoactive medication use (NE ⩾ 0.05 μg/kg per minute, EPI ⩾ 0.03 μg/kg per minute, V ⩾ 0.03 units/min or any vasopressor combination). Data are shown as patient number receiving vasopressor therapy at the anhepatic measurement point (15 min before reperfusion). *P* value was obtained using $\chi^2$ test. A *P*-value for < 0.05 was set for statistical significance. MAP/mPAP: Mean systemic-to-pulmonary artery pressure.

and severity of vasopressor support was not different between Group 1 and 2.

There were significant differences in duration of postoperative intubation, length of stay in the ICU and length of hospital stay between Group 1 and Group 2 (Table 2). Patients in Group 1 extubated on an average of 12 h earlier than the patients in Group 2 (16.1 ± 22.7 h *vs* 28.7 ± 32.2 h, respectively; $P = 0.04$). The duration of ICU stay was reduced by almost 8 d (Group 1: 3.9 ± 4.4 d, Group 2: 12.1 ± 19.2; $P < 0.01$). Total length of hospitalization was approximately 2 wk less in Group 1 than in Group 2 (12.0 ± 12.5 d *vs* 26.3 ± 33.2 d, respectively; $P < 0.01$).

In the immediate post OLT period (up to 30 d postoperatively) no mortality was reported in either group. In Group 1 four out of 66 patients (6%) required reintubation and five out of 66 patients (8%) received RRT; no other complications were reported in this group. In Group 2 three out of 25 patients (12%) required reintubation and seven out of 25 patients (28%) received RRT post OLT. One patient was readmitted to ICU and one patient developed seizures.

## DISCUSSION

The main finding of this study was that despite having similar preoperative pathophysiology, one quarter of patients undergoing OLT did not display the expected increase of MAP/mPAP ratio during the anhepatic phase. This lack of increased MAP/mPAP ratio in the anhepatic period by > 1 compared to baseline values was associated with: (1) longer duration of postoperative intubation; and (2) prolonged ICU stay and total hospitalization time when compared to patients with an increase in MAP/mPAP ratio during the anhepatic period. The changes in MAP/mPAP ratio were not mirrored by CI, thus the intraoperative pattern of MAP/mPAP may be predictive of patient clinical outcomes after liver transplantation. To our knowledge, this is the first study to describe the intraoperative pattern of MAP/mPAP ratio during liver transplant and its possible relationship with patient outcomes following OLT.

Advanced liver disease has been known to affect other organ functions, most importantly the cardiovascular and renal systems[8,9]. The connection between liver cirrhosis and cardiac dysfunction has been previously recognized[8,10-12] as recent studies have documented that cirrhosis is associated with biventricular systolic dysfunction[5,6]. It is standard practice for patients to undergo an extensive preoperative cardiac evaluation to risk stratify them prior to listing for liver transplantation. The echocardiographic assessment and stress testing is usually centered on left ventricular function. While right ventricular systolic pressure and or Tricuspid annular plane systolic excursion is usually measured to evaluate the patient for right heart function and pulmonary hypertension; neither of these parameters correlates well with right systolic function[5]. Advanced echocardiography techniques (strain analysis/right ventricle relative area change) are rarely included in the standard pre OLT echocardiography assessment[5,13].

Cardiologists commonly use the MAP/mPAP ratio to stratify the severity of pulmonary hypertension because it describes the close relationship between systemic and pulmonary circulations[14]. Preoperative value of MAP/mPAP < 4 would be of concern for cardiologists for possible pulmonary hypertension, and MAP/mPAP values < 4 have been correlated with lower survival rates after cardiac surgery[2,15]. We used the MAP/mPAP ratio in this study to assess its value to predict outcomes after liver transplantation, since this parameter has shown to be a useful predictor of hemodynamic complications after cardiac surgery[2]. Our data confirm the previous findings of the use of MAP/mPAP to identify patients at risk for adverse events after high-risk surgery. In this study, the intraoperative hemodynamic MAP/mPAP pattern of patients undergoing OLT indicated that an increase in MAP/mPAP during the anhepatic phase is associated with better outcomes. A novelty of this report is the analysis of the MAP/mPAP ratio intraoperative pattern and correlating it with postoperative outcomes.

While our reported data are truly observational, it is

not well established what significance the MAP/mPAP ratio represents, especially in patients with advanced liver disease. Cardiologists have set a normal value of > 4[14]. The observation that the majority of the included patients in our study had values < 4 in absence of pulmonary hypertension may be due to advanced liver disease or the effect of anesthetic medications, since the baseline measurements were taken post induction of anesthesia. Previous studies indicated only minor effect of anesthesia on the MAP/mPAP ratio[2]. However, patients with advanced liver disease may have lower numbers due to systemic vasodilation and may have an exaggerated vascular response to anesthetics.

A recent study from Bushyhead *et al*[16] investigated preoperative data of liver transplant recipients and found that the pulmonary artery systolic pressure correlates with posttransplant outcome and therefore emphasized the importance of right ventricular assessment and pulmonary vascular resistance for the morbidity and mortality associated with the procedure. However, the publication did not assess the value of the MAP/mPAP ratio for preoperative risk stratification. While our study did not obtain preoperative MAP/mPAP values prior to anesthesia induction and did not include patients with pre-existing pulmonary hypertension defined by elevated pulmonary artery pressures, the findings of our study support the need for more thorough assessment of right heart function and cardiac reserve prior to liver transplantation. With the scarcity of acceptable donor organs, the best surgical candidate with the least likelihood for postoperative complication needs to be identified. Including MAP/mPAP ratio into the preoperative assessment may provide useful information.

Minimizing the importance of a single measurement and focusing on the intraoperative patterns of the MAP/mPAP ratio, the trend of the parameter may be interpreted as an indication of contractility reserve. The hemodynamic hallmark of the anhepatic phase during liver transplantation is characterized by significant reduction in IVC flow and therefore blood return to the heart. The behavior of MAP/mPAP ratio during the anhepatic phase therefore may indicate the systemic and pulmonary circulatory response during reduced cardiac preload. An increase in MAP/mPAP ratio may suggest that circulatory systems are able to adjust to stress and hypovolemia by vasoconstriction and inotropic compensation. Therefore, the ability to increase the MAP/mPAP ratio in the anhepatic phase observed in Group 1 indicates better cardiovascular reserve than the lack of increase or decrease as observed in Group 2.

We chose to use the MAP/mPAP ratio difference between baseline and anhepatic phase because the anhepatic stage primarily represents a single hemodynamic alteration (preload reduction). Therefore, all patients received a similar type of cardiac stress and the MAP/mPAP ratio may be more representative for the cardiac ability to compensate for the preload reduction. Although hepatic reperfusion can cause significant cardiac strain, the cardiac response to reperfusion depends on multiple factors and some of them may be due to the donor organ. The duration of reperfusion is usually short and therefore changes in MAP/mPAP may be not reflecting the cardiac response to the changes in preload, cardiac contractility or afterload. Our study used single MAP/mPAP ratio measurements at predetermined measurement point representing the hemodynamic situation during the surgical stage. Due to the fluctuating nature of all hemodynamic parameters during OLT, a continuous assessment of MAP/mPAP ratio throughout the entire surgical procedure may be more desirable in future studies to describe the cardiac reserve.

In our study, vasoactive medications were not controlled during the surgical procedure, and were titrated to effect by the anesthesia provider to ensure hemodynamic stability. Variable doses of vasoactive medications were given in both study groups. However, drug selection and dosing did not appear to influence the MAP/mPAP pattern since there were no statistical differences in distribution between both groups.

In previous studies on cardiac patients without pre-existing pulmonary hypertension, a low MAP/mPAP ratio was found to be an independent predictor of difficult separation of cardiopulmonary bypass and right heart failure[2,17]. Robitaille *et al*[2] found that patients with lower MAP/mPAP ratios had more hemodynamic complications after cardiac surgery defined as cardiac arrest, vasopressor therapy > 24 h postop, and/or use of intra-aortic balloon pump postop. These findings are in agreement with our interpretation of MAP/mPAP ratio as a predictor of the ability of the cardiovascular system to provide hemodynamic compensation. If a MAP/mPAP increase during the anhepatic phase is interpreted as a positive cardiovascular response to stressors, the lack of such compensation would explain why the patients without such a response would have less favorable outcomes.

Robitaille *et al*[2] correlated the preoperative MAP/mPAP ratio with surgical outcome after cardiac surgery and reported that the preoperative MAP/mPAP ratio was significantly higher in survivors (3.9 ± 1.4) than in those who died (3.2 ± 1.4). Since the surgical procedure during liver transplantation has more complexity and varying hemodynamic challenges specific to each surgical phase, we chose (per expert consensus) observation points to describe the MAP/mPAP pattern throughout different surgical stages of the procedure. Our pattern analysis confirms the findings of the single preoperative measurement in the previous study[2]. However, our observation and current understanding of the ratio is that it is not a static parameter and, per our data, large ratio fluctuations can occur and should alert the clinician to initiate an adjustment in the treatment plan.

Our study has several limitations. First, all data was gathered as a retrospective study from only one institution without randomization or blinding. The normal hemodynamic response was defined by the authors

based on preliminary observations and understanding of hemodynamic response to the IVC flow alterations during the anhepatic phase. However, this categorization may be oversimplified to demonstrate the variety of possible dynamic responses in the anhepatic period. The study was also limited by data analysis. The data was not measured continuously, as we selected the ratio of mean systemic to pulmonary artery pressure at defined time points during the liver transplant. Our endpoint data (time to extubation in ICU, LOS ICU, *etc*.) could have also been affected by variability in ICU provider practice. Certain providers may not have been as aggressive as other providers in extubating patients. In addition, there are other factors that affect duration of endotracheal intubation, such as failure to extubate due to opioid induced apnea. All these variables are not factored into this study.

If future prospective trials confirm the value of intra-operative MAP/mPAP ratio patterns for postoperative outcome prediction, the question will arise if MAP/mPAP ratio manipulation may be able to alter the outcome after major surgery. Increasing MAP with vasopressor/inotropic medications or lowering mPAP with pulmonary vasodilators could be beneficial.

In conclusion, the data of this retrospective study raises awareness of the mean systemic to mean pulmonary artery pressure ratio during surgery as a potential indicator for poor patient outcome following OLT. To further delineate the significance of this parameter, a multi-center, randomized, blinded prospective study with more frequent measurement points is needed for validation.

## ACKNOWLEDGMENTS

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## COMMENTS

### *Background*

This study provides relevant information to identify patients at risk for complications after orthotopic liver transplantation.

### *Research frontiers*

With the scarcity of available livers for transplantation, it is crucial that patients are properly selected and every effort is made to have the best possible outcome after the procedure.

### *Innovations and breakthroughs*

The study is offering useful information for patient selection; the next step would be (after validation) to assess if intraoperative manipulation of this parameter would optimize patient outcomes.

### *Applications*

This study should be of interest to any care provider involved with the care of potential liver transplant recipients.

### *Peer-review*

Rebel *et al* described that the systemic to pulmonary artery pressure ratio can be a predictor of survival after liver transplantation.

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ORIGINAL ARTICLE

Observational Study

# Novel non-invasive biological predictive index for liver fibrosis in hepatitis C virus genotype 4 patients

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Informed consent statement: All patients signed informed consent to participate in the study.

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## Abstract

***AIM***

To investigate the diagnostic ability of a non-invasive biological marker to predict liver fibrosis in hepatitis C genotype 4 patients with high accuracy.

***METHODS***

A cohort of 332 patients infected with hepatitis C genotype 4 was included in this cross-sectional study. Fasting plasma glucose, insulin, C-peptide, and angiotensin-converting enzyme serum levels were measured. Insulin resistance was mathematically calculated using the homeostasis model of insulin resistance (HOMA-IR).

***RESULTS***

Fibrosis stages were distributed based on Metavir score as follows: F0 = 43, F1 = 136, F2 = 64, F3 = 45 and F4 = 44. Statistical analysis relied upon reclassification of fibrosis stages into mild fibrosis (F0-F) = 179, moderate fibrosis (F2) = 64, and advanced fibrosis (F3-F4) = 89. Univariate analysis indicated that age, log aspartate amino transaminase, log HOMA-IR and log platelet count were independent predictors of liver fibrosis stage ($P$ < 0.0001). A stepwise multivariate discriminant functional analysis was used to drive a discriminative model for liver fibrosis. Our index used cut-off values of $\geqslant$ 0.86 and $\leqslant$ -0.31 to diagnose advanced and mild fibrosis, respectively, with receiving operating characteristics of 0.91 and 0.88, respectively. The sensitivity, specificity, positive predictive value, negative predictive value and positive likelihood ratio were: 73%, 91%, 75%, 90% and 8.0 respectively for advanced fibrosis, and 67%, 88%, 84%, 70% and 4.9, respectively, for mild fibrosis.

***CONCLUSION***

Our predictive model is easily available and reproducible, and predicted liver fibrosis with acceptable accuracy.

**Key words:** Liver fibrosis; Insulin resistance; Aspartate amino transaminase; Platelets; Age



**Core tip:** This observational study included a cohort of 332 recruited patients with hepatitis C virus (HCV) genotype 4 infections. The study assessed the status of demographic and biological variables at different stages of liver fibrosis. Liver biopsy with Metavir scoring was the reference standard used to classify patients into five stages of liver fibrosis (F0-F4). Patient regrouping to include three levels of fibrosis, mild (F0-F1), moderate (F2), and advanced (F3-F4), was performed to conform with practical guidelines for the management and follow-up of HCV patients. Age, aspartate transaminase enzyme (AST), insulin resistance (HOMA-IR), and platelet count were significant predictors of liver fibrosis as shown on univariate analysis. Log AST, log HOMA-IR, log platelet count and age were introduced into stepwise multivariate discriminative analysis, and a model for the prediction of liver fibrosis level was derived. Our predictive index exhibited an area under the curve (AUC) of 0.91 for the diagnosis of advanced stages of fibrosis and an AUC of 0.88 for the diagnosis of mild stages of fibrosis. The index exhibited a lower AUC of 0.64 in the diagnosis of moderate stages of fibrosis.

Khattab M, Sakr MA, Fattah MA, Mousa Y, Soliman E, Breedy A, Fathi M, Gaber S, Altaweil A, Osman A, Hassouna A, Motawea I. Novel non-invasive biological predictive index for liver fibrosis in hepatitis C virus genotype 4 patients. *World J Hepatol* 2016; 8(32): 1392-1401 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i32/1392.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i32.1392

## INTRODUCTION

Hepatitis C virus (HCV) infection exhibits worldwide distribution with a global prevalence of 2.35%, and it affects 160-170 millions of chronically infected individuals[1]. Approximately three to four million peoples are infected annually[2]. Egypt has one of the highest prevalence rates worldwide, 14.9%, as estimated by the Egypt Demographic and Health Survey. HCV genotype 4 is the most common genotype in Egypt[3]. Liver fibrosis is the essential pathophysiological consequence of chronic liver injury regardless of injurious agent because it is the pathological outcome of chronic HCV infections[4].

Hepatic stellate cells (HSCs) are the major fibrogenic cells in the liver. Apoptotic HSCs regulate the balance between the synthesis and degradation of the extracellular matrix[5]. HCV-induced bioactive transforming growth beta 1 is critical for the induction of α-smooth muscle actin and type-1 collagen, which are markers for HSC activation and proliferation[6].

The assessment of liver fibrosis level (stage) is a major issue for the management and follows-up of patients with chronic hepatitis C infection. Liver biopsy is the gold standard for the assessment of fibrosis and grade of necro-inflammation and histological staging is based on semi-quantitative scoring systems (*e.g.*, Metavir and Ishak Scores)[7].

However, liver biopsy exhibits certain drawbacks, including sampling error, invasiveness with potentiality adverse effects, complications, such as haemorrhage in 0.3% of cases, pain in 30% of cases and mortality in 0.01% of cases, and inter and intra observer variability in the reading of biopsy specimens[8]. Therefore, liver biopsy is not a perfect assessment of liver fibrosis and there is a growing need to identify surrogate non-invasive markers of liver injury with its clinical consequences and future events.

HCV chronic infections are associated with insulin resistance and type 2 diabetes mellitus, which are more frequently observed in HCV infections compared with healthy controls and liver diseases of other aetiology. HCV infection promotes insulin resistance primarily *via* increased TNF-α production and enhanced suppressor of cytokine, which block $PI_3K$ and Akt phosphorylation[9]. Insulin resistance and geographical origin (Egyptian) are the major predictors of liver fibrosis and response to therapy in HCV-genotype 4[10].

Physiological hepatic angiogenesis occurs during liver regeneration and leads to the formation of new functional

sinusoids. However pathological angiogenesis occurs in fibrosis, and it is characterized by the appearance of capillaries vascular structures[11]. The resulting hypoxia in liver injury induces activation of the renin-angiotensin system (RAS), which plays a role in the pathogenesis of fibrosis in the heart, kidney, lung and liver[12].

Multiple markers using non-invasive methods to determine liver fibrosis are available. No single non-invasive test or model can match the information obtained from actual perfect histology, and there is a need to develop further tests or models that alleviate or that reduce the need for invasive liver biopsy.

We used simple biological parameters that are related to the development and progression of liver fibrosis, to obtain a model of acceptable accuracy that predicted levels of liver fibrosis in HCV-genotype 4 patients.

## MATERIALS AND METHODS

This cross-sectional observational study included a cohort of 352 recruited patients with chronic hepatitis C infection. Patients were attending liver clinics at Minia University, Egypt, from June 2011 to July 2013. Data from twenty patients were excluded because eight patients were not genotype 4, five patients had a small core of liver biopsy that required correct assessment, four patients were diabetic, and three patients failed to follow-up. Only data of 332 patients were subjected to statistical analyses. Included patients had HCV-genotype 4 infection. HCV infection was defined as positive second generation anti-HCV antibodies and detection of HCV RNA in serum using quantitative reverse transcription polymerase chain reaction during the study period (Abbott M 2000, United States; -lower limit of detection 12 IU/mL). HCV genotyping was performed using line probe assay or reverse hybridisation and commercially available kits (Innolipa, Innogenetic, and Genetics, Belgium).

Exclusion criteria included co-infection with hepatitis B virus, human immunodeficiency virus or schistosomal infections, regular alcohol intake greater than 10 g/d, previous interferon therapy, other aetiologies of liver disease such as immune-mediated liver diseases, clinical evidence of liver decompensation and use of drugs that may alter insulin resistance, such as insulin sensitizers. Obesity determined as body mass index > 30 [body mass index (BMI) > 30] and frank diabetes mellitus diagnosed according to the American Diabetes Association diagnosis criteria[13] were exclusion criteria from the study because these conditions may confound the results. Associated lung disease was also excluded because it may confound angiotensin converting enzyme (ACE) levels.

### *Informed consent*

The Institutional Ethics Committee of participating units approved the study protocol, and all patients signed informed consent. The study was conducted in accordance with the ethical guidelines of the 1975 Helsinki Declaration.

### *Liver histopathology*

Sonographic-guided liver biopsy was performed on the second day of blood withdrawal for tests using disposable true cut needles (14 gauge) to obtain a sufficient liver tissue core. Liver biopsy specimens not less than 15 mm in length or the presence of at least 10 complete portal tracts were required for data inclusion.

Liver biopsy specimens were fixed and paraffin embedded, stained with the routinary haematoxylin and eosin (H and E) and mason trichrome stain to define fibrosis in combination with Prussian blue for iron staining. A single experienced pathologist who was blinded to clinical and laboratory data examined liver biopsy specimens.

Fibrosis staging and necroinflammatory grading were scored according to Metavir scores, which scores fibrosis as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis). Necroinflammatory activity was graded as A0 (absent), A1 (mild), A2 (moderate) and A3 (severe)[14].

### *Demographic and laboratory assessment*

The following data were collected from all patients at baseline: Age, sex, weight (W) in kilograms, height (H) in meteres, waist and hip circumferences in centimeteres, and BMI calculated as W/H2, and Waist/Hip ratio. Venous blood was withdrawn after an 8-h overnight fast and was analysed for fasting plasma glucose.

Other sample of venous blood was withdrawn after a 12-h overnight fast and collected in three tubes, one of which contained EDTA-K3 for haemogram assessment. Serum from the other two tubes was distributed as follows: One sample was frozen in a -70 ℃ refrigerator for later assessments of insulin, C-peptide and ACE. Serum from the third tube was analysed on the same day for, cholesterol, triglycerides, and liver biochemical and renal profiles.

### *Laboratory methods*

Serum insulin and C-peptide were assayed using a sandwich ELISA technique and kits from Monobind Inc (Lake Forest, CA, United States); Serum ACE was assayed using kits from R and D systems (R and D Systems, Inc. United States and Canada) that employ a quantitative sandwich immunoassay technique; Liver function tests [serum total and conjugated bilirubin, alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphates, total proteins and albumin], kidney function tests (urea and creatinine), and total cholesterol and triglycerides were performed using a Synchron $CX^{-9}$ auto-analyser using Beckman reagents (Beackman Instruments; Scientific Instruments Division, Fullerton, CA, United States); Complete blood count was performed on using Coulter Counter T 660 (Beckman Coulter, Inc., Harbor Blvd., Fullerton, CA, United States); Prothrombin time was assessed on an STA-Stago Compact CT autoanalyser (Diagnostic Stago,

Inc., Parsippany, NJ, United States) using reagents from Dade Behring (Dade Behring Holdings Inc., IL, United States); hepatitis B surface antigen and C-antibody were measured using Roche Cobase 411 (Roche Diagnostic Gmblt); insulin resistance (IR) was determined using the homeostasis model assessment for insulin resistance (HOMA-IR) method and the following equation: HOMA-IR = Fasting insulin (mU/mL) × Fasting plasma glucose (mmol/L)/22.5. Insulin resistance as calculated using this method correlates closely with the gold standard hyperinsulinemic/euglycemic clamp method in diabetic and non-diabetic subjects[15,16].

### *Statistical analysis*

Qualitative data are presented as numbers, (%). Normally distributed variables are presented as the means ± SD and non-parametric data are presented as the medians and interquartile range. The distribution of qualitative variables was evaluated using the $\chi^2$ test or Fisher's exact test, as indicated. The means were compared between groups using the non-parametric independent-samples Kruskal-Wallis test, and the level of significance following pairwise comparisons was adjusted for the number of comparisons made.

Fibrosis stages based on Metavir scores were distributed into 5 classes: F0, F1, F2, F3 and F4. Patients were further regrouped into 3 stages of mild (F0-F1), moderate (F2) and advanced fibrosis (F3-F4) for statistical analyses. Univariate analyses identified patient' age, AST and platelet count added to HOMA-IR as significantly different between the 3 levels of fibrosis in overall and pairwise comparisons. All variable were introduced in a stepwise discriminative functional analysis model for the three levels of fibrosis after normalising HOMA-IR, AST and platelet count into their $\log^{10}$ values. Diagnostic accuracy is expressed as area under the curve of receiving operating characteristic (AUROC) (asymptomatic 95%CI), sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios. All tests were bilateral, and a *P* value of 0.05 was the limit of statistical significance. Statistical analyses were performed using the IBM SPSS statistical software package for MAC version 22.

## RESULTS

A total of 332 HCV-genotype 4 Egyptian patients were included to statistical analysis. Patients exhibited a mean age of 42 ± 10.7 years and male to female ratio of 180/146 (65/44%). Gender showed no statistically significant difference between levels of liver fibrosis. Mean BMI and Waist/Hip ratio were 26.7 ± 4.4 and 0.89 ± 0.08, respectively, which indicates that none of our patients was obese. None of the study patients consumed alcohol or had history of drug abuse. A total of 69.6% were non-smokers, 19% were moderate smokers and 11.4% were heavy smokers. The Metavir scoring system identified F0 = 43, F1= 136, F2 = 64, F3 = 45 and F4 = 44 patients.

Table 1 presents quantitative variables such as the mean, SD, median and quartile range in the five stages of liver fibrosis. Table 2 presents pairwise comparisons of significant variables between the three levels of fibrosis. Table 3 presents the overall significant variables using independent - samples Kruskal-Wallis tests which indicated that age, ACE, blood glucose, ALT, AST, platelet count, fasting serum insulin, serum creatinine, total and direct bilirubin, and serum albumin were significant predictors of liver fibrosis stage. Viral load showed no statistically significant difference among stages and levels of liver fibrosis.

Statistically significant variables that discriminated between the 3 levels of fibrosis on univariate analysis, namely AST, platelet count and age and HOMA-IR were introduced to a stepwise multivariate discriminant analysis. This analysis requires a normal distribution of the dependent variables and equality of variance. Therefore; HOMA-IR, AST and platelet count were transformed into $\log^{10}$ values.

Table 4 indicates that all variables were statistically significant before being introduced in the model. These variables were introduced into a model that significantly predicted liver fibrosis. Stepwise analysis derived the following equation.

Outcome = 0.514 (age) + 0.373 (Log HOMA-IR) + 0.49 (Log AST) + (-0.532) Log platelet count.

The interpretation of outcome is dependent on the functions of group centroids as: (1) mild fibrosis if outcome is $\leqslant$ -0.31 or more negative; (2) moderate fibrosis if outcome is > -0.31 (more positive) and up to +0.86; and (3) advance fibrosis if outcome is > 0.86.

Table 5 presents accuracy indices of the model in the discrimination of fibrosis stages. In mild fibrosis and at a cut-off value -0.31 or more negative, AUC was 0.88 with sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratios and negative likelihood ratios were 67.2%, 86.3%, 83.6%, 69.5%, 4.9 and 0.38, respectively, Figure 1. In advanced fibrosis and at a cut-off value > 0.86, AUC was 0.91 with sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratios and negative likelihood ratios were 73%, 90.9%, 74.4%, 90.1%, 8.0 and 0.3, respectively (Figure 2). While, in moderate fibrosis and at a cut-off value > -0.31 up to +0.86, AUC was 0.64 with sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratios and negative likelihood ratios were 53.1%, 74.1%, 33%, 86.8%, 2.0 and 0.63, respectively, Figure 3.

The obtained model was validated by applying the model to the selected studied groups. Table 6 shows the results of this validation which indicated that two-thirds of the cases were correctly classified by the model (66.1%). This sensitivity increased to 67.2% and 73% in mild and advanced fibrosis, respectively, but dropped to 53.1% in moderate fibrosis.

## DISCUSSION

The prediction of liver fibrosis is a major issue for management and follow-up of patients with chronic

**Table 1 Presentation of quantitative variable as means, standard deviation, median and quartile range by stages of fibrosis**

| Variant | Total (*n* = 332) | F0 (*n* = 43) | F1 (*n* = 136) | F2 (*n* = 64) | F3 (*n* = 45) | F4 (*n* = 44) |
|---|---|---|---|---|---|---|
| Age (yr) | | | | | | |
| Mean ± SD | 42 ± 9.8 | 31.6 ± 7.4 | 41.1 ± 9.2 | 42 ± 7.4 | 48.5 ± 8.9 | 49.7 ± 7.5 |
| Median ± QR | 42 ± 15 | 31 ± 11 | 40.5 ± 15 | 42 ± 10 | 50 ± 11 | 49 ± 13 |
| Gender (male) | | | | | | |
| *n* (%) | 184 (55.4) | 24 (55.8) | 77 (56.6) | 40 (62.5) | 22 (48.9) | 21 (47.7) |
| HOMA-IR | | | | | | |
| Mean ± SD | 3.1 ± 1.3 | 2.4 ± 0.9 | 2.7 ± 1 | 3.4 ± 1.2 | 4.1 ± 1.7 | 4.0 ± 1.2 |
| Median ± QR | 2.9 ± 1.6 | 2.4 ± 1.8 | 2.7 ± 1.4 | 3.4 ± 1.4 | 4.1 ± 2.5 | 4.2 ± 2 |
| ACE (U/mL) | | | | | | |
| Mean ± SD | 286.7 ± 132.9 | 248.9 ± 122.3 | 277.3 ± 129 | 287.2 ± 122.5 | 325.3 ± 173.3 | 320.4 ± 122.4 |
| Median ± QR | 260 ± 180 | 235 ± 127.5 | 260 ± 195 | 300 ± 190 | 275 ± 171.3 | 285 ± 138.8 |
| Glucose (mmol) | | | | | | |
| Mean ± SD | 5.1 ± 0.9 | 5 ± 0.6 | 5.1 ± 0.9 | 4.8 ± 0.8 | 5.3 ± 0.9 | 5.7 ± 1.1 |
| Median ± QR | 5.1 ± 1.22 | 5.1 ± 1.1 | 4.9 ± 1.1 | 4.7 ± 1.2 | 5.3 ± 1.6 | 5.4 ± 1.7 |
| ALT (U/L) | | | | | | |
| Mean ± SD | 58.4 ± 36.9 | 37 ± 16.6 | 53.6 ± 37.7 | 55.5 ± 30.4 | 79.8 ± 42.6 | 82 ± 33.9 |
| Median ± QR | 44 ± 52 | 36 ± 24.3 | 43 ± 43 | 47 ± 53 | 81.5 ± 57.5 | 90 ± 41.5 |
| AST (U/L) | | | | | | |
| Mean ± SD | 53.2 ± 37.6 | 27.5 ± 10.9 | 41.1 ± 21.3 | 55 ± 35.4 | 87.2 ± 53.9 | 88.6 ± 42.5 |
| Median ± QR | 36 ± 43 | 23.5 ± 18.3 | 34 ± 25.8 | 36 ± 39 | 89 ± 61.3 | 85 ± 67.3 |
| Platelet ($\times 10^9$/L) | | | | | | |
| Mean ± SD | 213.6 ± 70 | 225.8 ± 49.4 | 240 ± 65.4 | 207.1 ± 70.2 | 164.8 ± 73.8 | 158.3 ± 36.2 |
| Median ± QR | 215 ± 105 | 221.5 ± 77.5 | 233 ± 82.5 | 226 ± 120 | 150.5 ± 90.3 | 162.5 ± 38.8 |
| BMI | | | | | | |
| Mean ± SD | 27.4 ± 4.5 | 25.7 ± 4.3 | 27.9 ± 5.1 | 27.8 ± 4.3 | 27.2 ± 3.6 | 26.6 ± 3.1 |
| Median ± QR | 27.7 ± 5.8 | 25.9 ± 7.5 | 28.3 ± 7.8 | 27.7 ± 5.3 | 27.6 ± 4.6 | 27.8 ± 4.2 |
| Waist: Hip ratio | | | | | | |
| Mean ± SD | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 |
| Median ± QR | 0.9 ± 0.1 | 0.9 ± 0.04 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 |
| Insulin (uU/mL) | | | | | | |
| Mean ± SD | 13.9 ± 5.3 | 11.1 ± 4.2 | 12.2 ± 4.5 | 15.8 ± 4.9 | 17.7 ± 7.1 | 15.7 ± 3.3 |
| Median ± QR | 13.6 ± 6.7 | 12.6 ± 7.2 | 12.3 ± 6.3 | 14.6 ± 3.4 | 16.7 ± 10.2 | 16.6 ± 5.8 |
| Albumin (g/dL) | | | | | | |
| Mean ± SD | 4.2 ± 0.5 | 4.2 ± 0.3 | 4.4 ± 0.5 | 4 ± 0.8 | 4 ± 0.5 | 4 ± 0.3 |
| Median ± QR | 4.2 ± 0.6 | 4.1 ± 0.5 | 4.4 ± 0.5 | 4.2 ± 0.8 | 3.9 ± 0.7 | 4 ± 0.6 |
| Viral load (IU/mL) | | | | | | |
| Mean ± SD | 372826.7 ± 902784.9 | 338113.1 ± 624770.4 | 409890.1 ± 941388.1 | 283586 ± 858939.26 | 264338.4 ± 452377.9 | 119830.8 ± 162200.8 |
| Median ± QR | 78000 ± 280088 | 117466.5 ± 358614 | 59112 ± 310055 | 156797 ± 251419 | 47546 ± 278956 | 97133 ± 167712 |

SD: Standard deviation; QR: Quartile range; HOMA-IR: Homeostasis model for insulin resistance, a mathematically calculated formula; ACE: Angiotensin converting enzyme; AST: Aspartate transaminase enzyme; ALT: Alanine amino transferase; BMI: Body mass index.

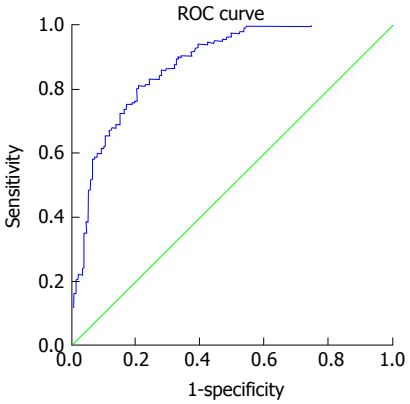


**Figure 1 Receiving operating characteristic curve for discriminating mild fibrosis.** At cut off value: -0.31 or more negative: AUC 0.88, 95%CI: 0.84 -0.91, sensitivity 67.2%, specificity 6.3%, PPV 83.6%, NPV 69.5%, PLR 4.9 and NLR 0.38. AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; ROC: Receiving operating characteristic.

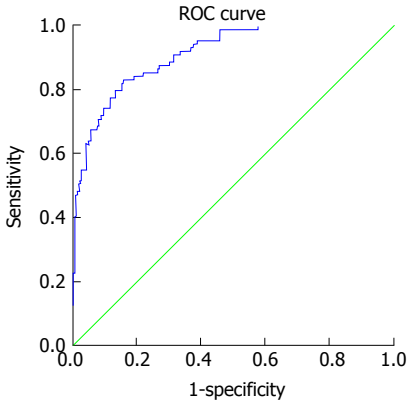


**Figure 2 Receiving operating characteristic curve for discriminating advanced fibrosis.** At cut off value > 0.86: AUC 0.91, 95%CI: 0.88-0.94, sensitivity 73%, specificity 90.9%, PPV 74.4%, NPV 90.1%, PLR 8.0 and NLR 0.3. PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; ROC: Receiving operating characteristic.

**Table 2 Presentation of quantitative variables and their significance among three levels of fibrosis and significance between groups**

| Variant | Mild fibrosis (F0-F1) (*n* = 179) | Moderate fibrosis (F2) *n* = 64) | Advanced fibrosis (F3-F4) (*n* = 89) | *P*1 | *P*2 | *P*3 |
|---|---|---|---|---|---|---|
| Age (yr) | | | | | | |
| Mean ± SD | 39.1 ± 9.6 | 42 ± 7.4 | 49 ± 8.2 | 0.001 | 0.001 | 0.001 |
| Median ± QR | 38.5 ± 16 | 42 ± 10 | 50 ± 11 | | | |
| Gender (male) | | | | | | |
| *n* (%) | 101 (56) | 40 (62) | 43 (48) | 0.100 | 0.060 | 0.832 |
| HOMA-IR | | | | | | |
| Mean ± SD | 2.6 ± 0.9 | 3.4 ± 1.2 | 4.1 ± 1.5 | 0.027 | 0.001 | 0.008 |
| Median ± QR | 2.5 ± 1.4 | 3.4 ± 1.4 | 4.1 ± 2.2 | | | |
| ACE (U/mL) | | | | | | |
| Mean ± SD | 271.5 ± 127.3 | 287.2 ± 122.5 | 323.2 ± 150.9 | 0.051 | 0.001 | 0.022 |
| Median ± QR | 255 ± 187.5 | 300 ± 190 | 275 ± 142.5 | | | |
| Glucose (mmol) | | | | | | |
| Mean ± SD | 5.1 ± 0.9 | 4.8 ± 0.8 | 5.4 ± 1 | 0.629 | 0.013 | 0.017 |
| Median ± QR | 5 ± 1 | 4.6 ± 1.1 | 5.3 ± 1.5 | | | |
| ALT (U/L) | | | | | | |
| Mean ± SD | 50.2 ± 34.9 | 55.5 ± 30.3 | 80.7 ± 38.4 | 0.004 | 0.001 | 0.022 |
| Median ± QR | 40 ± 43 | 47 ± 53 | 85.5 ± 53.3 | | | |
| AST (U/L) | | | | | | |
| Mean ± SD | 38.3 ± 20.3 | 55 ± 35.4 | 87.8 ± 48.5 | 0.001 | 0.001 | 0.001 |
| Median ± QR | 34 ± 20.5 | 36 ± 39 | 87 ± 61 | | | |
| Platelet (× $10^9$/L) | | | | | | |
| Mean ± SD | 237.1 ± 62.4 | 207.1 ± 70.2 | 162 ± 59.8 | 0.003 | 0.001 | 0.001 |
| Median ± QR | 231 ± 81.3 | 226 ± 120 | 160.5 ± 64.3 | | | |
| Insulin (uU/mL) | | | | | | |
| Mean ± SD | 11.9 ± 4.4 | 15.8 ± 4.9 | 16.8 ± 5.8 | 0.016 | 0.001 | 0.071 |
| Median ± QR | 12.4 ± 6.2 | 14.6 ± 3.4 | 16.7 ± 6.4 | | | |
| Creatinine (mg/dL) | | | | | | |
| Mean ± SD | 0.7 ± 0.2 | 0.6 ± 0.1 | 0.7 ± 0.2 | 0.999 | 0.009 | 0.039 |
| Median ± QR | 0.7 ± 0.2 | 0.6 ± 0.3 | 0.6 ± 0.3 | | | |
| Albumin (g/dL) | | | | | | |
| Mean ± SD | 4.3 ± 0.43 | 4 ± 0.8 | 4 ± 0.41 | 0.044 | 0.001 | 0.005 |
| Median ± QR | 4.4 ± 0.54 | 4.2 ± 0.84 | 4 ± 0.67 | | | |
| Viral load[1] | | | | | | |
| Range | 45979 (2.47-6570282) | 36355.5 (2.46-6403601) | 55000 (6.00-5600790) | 0.37 | 0.96 | 0.52 |
| Mean ± SD | 358316.6 ± 909311.81 | 283586 ± 858939.26 | 331799.1 ± 863675.2 | | | |

[1]Comparison was done using non-parametric Mann-Whitney test. SD: Standard deviation; QR: Quartile range; HOMA-IR: Homeostasis model for insulin resistance, a mathematically calculated formula; ACE: Angiotensin converting enzyme; AST: Aspartate transaminase enzyme; ALT: Alanine amino transferase; *P*1: Significance between mild and moderate fibrosis; *P*2: Significance between mild and advanced fibrosis; *P*3: Significance between moderate and advanced fibrosis.

**Table 3 The overall significant variables among the studied variables: Using independent-samples Kruskal-Wallis test**

| Variables | *P* value |
|---|---|
| Age (yr) | 0.001 |
| HOMA-IR | 0.001 |
| ACE (U/mL) | 0.001 |
| Glucose (mmol) | 0.021 |
| ALT (U/L) | 0.001 |
| AST (U/L) | 0.001 |
| Platelet (× 109/L) | 0.001 |
| Insulin (uU/mL) | 0.001 |
| Creatinine (mg/dL) | 0.024 |
| Total Bilirubin (mg/dL) | 0.001 |
| Direct Bilirubin (mg/dL) | 0.002 |
| Albumin (mg/dL) | 0.001 |
| Portal vein diameter | 0.004 |
| Splenic diameter | 0.001 |

HOMA-IR: Homeostasis model for insulin resistance, a mathematically calculated formula; ACE: Angiotensin converting enzyme; AST: Aspartate transaminase enzyme; ALT: Alanine amino transferase.

**Table 4 Multivariate discriminant functional analysis among the significant predictive variables**

| Variable | Statistic | *P* value |
|---|---|---|
| Log AST | 61.295 | 0.001 |
| Log platelet | 44.331 | 0.001 |
| Age (yr) | 39.635 | 0.001 |
| Log HOMA-IR | 33.682 | 0.001 |

HOMA-IR: Homeostasis model for insulin resistance, a mathematically calculated formula; AST: Aspartate transaminase enzyme.

hepatitis C. Liver biopsy provides the best for evaluation of liver fibrosis stages[17], but this technique has its drawbacks. Liver biopsy is not the perfect tool for follow up assessments of fibrosis in patients with chronic hepatitis C with or without virological cure[18].

The limitations of liver biopsy disclosed the need for the development of non-invasive tests to assess liver fibrosis. Currently available methods to predict liver fibrosis rely on two different but complementary approaches: (1) a biological approach based on measurements of serum levels of biological markers that

**Table 5 Accuracy indices of the discriminant score in the prediction of fibrosis**

| Stage of fibrosis | Cut-off value | AUC | 95%CI | Sens | Specific | PPV | NPV | PLR | NLR |
|---|---|---|---|---|---|---|---|---|---|
| Mild fibrosis (F0-F1) | -0.31 or more negative | 0.88 | 0.84-0.91 | 67.2% | 86.3% | 83.6% | 69.5% | 4.9 | 0.38 |
| Moderate fibrosis (F2) | > -0.31 up to +0.86 | 0.64 | 0.61-0.74 | 53.1% | 74.1% | 33% | 86.8% | 2.0 | 0.63 |
| Advanced fibrosis (F3-F4) | > 0.86 | 0.91 | 0.88-0.94 | 73% | 90.9% | 74.4% | 90.1% | 8.0 | 0.3 |

Sens: Sensitivity; Specific: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; AUC: Area under the curve.

**Table 6 Validation results done on the studied selected groups**

| Stage of fibrosis | Predicted group membership | | | Total |
|---|---|---|---|---|
| | Mild fibrosis | Moderate fibrosis | Advanced fibrosis | |
| Count | | | | |
| Mild fibrosis | 119 | 50 | 8 | 177 |
| Moderate fibrosis | 16 | 34 | 14 | 64 |
| Advanced fibrosis | 5 | 19 | 65 | 89 |
| Percent | | | | |
| Mild fibrosis | 67.2 | 28.2 | 4.5 | 100.0 |
| Moderate fibrosis | 25.0 | 53.1 | 21.9 | 100.0 |
| Advanced fibrosis | 5.6 | 21.3 | 73.0 | 100.0 |

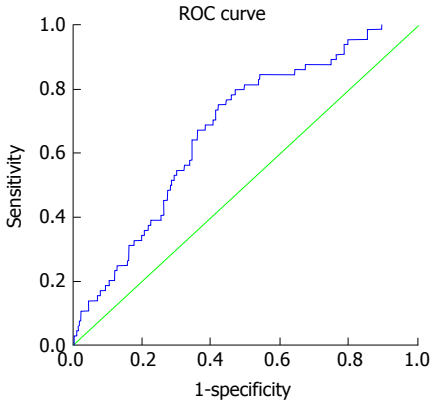


**Figure 3 Receiving operating characteristic curve for discriminating moderate fibrosis.** At cut off value > -0.31 up to +0.86: AUC 0.64, 95%CI: 0.61-0.74, sensitivity 53.1%, specificity 74.1%, PPV 33%, NPV 86.8%, PLR 2.0 and NLR 0.63. AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; ROC: Receiving operating characteristic.

are independent predictors of liver fibrosis[19]; and (2) a "physical" approach based on the measurement of liver stiffness using transient elastography or other recent radiological tools[20].

Many biomarkers have been developed and validated, but none of these markers provide the perfect test. This result may be due to the relatively reduced accuracy of otherwise the sophisticated techniques and the high costs of these tests[21]. We developed a non-invasive biomarker using variables that are biologically relevant to the development and progression of liver fibrosis, because of limitations of the available methods of non-invasive markers for assessment of liver fibrosis[22].

Our study demonstrated on univariate analysis that age significantly ($P < 0.001$) correlated with the stage of liver fibrosis. Age is used with some of the current biomarkers as an independent determinant of liver fibrosis, such as Forn's Index[23] and Fib 4[24].

The results of univariate and multivariate analyses demonstrated that AST was a highly significant ($P < 0.0001$) independent predictor of liver fibrosis stage. AST is used in many available biomarker tests as an independent predictor for liver fibrosis, such as Fib-4[24], APRI test[25], Fibro index[26], and Fibrometere[27].

Our results indicated that platelets were significantly negatively correlated with the advancement of liver fibrosis stage ($P < 0.0001$). Platelet count was reported previously to progressively decrease with the progression of liver fibrosis[28], which makes it to be included in some currently available biomarker evaluations of liver fibrosis stage including Forn's index[23], and Fib-4[24], APRI test[25], and Fibro index[26].

Our univariate analysis results demonstrated a significantly increasing level of HOMA-IR with the progression of liver fibrosis stage ($P < 0.0001$). Insulin resistance is a powerful promoter of fibrogenesis *via* direct HSC stimulation, TNF-α, connective growth factor production and ductular reaction induction[29]. However, only the Sud index included insulin resistance as a variable to evaluate liver fibrosis[30].

Our study is the first report of the correlation of the progressive rise of serum ACE levels with the advancement of liver fibrosis stage ($P < 0.0001$). Multivariate analysis of ACE serum level significantly predicted the stage of liver fibrosis ($P < 0.001$).

ACE is the key rate-limiting enzyme for activation of the RAS, which results in the production of angiotensin Ⅱ. Angiotensin Ⅱ induces the contraction and proliferation of the human HSCs that are responsible for hepatic fibrogenesis[31]. However, it was excluded from our discriminating analysis to avoid the possible confounding effect of some disease states that may alter ACE serum level.

Stepwise multiple discriminative functional analysis indicated that platelets, age, AST, and HOMA-IR variables, in this order of frequency, were independent predictors of liver fibrosis with highly significant values ($P < 0.0001$).

Log AST, log platelet count, log HOMA-IR and age were introduced in a stepwise discriminant analysis model. Our discriminating index for the prediction of liver fibrosis was processed into three levels based on 2014 EASL recommendations for the management of HCV patients to discriminate fibrosis in chronic hepatitis C: 1-No to Mild Fibrosis = F0-F1. 2-Moderate fibrosis = F2 3-Advanced fibrosis = F3-F4 according to Metavir staging

score.

All variables were statistically significant before introduction into the model. The following discriminative outcome was obtained using multiple stepwise analysis: Outcome = 0.514 (age) + 0.373 (Log HOMA-IR) + 0.49 (Log AST) + (-0.532) Log platelet count.

Where the level of fibrosis was predicted using the following cut-off values: (1) mild fibrosis = -0.31 or more negative; (2) moderate fibrosis if outcome > -0.31 (more positive) and up to +0.86; and (3) advance fibrosis if outcome > 0.86.

Our index with a cut-off value ⩾ 0.86 exhibited an AUROC of 0.91 for predicting advanced stages of liver fibrosis (F3-F4) with a sensitivity, specificity, positive predictive value, negative predictive value and positive likelihood ratio of 73%, 90.9%, 74.7%, 90.0% and 8.0, respectively. The diagnostic accuracy of our index for the predicting of advanced liver fibrosis (F3-F4) was more effective than other scores such as the Fibrotest, APRI, Fibrometere, Hepascore. Degos *et al*[32] performed a large study (*n* = 1307) that compared transient elastography with patented and non-patented biomarkers (*e.g.*, Fibrotest, Fibrometere, Hepascore and APRI) compared to liver biopsy. They reported an AUROCs of 0.76 for transient elastography, which did not differ from the AUROCs of the serum markers (0.72-0.78) for the diagnosing of significant fibrosis (F2-F3). However, they reported an AUROC of 0.90 for transient elastography compared to 0.82, 0.86, 0.77 and 0.86 for the Fibrotest, Fibrometere, APRI and Hepascore respectively, for the diagnosing of F4.

Our discriminating index using a cut-off value < 0.31 exhibited an AUROC of 0.88 in the diagnosing of no or mild fibrosis (F0-F2) with a sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio of 67.2%, 86.3%, 83.6%, 69.5% and 4.9, respectively, which indicates high diagnostic performance in the diagnosing of this group of patients. Most currently available scores did not diagnose this group of patients. Poynard *et al*[33] performed a meta-analysis of 30 studies that assessed the diagnostic value of the Fibrotest compared to liver biopsy and found that the AUROC for Fibrotest in the diagnosing of adjacent stages of fibrosis (F1 *vs* F2) was 0.77 (0.75-0.79), and the AUROC was 0.83 (0.81-0.85) for advanced fibrosis (F3-F4). These figures for Fibrotest in the diagnosing of mild and advanced fibrosis are lower in performance than in our index.

Koda *et al*[26] formulated their Fibroindex and reported its accuracy compared to APRI and Forns indices. Their data indicated that the AUROCs of APRI, Forns index, and Fibronectin were 0.78, 0.78 and 0.83, respectively, in discriminating mild degrees of fibrosis (F0-F1) *vs* significant stages of fibrosis (F2-F3), but the AUROCs were 0.81, 0.83 and 0.85, respectively, for discriminating F3-F4.

These data indicate that our index exhibited higher AUROCs for predicting advanced and mild stages of fibrosis than the currently available scores with higher performance accuracy.

Attallah *et al*[34] reported the Fibronectin discriminant score (FDS), using fibronectin, APRI and albumin. FDS exhibited an AUROC of 0.91 in discriminating F0-F1 *vs* F2-F4 and an AUROC of 0.86 in discriminating F0-F2 *vs* F3-F4. These data are nearly equal to the values of our discriminating index.

However, one limitation of our index is the low performance in the diagnosing of F2. The AUROCs for the diagnosing of F2 was 0.64 with cut-off values of ⩾ -0.31 up to +0.86 with a sensitivity, specificity, positive predictive value, negative predictive value, and a positive likelihood ratio of 53.1%, 74.1%, 33.0%, 86.8% and 2.0, respectively.

Crisan *et al*[35] validated the performance of six blood scores (APRI, Forns, Fib-4, Hepascore, Fibrotest and Fibrometere) using transient elastography compared to liver biopsy. Their data indicated that significant fibrosis F > 2 was predicted with AUROCs of 0.727, 0.680, 0.714, 0.778, 0.688, 0.797 and 0.751 for APRI, Forns, Fib-4, Fibrotest, Hepascore, Fibrometere and transient elastography, respectively, and AUROCs were 0741, 0.737, 0.767, 0.705, 0.811, 0.782 and 0.809 in the diagnosis of severe fibrosis (F3-F4). These data provide further support to the higher performance of our index compared to these six serum scores.

Chisti *et al*[36] performed a prospective study to validate three biological scores (Fibrotest, Fibrometere and Hepascore) and reported AUROCs for the predicting of mild to moderate fibrosis of 0.81, 0.85, and 0.77, respectively and AUROCs for the diagnosing of F4 of 0.84, 0.92 and 0.88 respectively. These figures approximate our discriminating scores in the predicting of mild and advanced stages of fibrosis.

Our discriminating index was validated *via* application to originally selected patients. The results indicated that the model correctly classified two-thirds of the cases (66.1%). This sensitivity increased to 67.2% and 73% in mild and advanced fibrosis, respectively, but dropped to 53.1% in moderate fibrosis.

Our discriminating score exhibited higher performance in the diagnosing of mild or no fibrosis and advanced stages of liver fibrosis than the currently available blood tests, but our study and others evaluations of biological scores used liver biopsy as the reference standard.

Pyonard *et al*[37] investigated the performance of liver biopsy itself compared to two non-invasive tests (Fibrotest and Fibroscan) in the absence of the gold standard. The authors reported a relatively lower level of performance for liver biopsy even with the use of 20 mm length for the diagnosis of significant fibrosis (F2-F3). The specificity and sensitivity were 0.67 and 0.63, respectively, for liver biopsy compared to 0.93 and 70 and 0.95 and 0.50 for the Fibrotest and Fibroscan, respectively. These reported data suggested that the discordance between a non-invasive blood test and liver biopsy may be due to the lower diagnostic efficiency of the liver biopsy itself.

The end point of treatment of patients with HCV infections is virus eradication, improvements in liver

histology and prevention of the development of complications. Lee *et al*[38] recently reported on the regression, maintenance and progression of liver fibrosis after virological cure. Pyonard *et al*[39] validated the use of non-invasive markers (Fibrotest and Fibroscan) in a prospective longitudinal study for the prediction of fibrosis regression and development of complications.

The current policy is to follow-up with hepatitis C patients even if these patients are cured virologically. Here, the availability of an easily assessed, less expensive, reproducible blood test with high performance may alleviate or reduce the need for liver biopsy.

In conclusion, our discriminating index for liver fibrosis in hepatitis C genotype 4 patients is a simple, easily reproducible test with accepted accuracy. The index is based on biomarkers that are related to the development and progression of liver fibrosis.

### Limitation of the study

The lack of external validation of the obtained discriminating index is a limitation of this study. Our index is a candidate for multicenter external validation. This index may also be subjected to longitudinal studies to validate its prediction of future complications in HCV patients. Other limitations are the lack of two pathological observers for each specimen and the lack of determination of elastin connective tissue added to collagens.

## COMMENTS

### Background

Hepatitis C virus (HCV) induces liver fibrosis through transforming hepatic stellate cells and other intrahepatic cells to fibrous tissue laying cells. The severity of liver fibrosis is related to multiple host and viral factors. These factors are reflected on changes on biological variables. Studying levels of serum levels of some of these biological markers may provide a non-invasive test that can predict the liver fibrosis stage.

### Research frontiers

Multiple studies have reported about the increased insulin resistance in HCV infections, possibly as a part of HCV-induced metabolic syndrome. Also, there are available data about the impact of increased activity of hepatitis on the development and progression of liver fibrosis. The authors studied multiple biological and host factors in a cohort of genotype 4 Egyptian patients to assess the predictive ability of these variables in diagnosis of liver fibrosis stage.

### Innovations and breakthroughs

This study identified insulin resistance as estimated by homeostasis model of insulin resistance, aspartate transaminase enzyme, platelet count, and age as significant predictors of liver fibrosis stage. A model could be obtained utilizing these markers that could predict liver fibrosis stage with accuracy performance higher than available biological tests. The index is easily applicable and with low expenses.

### Applications

The non-invasive test for diagnosis of liver fibrosis stage can alleviate or reduce the need of the invasive liver biopsy to determine the level of liver fibrosis at basal level before starting antiviral treatment. Because liver biopsy cannot be done sequentially to follow-up HCV patients with or without virus cure, the non-invasive test may provide acceptable tool to do this task.

### Terminology

HOMA-IR: Homeostasis model for insulin resistance, a mathematically calculated formula; ACE: Angiotensin converting enzyme; AST: Aspartate transaminase enzyme; BMI: Body mass index; W/H: Waist/hip ratio.

### Peer-review

The study is interesting and shows a good bibliographic study.

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ORIGINAL ARTICLE

Randomized Clinical Trial

# Telbivudine *vs* tenofovir in hepatitis B e antigen-negative chronic hepatitis B patients: OPTIMA roadmap study

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Informed consent statement: This study was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. Written informed consent was obtained from each patient before enrolment.

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## Abstract

***AIM***

To make efficacy and safety comparison of telbivudine-raodmap and tenofovir-roadmap in hepatitis B e antigen (HBeAg)-negative chronic hepatitis B (CHB) patients.

***METHODS***

This was the first prospective, randomised, two-arm, open-label, non-inferiority study in HBeAg-negative CHB patients that compared telbivudine and tenofovir administered as per roadmap concept. Patients were treated up to 24 wk and, depending on virologic response, continued the same therapy or received add-on therapy up to 104 wk. Eligible patients received an additional 52 wk of treatment in the extension period (*i.e.*, up to 156 wk). Patients who developed virologic breakthrough (VB) while on monotherapy also received add-on therapy. The primary efficacy endpoint was the rate of patients achieving hepatitis B virus (HBV) DNA < 300 copies/mL at week 52. Secondary efficacy endpoints included the rates of HBV DNA < 300 and < 169 copies/mL, HBV DNA change from baseline, alanine aminotransferase normalisation, hepatitis B surface antigen (HBsAg) loss, HBsAg seroconversion, VB, and emergence of resistance at various timepoints throughout the study. Safety and estimated glomerular filtration rate (eGFR) were also analysed.

***RESULTS***

A total of 241 patients were randomised. Non-inferiority of telbivudine arm to tenofovir arm was demonstrated at week 52 (± 7 d window), with over 91% of patients in each treatment arm achieving HBV DNA level < 300 copies/mL. Both arms were similar in terms of key secondary efficacy variables at weeks 104 and 156. The percentage of patients achieving HBV DNA < 300 copies/mL remained high and was similar in the telbivudine and tenofovir arms at both weeks 104 and 156. Over 82% of patients in both arms achieved alanine aminotransferase normalisation at week 52, and this percentage remained high at weeks 104 and 156. Telbivudine treatment progressively reduced serum HBsAg levels from baseline while no change was reported in quantitative HBsAg during therapy with tenofovir. Both treaments showed acceptable safety profiles. The telbivudine arm showed eGFR improvement unlike the tenofovir arm.

***CONCLUSION***

Efficacy was shown for both telbivudine-roadmap and tenofovir-roadmap regimens in HBeAg-negative CHB patients over 156 wk. Telbivudine arm was associated with renal improvement.


**Key words:** Chronic hepatitis B; Glomerular filtration rate; Telbivudine; Tenofovir; Roadmap concept

**Core tip:** This was the first prospective, randomised, non-inferiority study in hepatitis B e antigen-negative chronic hepatitis B patients that compared telbivudine and tenofovir administered as per roadmap concept. Both treatments based on the roadmap approach were effective over a 156 wk treatment period. Non-inferiority of telbivudine arm to tenofovir arm was demonstrated at week 52, with over 91% of patients in each treatment arm achieving hepatitis B virus DNA level < 300 copies/mL. Both treaments showed acceptable safety profiles. Moreover, telbivudine showed an improvement in estimated glomerular filtration rate from baseline.

Krastev Z, Petrova D, Kotzev I, Celen MK, Mendelson M, Chandra R, Pandey P, Hamed K. Telbivudine *vs* tenofovir in hepatitis B e antigen-negative chronic hepatitis B patients: OPTIMA roadmap study. *World J Hepatol* 2016; 8(32): 1402-1413 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i32/1402.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i32.1402

## INTRODUCTION

Approximately 240-400 million people worldwide are chronically infected with hepatitis B virus (HBV), with a wide variation of prevalence among countries, and more than 780000 people die every year due to acute or chronic hepatitis B (CHB)[1-3]. Although CHB may be treated with interferon or nucleos(t)ide analogue (NA) antivirals, emergence of resistance due to prolonged NA therapy or incomplete suppression of HBV still remains an important concern[4]. Several studies have suggested that the use of response-guided add-on therapy is associated with a higher rate of virologic response and reduced antiviral resistance as compared to sequential monotherapy[5,6].

Early virologic response has been used as a guide to predict better outcomes and to reduce the risk of antiviral resistance[7,8]. As previously reported[9,10], the roadmap concept uses early virologic response at week 24 to individualize ongoing management of CHB patients. Patients with a complete response at week 24 can remain on their initial therapy, whereas treatment modification that may include the addition of a second drug is done for those with an inadequate virologic response. This strategy is relevant mainly in patients receiving NA with a low genetic barrier to resistance (clevudine, emtricitabine, lamivudine, telbivudine)[10]. In hepatitis B e antigen (HBeAg)-positive CHB patients treated with telbivudine, a response-guided treatment optimization strategy with telbivudine based on the roadmap concept has been demonstrated to improve the clinical outcomes of patients with a suboptimal antiviral response[11,12].

The aim of this study, OPTIMA, was to assess the efficacy and safety of telbivudine and tenofovir regimens,

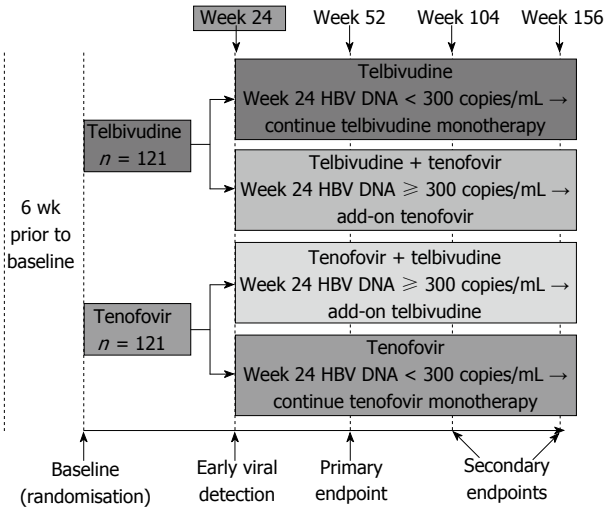


**Figure 1 Study design.** HBV: Hepatitis B virus.

when administered using the roadmap concept, in HBeAg-negative patients with CHB. This was the first study that compared efficacy of the 2 regimens in a prospective manner. The safety of the combination of telbivudine and tenofovir, for which limited data are currently available, was also evaluated.

## MATERIALS AND METHODS

### *Study design and conduct*

OPTIMA was a prospective, randomised, 2-arm, open-label study (ClinicalTrials.gov ID: NCT01379508) that enrolled patients between February 2011 and October 2012 in 8 countries (Austria, Bulgaria, Germany, Greece, Italy, Russia, Spain and Turkey). This study was approved by the Institutional Review Board at each participating centre, and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from each patient before enrolment.

Eligible patients were randomised *via* an interactive voice response system in a 1:1 ratio to either telbivudine arm (600 mg/d) or tenofovir arm (300 mg/d) (Figure 1). Randomisation was stratified by the screening HBV DNA level (< 7 $\log_{10}$ copies/mL or ⩾ 7 $\log_{10}$ copies/mL) and alanine aminotransferase (ALT) level [< 3 × upper limit of normal (ULN) or ⩾ 3 × ULN].

This study used the response-guided add-on strategy (roadmap concept). For patients with HBV DNA ⩾ 300 copies/mL (⩾ 51 IU/mL) at week 24, tenofovir was added to telbivudine by week 26 in the telbivudine arm, and telbivudine was added to tenofovir by week 26 in the tenofovir arm. For patients with HBV DNA < 300 copies/mL at week 24, telbivudine and tenofovir monotherapies in the respective arms were continued. Patients who developed virologic breakthrough (VB) while on monotherapy received add-on therapy. However, patients who developed VB after week 24 while on combination therapy were discontinued from the study.

### *Patients*

Eligible patients were male or female ⩾ 18 years of age, with detectable hepatitis B surface antigen (HBsAg) for ⩾ 6 mo, HBeAg-negative with positive hepatitis B e antibody, available liver histology report within 12 mo before screening compatible with CHB (patients without evaluable liver histology were eligible if they had clinical evidence of compensated liver cirrhosis or non-invasive methods that support the diagnosis of moderate to severe liver inflammation and/or fibrosis), serum HBV DNA > 2000 IU/mL, and serum ALT level > 1 × ULN and < 10 × ULN at the screening visit. Patients with ALT ⩽ 1 × ULN at screening were eligible if they had at least moderate liver inflammation or fibrosis, clinical evidence of compensated cirrhosis, or ALT level > 1 × ULN within the last 6 mo.

Main exclusion criteria included co-infection with hepatitis C virus, hepatitis D virus or human immunodeficiency virus; hepatic decompensation; liver disease other than CHB; any nucleos(t)ide or interferon/immunomodulator treatment in the previous 6 mo; chronic renal insufficiency or serum creatinine clearance < 50 mL/min; history of myopathy, myositis, or persistent muscle weakness; pregnant or nursing (lactating) women; or history of malignancy of any organ system (other than localized basal cell carcinoma of the skin).

Patients were allowed to recieve an additional 52 wk of treatment in the extension period (*i.e.*, up to 156 wk) if they had HBV DNA < 300 copies/mL at both weeks 92 and 104, and serum creatinine clearance ⩾ 50 mL/min at two consecutive visits including week 104.

### *Efficacy and safety analyses*

The primary efficacy endpoint was the rate of patients achieving HBV DNA < 300 copies/mL (51 IU/mL) at week 52. Secondary efficacy endpoints included the rates of patients with HBV DNA < 300 copies/mL at weeks 104 and 156, and HBV DNA < 169 copies/mL (29 IU/mL) (lower limit of detection) at weeks 24, 52, 104 and 156; change from baseline in HBV DNA; ALT normalisation at weeks 52, 104 and 156; HBsAg loss and HBsAg seroconversion; VB; and emergence of resistance. In addition, subgroup analyses were performed for secondary efficacy endpoints by baseline HBV DNA (*i.e.*, < 7 $\log_{10}$ copies/mL or ⩾ 7 $\log_{10}$ copies/mL).

VB was defined as an increase of HBV DNA by at least 1 $\log_{10}$ copies/mL (or 1 $\log_{10}$ IU/mL) above nadir on 2 consecutive visits, or at the last on-treatment visit in patients who did not have a primary non-response. Emergence of resistance was assessed as the rate of confirmed treatment-emergent genotypic resistance and was assessed at the time of confirmed VB and at week 24 in patients with viral load ⩾ 300 copies/mL, it was calculated cumulatively at weeks 52, 104 and 156.

HBV DNA detection and quantification were performed at a central laboratory using the COBAS TaqMan real-time polymerase chain reaction assay (Roche Molecular Systems, Branchburg, NJ, United States).

Safety assessments included monitoring of adverse events (AEs), vital signs, and graded laboratory abnormalities. Estimated glomerular filtration rate (eGFR), calculated by the modification of diet in renal disease formula was recorded. AEs of special interest (muscle and renal function related events) were also reported.

### *Statistical analysis*

For the primary efficacy analysis, study treatments were compared for non-inferiority.

Based on the assumptions of 96% and 97% HBV DNA < 300 copies/mL at week 52 in the telbivudine arm and the tenofovir arm, respectively, and an approximately 10% dropout rate, it was estimated that 120 randomised patients per arm would provide 87% power for the non-inferiority testing on the primary analysis. Non-inferiority in efficacy of telbivudine arm to tenofovir arm was to be claimed if the lower limit of the 2-sided confidence interval (CI) for the difference was above the pre-determined non-inferiority margin (-10%).

A weighted Cochran-Mantel-Haenszel method, adjusting for randomisation strata [HBV DNA (< or ≥ 7 $\log_{10}$ copies/mL) and ALT (< or ≥ 3 × ULN) levels], was used to assess comparative therapeutic response rates.

For continuous variables, summary statistics of absolute value and of change from baseline, including mean, standard deviation (SD), median, minimum, and maximum were used. For dichotomous endpoints, statistical summaries included count and percentage of patients with a positive response (response rate) and also 95%CI for the response rate.

The intent-to-treat (ITT) population consisted of all patients who received at least one dose of study drug and had at least one post-baseline assessment of serum HBV DNA. The roadmap ITT (rITT) population consisted of all patients who did not discontinue before week 24 and did not deviate from the protocol defined rules of receiving add-on at week 24 (*i.e.*, patients who received the add-on therapy at week 24 if they had HBV DNA ≥ 300 copies/mL, or did not receive the add-on at week 24 if they had HBV DNA < 300 copies/mL). The modified ITT (mITT) population consisted of all patients in the ITT population who were eligible and enrolled in the extension period beyond week 104. The per-protocol population consisted of all patients in the ITT population who had no major protocol deviations.

All efficacy observations on or after censoring date were treated as missing. A patient's censoring date was the date of the first occurrence of: One day after the last dose of the study drug, the start of first prohibited CHB-related medication, pregnancy, or a specific major protocol deviation. To assess the robustness of the results due to missing data, the analysis of primary and all secondary efficacy endpoints were performed based on the rITT and ITT analysis populations. The mITT population was used only for the week 156 analysis.

The primary efficacy endpoint (week 52) analysis was performed on the rITT population. The analyses presented include: (1) assessments within the ± 7 d protocol-specified visit window around the scheduled week 52 date; (2) missing data at week 52 treated as failure; (3) missing data imputed using the earliest available assessment within the 28 d window starting from the scheduled week 52 date; and (4) missing data imputed using the last observation carried forward (LOCF).

Secondary efficacy parameters including HBV DNA, ALT normalisation, HBsAg loss, and HBsAg seroconversion were analysed using two imputation methods for missing data: (1) missing data treated as failure; and (2) missing data imputed using the earliest available assessment within the 28 d window starting from the scheduled visit for weeks 52 (except HBV DNA < 300 copies/mL), 104 and 156. VB and eGFR were analysed using the LOCF imputation method for missing data. Treatment-emergent genotypic resistance was analysed using cumulative imputation method for missing data. Missing eGFR assessments were imputed using the LOCF method.

Analyses of endpoints using LOCF imputation at weeks 104 and 156 are presented for the rITT and mITT populations, respectively.

## RESULTS

### *Study patients*

A total of 241 patients (121 in the telbivudine arm and 120 in the tenofovir arm) were randomised in this study. A total of 22 (18.2%) patients in the telbivudine arm and 13 (10.8%) patients in the tenofovir arm discontinued prematurely from the study. The most common reasons for discontinuation in the telbivudine arm were consent withdrawal ($n$ = 7), lost to follow-up ($n$ = 5), and administrative reasons ($n$ = 4). In the tenofovir arm, the most common reasons for discontinuation were AEs ($n$ = 5), consent withdrawal ($n$ = 4), and lost to follow-up ($n$ = 3).

Major protocol deviations were reported in 11 (9.1%) patients in the telbivudine arm and 8 (6.7%) patients in the tenofovir arm. The most commonly reported major deviations were patients on monotherapy with confirmed VB not starting add-on therapy within 2 wk of laboratory confirmation of VB ($n$ = 9), patients with a positive HBeAg result ($n$ = 6), and patients not completing 3 wk of treatment before the third visit ($n$ = 4).

The safety population comprised 120 patients in each of the 2 treatment arms. One patient in the telbivudine arm was excluded from the safety population as this patient did not receive any study treatment. Of the 241 randomized patients, 235 patients were included in the ITT population, with 117 (96.7%) in the telbivudine arm and 118 (98.3%) in the tenofovir arm. Six patients were excluded from the ITT population (4 patients in the telbivudine arm due to no post-baseline HBV DNA assessments, non-compliance with the study conduct, or no study treatment received; and 2 patients in the tenofovir arm because of no post-baseline HBV DNA assessments and viral resistance at baseline). A total of 113 (93.4%) patients in the telbivudine arm and 117 (97.5%) patients in the tenofovir arm comprised the

**Table 1 Demographic and baseline characteristics, randomised population**

| Patients characteristics | Telbivudine ($n$ = 121) | Tenofovir ($n$ = 120) |
|---|---|---|
| Age, mean (SD), yr | 42.1 (11.5) | 43.3 (12.6) |
| Median (min-max) | 42.0 (19-70) | 44.0 (18-73) |
| Male gender, $n$ (%) | 86 (71.1) | 82 (68.3) |
| Race, Caucasian, $n$ (%) | 117 (96.7) | 118 (98.3) |
| Body mass index, mean (SD), kg/m$^2$ | 25.8 (4.1) | 25.7 (4.0) |
| Median (min-max) | 25.6 (16.5-40.4) | 25.2 (18.4-39.8) |
| Genotype, $n$ (%) | | |
| A | 6 (5.0) | 2 (1.7) |
| B | 1 (0.8) | 0 (0.0) |
| C | 0 (0.0) | 1 (0.8) |
| D | 104 (86.0) | 110 (91.7) |
| G | 1 (0.8) | 0 (0.0) |
| Other | 1 (0.8) | 0 (0.0) |
| Unknown | 8 (6.6) | 7 (5.8) |
| HBV DNA, mean (SD), $\log_{10}$ copies/mL | 6.2 (1.5) | 6.0 (1.4) |
| Median (min-max) | 6.1 (3.2-9.5) | 5.9 (2.5-9.9) |
| < 7 $\log_{10}$, $n$ (%) | 85 (70.2) | 86 (71.7) |
| ⩾ 7 $\log_{10}$, $n$ (%) | 36 (29.8) | 34 (28.3) |
| Serum alanine aminotransferase, mean (SD), IU/L | 79.8 (84.1) | 78.2 (86.1) |
| Median (min-max) | 53.0 (13-494) | 49.0 (5-568) |
| Serum aspartate aminotransferase, mean (SD), IU/L | 54.0 (52.8) | 52.5 (47.1) |
| Median (min-max) | 35.0 (13-347) | 35.0 (13-322) |
| Creatine phosphokinase, mean (SD), IU/L | 118.6 (64.4) | 160.1 (299.3) |
| Median (min-max) | 104.0 (35-430) | 111.0 (36-2976) |
| eGFR[1], mean (SD), (mL/min per 1.73 m$^2$) | 97.4 (17.9) | 95.8 (16.4) |
| Median (min-max) | 96.6 (60.9-147.1) | 94.2 (60.5-138.4) |

[1]eGFR: Estimated glomerular filtration rate (modification of diet in renal disease formula). HBV: Hepatitis B virus; SD: Standard deviation.

rITT population. Five patients (4 in the telbivudine arm and 1 in the tenofovir arm) that were included in the ITT population were excluded from the rITT population because they discontinued before week 24 and were not eligible for or enrolled into the roadmap concept period (weeks 24 to 104).

The per-protocol population consisted of 107 (88.4%) patients in the telbivudine arm and 111 (92.5%) patients in the tenofovir arm. A total of 17 patients (10 in the telbivudine arm and 7 in the tenofovir arm) were included in the ITT and rITT populations but were excluded from the per-protocol population because of major protocol deviations. The mITT population consisted of 79 (65.3%) patients in the telbivudine arm and 89 (74.2%) patients in the tenofovir arm.

Treatment arms were balanced with respect to demographics and baseline characteristics, with no clinically meaningful differences between the telbivudine and tenofovir arms (Table 1). Most (86.0% telbivudine, 91.7% tenofovir) patients were infected with HBV genotype D, and the mean HBV DNA at baseline was 6.2 $\log_{10}$ copies/mL in the telbivudine arm and 6.0 $\log_{10}$ copies/mL in the tenofovir arm, with 70.2% and 71.7% of patients, respectively, having a baseline HBV DNA < 7 $\log_{10}$ copies/mL.

### *Primary efficacy endpoint*

Virologic response (HBV DNA < 300 copies/mL) at week 52 was achieved in more than 91% of patients in each treatment arm (Figure 2A). The primary endpoint analysis showed that the antiviral efficacy of telbivudine-roadmap was non-inferior to that of tenofovir-roadmap application at week 52 in the rITT population; the lower bound of the 95%CI for the difference between the 2 treatment arms was above the non-inferiority margin of -10%: -9.4% (utilizing assessments within the ±7 d protocol-prespecified visit window); -8.3% for the 28 d window imputation; and -7.9% for the LOCF imputation. Using missing data as treatment failure, non-inferiority was not demonstrated (lower bound of the 95%CI: -10.5%, just below the protocol defined non-inferiority margin) (Table 2). In this analysis, HBV DNA samples from 6 patients (4 in the telbivudine arm and 2 in the tenofovir arm), although resulted in < 300 copies/mL, were considered as missing because they were not obtained at the week 52 visit date itself (*i.e.*, patients were counted as treatment failures).

The primary endpoint analysis at week 52 in the per-protocol population supported the non-inferiority of the telbivudine arm to the tenofovir arm (98.0% in the telbivudine arm and 99.0% in the tenofovir arm, lower bound of the 95%CI: -4.3%).

### *Secondary efficacy endpoints*

**Virologic responses:** Percentage of patients achieving HBV DNA < 300 copies/mL (51 IU/mL) at weeks 24 and 104, and by baseline viral load at weeks 24, 52 and 104 in the rITT population: The percentage of patients achieving HBV DNA < 300 copies/mL in the telbivudine and tenofovir arms at week 24 was 80.5% and 89.7%,

**Table 2 Virologic response, roadmap intent-to-treat population**

| Parameters | Telbivudine ($n$ = 113) | Tenofovir ($n$ = 117) | Difference between arms and 95%CI |
|---|---|---|---|
| Patients achieving HBV DNA < 300 copies/mL (51 IU/mL) at week 52, $n$ (%) | | | |
| ± 7 d protocol-prespecified visit window | 104 (91.9) | 111 (95.0) | -3.1% (-9.4%, 3.1%)[1] |
| Treating missing as failure | 103 (91.0) | 111 (95.0) | -4.0% (-10.5%, 2.5%)[1] |
| 28 d imputation | 105 (92.7) | 111 (95.0) | -2.3% (-8.3%, 3.8%)[1] |
| Last observation carried forward | 108 (95.4) | 116 (99.2) | -3.8% (-7.9%, 0.4%)[1] |
| Change from baseline in HBV DNA levels ($log_{10}$ copies/mL) by visit, mean (SD) | | | $P$-value |
| Week 24 | -4.001 (1.256) | -4.122 (1.165) | $P < 0.0001$[2] |
| Week 52 | -4.356 (1.473) | -4.305 (1.343) | $P < 0.0001$[2] |
| Week 104 | -4.281 (1.753) | -4.349 (1.382) | $P < 0.0001$[2] |

[1]Percentages and 95%CIs were calculated using Mantel-Haenszel weighted estimates stratified by baseline HBV DNA and alanine aminotransferase levels; [2]$P$-values were calculated using paired $t$-test comparing post-baseline timepoints to baseline timepoints. CI: Confidence interval; HBV: Hepatitis B virus; SD: Standard deviation.

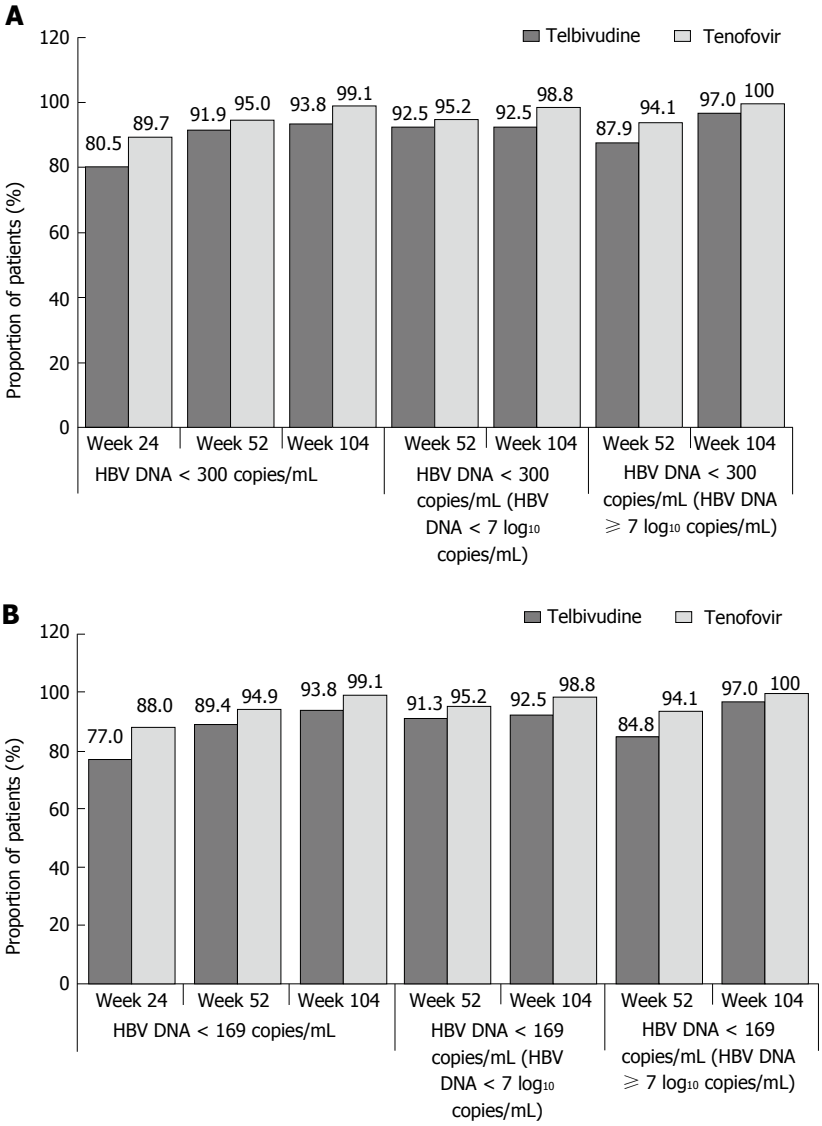


**Figure 2 Proportions of patients achieving hepatitis B virus DNA < 300 (A) or < 169 copies/mL (B), by visit and by baseline hepatitis B virus DNA levels (< 7 or ⩾ 7 $log_{10}$ copies/mL), roadmap intent-to-treat population.** HBV: Hepatitis B virus.

and at week 104, 93.8% and 99.1%, respectively (Figure 2A).

In patients with lower baseline viral load (HBV DNA level < 7 $log_{10}$ copies/mL) at week 24, telbivudine and tenofovir regimens were similar in terms of viral load reduction with 93.8% and 95.2% of patients achieving HBV DNA levels < 300 copies/mL in the telbivudine and tenofovir arms, respectively. At weeks 52 and

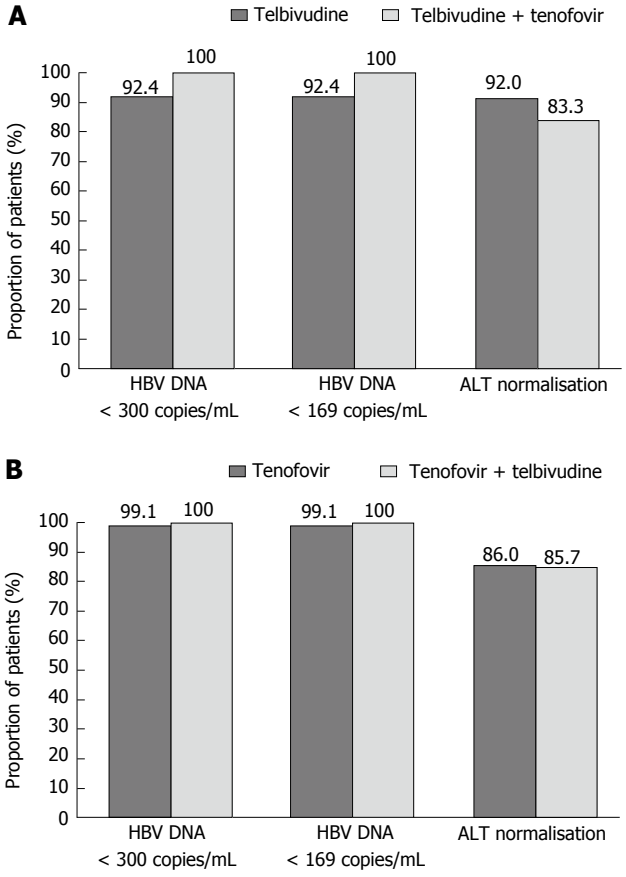


**Figure 3 Intensification with tenofovir (A) or telbivudine (B), virologic response and aminotransferase normalisation at week 104, roadmap intent-to-treat population.** ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

104, telbivudine and tenofovir regimens seemed to be similar in terms of viral load reduction, with over 92% of patients achieving HBV DNA levels < 300 copies/mL at weeks 52 and 104 (Figure 2A). The proportion of patients in each arm with higher baseline viral load ($\geqslant$ 7 $\log_{10}$ copies/mL) was relatively small to make any meaningful interpretation.

Change from baseline in HBV DNA levels from week 24 to week 104 in the rITT population: A statistically significant ($P < 0.0001$) reduction in HBV DNA levels *vs* baseline was achieved in both treatment arms at week 24 and was sustained through week 104 (Table 2).

Intensification with tenofovir or telbivudine for HBV DNA $\geqslant$ 300 copies/mL at week 24 or for VB post week 24 through week 104 in the rITT population; response at week 104 (HBV DNA < 300 copies/mL) according to the requirement for add-on therapy at week 24: A greater number of patients in the telbivudine arm required add-on therapy compared with the tenofovir arm (35 patients in the telbivudine arm including 22 patients requiring add-on therapy at week 24 and 13 requiring add-on therapy post week 24 *vs* 11 patients in the tenofovir arm, all requiring add-on therapy at week 24).

The proportion of patients in the telbivudine arm achieving HBV DNA < 300 copies/mL at week 104 was greater in those who required tenofovir add-on therapy at week 24 (100%, 21/21 patients) than patients who were in the telbivudine monotherapy group following the week 24 visit (92.4%, 85/92 patients) (Figure 3A).

The proportion of patients in the tenofovir arm achieving HBV DNA < 300 copies/mL at week 104 was similar in those who required telbivudine add-on therapy at week 24 (100%, 11/11 patients) to those who were in the tenofovir monotherapy group following the week 24 visit (99.1%, 105/106 patients) (Figure 3B).

Percentage of patients achieving HBV DNA < 169 copies/mL (29 IU/mL) at weeks 24, 52 and 104 in the rITT population: The rate of patients achieving HBV DNA < 169 copies/mL at weeks 24, 52 and 104 was consistent with that observed for the endpoint of HBV DNA < 300 copies/mL (Figure 2B).

Percentage of patients achieving HBV DNA < 169 copies/mL at week 104 in the rITT population according to the requirement for add-on therapy at week 24: The proportion of patients in the telbivudine and tenofovir arms achieving HBV DNA < 169 copies/mL at week 104 and receiving add-on therapy were 7.6 and 0.9 percentage points greater, respectively, than patients who received monotherapy (Figure 3).

Maintained virologic responses at week 156 in the mITT population: The percentage of patients who maintained HBV DNA < 300 copies/mL at week 156 was similar in the telbivudine and tenofovir arms: 91.1% (72/79 patients) and 100% (89/89 patients), respectively, using LOCF imputation. Similar results were found in patients maintaining HBV DNA < 169 copies/mL [91.1% (72/79 patients) and 96.6% (86/89 patients), respectively].

**HBsAg loss and HBsAg seroconversion:** HBsAg loss and HBsAg seroconversion were not observed in any patient from either treatment arm at weeks 52, 104 or 156. Telbivudine treatment progressively reduced serum HBsAg levels (mean ± SD) from baseline in the rITT population [-0.116 ± 0.581 $\log_{10}$ IU/mL at week 52 ($P = 0.0368$) and -0.179 ± 0.633 $\log_{10}$ IU/mL at week 104 ($P = 0.0032$)]. In contrast, no change was reported in quantitative HBsAg during therapy with tenofovir [-0.038 ± 0.349 $\log_{10}$ IU/mL at week 52 ($P = 0.2399$) and -0.030 ± 0.385 $\log_{10}$ IU/mL at week 104 ($P = 0.4063$)]. At week 156, change from baseline in HBsAg levels in the mITT population was -0.204 ± 0.759 $\log_{10}$ IU/mL ($P = 0.0193$) in the telbivudine arm and -0.031 ± 0.412 $\log_{10}$/mL ($P = 0.4760$) in the tenofovir arm.

**Biochemical response:** ALT normalisation at weeks 52 and 104 in the rITT population: ALT levels significantly improved *vs* baseline in both treatment arms, with over 82% of patients in both arms achieving ALT normalisation at week 52 that was sustained up until week 104 (89.7% and 85.9% in the telbivudine and tenofovir arms, respectively) (Figure 4).

The results at week 104 by baseline viral load are presented in Figure 4.

ALT normalisation at week 104 in the rITT population according to the requirement for add-on therapy at week 24: The proportion of patients who achieved ALT

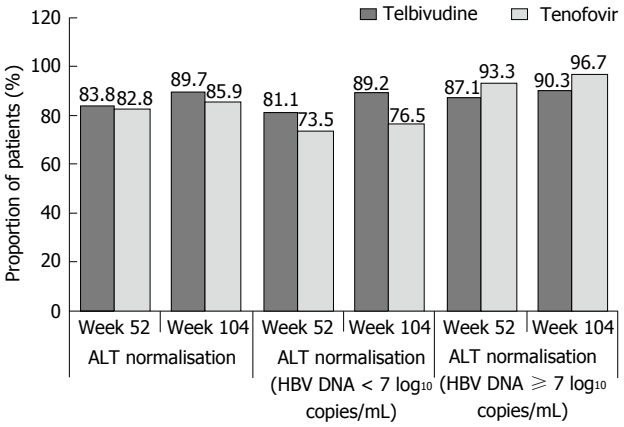


**Figure 4 Proportions of patients achieving aminotransferase normalisation, by visit and by baseline hepatitis B virus DNA levels (< 7 or ≥ 7 $log_{10}$ copies/mL), roadmap intent-to-treat population.** ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

nornalization at week 24 was higher (telbivudine arm) or similar (tenofovir arm) in patients who recevived add-on therapy (Figure 3).

Maintained biochemichal response at week 156 in the mITT population: ALT normalisation was maintained in 92.0% of patients in the telbivudine arm and 91.1% of patients in the tenofovir arm.

**Patients experiencing VB and emergence of resistance in the rITTand mITT populations:** At weeks 52 and 104, respectively, in the rITT population, cumulative rates of VB were reported in 2.7% (3/113) and 9.7% (11/113) of patients in the telbivudine arm (3.3% and 12.4% in the monotherapy group, none in the add-on treatment group). In the tenfovir arm, no patients developed VB cumulatively at week 52 and 1.7% (2/117) of patients developed VB cumulatively at week 104.

At week 52, cumulative emergence of resistance was reported in 2.7% (3/113) of patients in the telbivudine arm (3.3% in the monotherapy group, none in the add-on treatment group) and no treatment-emergent resistance was observed in the tenofovir arm. At week 104, cumulative emergence of resistance was reported in 7.4% (8/108) of patients in the telbivudine arm (9.2% in the monotherapy group, none in the add-on treatment group) and none in the tenofovir arm.

In the telbivudine arm, 10 patients experienced VB and 5 had emergence of resistance between weeks 104 and 156 in the mITT population. In the tenofovir arm, only 1 patient had VB and none developed viral resistance. The cumulative rate of VB at week 156 was 16.5% (13/79) in the telbivudine arm, and 1.1% (1/89) in the tenofovir arm. Cumulative rates of resistance were 10.8% (8/74) in the telbivudine arm (14.0% in the monotherapy group, none in the add-on treatment group) and none in the tenfovir arm.

### *Safety*

No patients died or experienced ALT flare during the study. The overall incidence of serious AEs (SAEs) was similar in the telbivudine arm and in the tenofovir arm [11 (9.2%) patients and 13 (10.8%) patients, respectively]. One patient in the tenofovir arm reported drug-related SAEs [moderately increased blood creatine phosphokinase (CPK), mild arthralgia, and moderate fatigue], which led to temporary interruption of the study drug (Table 3). There were no cases of myositis or myopathy.

Two patients in the telbivudine arm and 5 patients in the tenofovir arm discontinued due to AEs [myalgia and hepatocellular carcinoma (HCC) for telbivudine; headache, HCC, hepatic cirrhosis, cholestatic jaundice, and breast cancer for tenofovir], which were assessed by the investigator as unrelated to the study drugs. Most AEs were mild to moderate in severity. The proportion of patients reporting at least 1 AE, regardless of study drug relationship, was similar for telbivudine and tenofovir arms. The overall incidence of AEs suspected to be related to study drug was somewhat higher in the telbivudine arm compared with the tenofovir arm. The most frequent (≥ 2%) drug-related AEs reported in both arms are described in Table 3. Increased blood CPK levels [31 (25.8%) patients], myalgia [8 (6.7%) patients, and nausea 8 (6.7%) patients] were the drug-related AEs that were observed more frequently in the telbivudine arm compared with the tenofovir arm [16 (13.3%), 0, and 2 (1.7%) patients, respectively]. AEs of special interest were observed in 46 (38.3%) patients in the telbivudine arm and 27 (22.5%) patients in the tenofovir arm. These included elevated blood CPK and myalgia as the most commonly reported AEs in the telbivudine arm, and elevated blood CPK and ALT as the most commonly reported AEs in the tenofovir arm. Myalgia suspected to be drug related was reported in the telbivudine arm. The number of patients experiencing at least 1 muscle event along with 1 new-onset abnormal CPK episode during the study was greater in the telbivudine arm (Table 3).

The telbivudine arm showed a higher incidence of Grade 3/4 CPK elevations during the study than the tenofovir arm [19 (15.8%) patients *vs* 5 (4.2%) patients, respectively]. All Grade 3/4 CPK elevations were resolved (Table 3).

Telbivudine monotherapy (as of week 24) was associated with a significant improvement in eGFR as compared with tenofovir monotherapy (as of week 24). At week 24, the telbivudine monotherapy showed a statistically significant ($P$ = 0.0798) improvement from baseline in eGFR compared to worsening with tenofovir monotherapy, with least squares mean percentage changes from baseline of 2.46% *vs* -1.17%, respectively. Further improvement in eGFR in the telbivudine monotherapy group (as of week 24) was observed at weeks 52 (4.90% *vs* -2.68% with tenofovir, $P$ = 0.0098), 104 (5.54% *vs* -5.36%, $P$ < 0.0001, respectively), and 156 (9.55% *vs* -6.23%, $P$ < 0.0001, respectively) (Figure 5).

There was no significant change in vital signs from baseline for either treatment arm.

**Table 3 Summary of safety results, safety population *n* (%)**

| Safety parameters | Telbivudine | | | Tenofovir | | |
|---|---|---|---|---|---|---|
| | Monotherapy (*n* = 98) | Intensification with tenofovir (*n* = 22) | Overall (*n* = 120) | Monotherapy (*n* = 109) | Intensification with telbivudine (*n* = 11) | Overall (*n* = 120) |
| Any AE | 69 (70.4) | 17 (77.3) | 86 (71.7) | 75 (68.8) | 8 (72.7) | 83 (69.2) |
| AE related to drug | 36 (36.7) | 11 (50.0) | 47 (39.2) | 21 (19.3) | 6 (54.5) | 27 (22.5) |
| AE leading to drug discontinuation | 2 (2.0) | 0 (0.0) | 2 (1.7) | 5 (4.6) | 0 (0.0) | 5 (4.2) |
| Any SAE | 6 (6.1) | 5 (22.7) | 11 (9.2) | 11 (10.1) | 2 (18.2) | 13 (10.8) |
| SAE related to drug | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (9.1) | 1 (0.8) |
| Death | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| AEs related to drug occurring in ⩾ 2% of patients in any treatment arm | | | | | | |
| Blood CPK increased | 23 (23.5) | 8 (36.4) | 31 (25.8) | 13 (11.9) | 3 (27.3) | 16 (13.3) |
| Nausea | 6 (6.1) | 2 (9.1) | 8 (6.7) | 0 (0.0) | 2 (18.2) | 2 (1.7) |
| Myalgia | 7 (7.1) | 1 (4.5) | 8 (6.7) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Alanine aminotransferase increased | 2 (2.0) | 0 (0.0) | 2 (1.7) | 3 (2.8) | 1 (9.1) | 4 (3.3) |
| Proteinuria | 2 (2.0) | 0 (0.0) | 2 (1.7) | 4 (3.7) | 0 (0.0) | 4 (3.3) |
| Aspartate aminotransferase increased | 3 (3.1) | 0 (0.0) | 3 (2.5) | 2 (1.8) | 0 (0.0) | 2 (1.7) |
| Any AE of special interest | 35 (35.7) | 11 (50.0) | 46 (38.3) | 23 (21.1) | 4 (36.4) | 27 (22.5) |
| AEs of special interest occurring in ⩾ 2% of patients in any treatment arm | | | | | | |
| Blood CPK increased | 24 (24.5) | 10 (45.5) | 34 (28.3) | 17 (15.6) | 3 (27.3) | 20 (16.7) |
| Myalgia | 10 (10.2) | 2 (9.1) | 12 (10.0) | 2 (1.8) | 1 (9.1) | 3 (2.5) |
| Alanine aminotransferase increased | 5 (5.1) | 0 (0.0) | 5 (4.2) | 5 (4.6) | 1 (9.1) | 6 (5.0) |
| Proteinuria | 3 (3.1) | 0 (0.0) | 3 (2.5) | 4 (3.7) | 0 (0.0) | 4 (3.3) |
| Any patient with muscle event | 12 (12.2) | 2 (9.1) | 14 (11.7) | 2 (1.8) | 1 (9.1) | 3 (2.5) |
| Experiencing new-onset Grade 3/4 abnormal CPK within the study | 4 (4.1) | 1 (4.5) | 5 (4.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Experiencing new-onset Grade 1/2 abnormal CPK within the study | 6 (6.1) | 1 (4.5) | 7 (5.8) | 1 (0.9) | 1 (9.1) | 2 (1.7) |
| Any patient with new-onset Grade 3/4 CPK episode within the study | 17 (17.3) | 2 (9.1) | 19 (15.8) | 3 (2.8) | 2 (18.2) | 5 (4.2) |
| Episode not resolved | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

AE: Adverse event; CPK: Creatine phosphokinase; SAE: Serious adverse event.

## DISCUSSION

NAs given as a single daily oral dose are considered the mainstay of CHB treatment[13]. In clinical practice, attaining optimal efficacy with a low emergence of drug resistance remains an important goal[14]. The roadmap concept utilizing add-on therapy for patients who do not achieve HBV DNA < 300 copies/mL at week 24 (in particular for agents with lower barriers to resistance) has been identified as a strategy to achieve this goal. This study was the first prospective, randomised clinical trial using the roadmap concept in HBeAg-negative CHB patients comparing efficacy and safety of telbivudine with tenofovir. As previously reported[15], early detection of virologic response may be a useful guide to individualize CHB treatment. This study confirmed that monitoring virologic response at week 24 is a strong predictor of the treatment response by week 104[16]. These data were consistent with an earlier study comparing telbivudine with lamivudine[15].

In the real-world setting, use of the roadmap concept may offer several advantages such as early identification of patients with suboptimal responses to initiate an appropriate change in therapy[10,11], and to provide clinicians with options for individualized treatment decisions[5]. Although emergence of resistance had been identified as an issue for HBeAg-negative CHB patients treated with telbivudine monotherapy[15,17], the data from our study suggest that the risk for resistance is lower if telbivudine is administered using the roadmap concept, as compared to the GLOBE trial showing higher rates of resistance[15]. Moreover, despite a somewhat higher percentage of patients requiring add-on therapy in the telbivudine arm, the overall efficacy profile of the 2 roadmap approach arms was comparable, as assessed by the percentages of patients achieving HBV DNA levels < 300 or < 169 copies/mL, and ALT normalisation at weeks 52, 104 and 156. Moreover, telbivudine treatment resulted in a statistically significant reduction in serum HBsAg levels from baseline while no change was reported in quantitative HBsAg during therapy with tenofovir.

Overall, both treatments based on the roadmap concept were well tolerated over the 156 wk treatment period in HBeAg-negative patients. Although myalgia and elevated blood CPK levels were reported in a higher number of patients in the telbivudine arm, the rates were consistent with the findings reported earlier in the literature[12,15,18,19]. It is recommended that serum CPK levels should be monitored closely during treatment with telbivudine[20].

Renal safety issues with oral NAs have been well-documented[21-23]. Particularly, adefovir is considered to

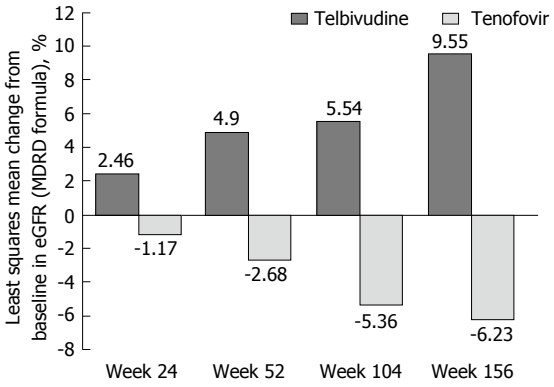


**Figure 5 Changes in estimated glomerular filtration rate over time with telbivudine and tenofovir, safety population.** eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

have high potential for nephrotoxicity and tenofovir has been associated with this risk[24]. In our study, telbivudine was associated with improvement in eGFR from baseline to week 156 compared to the increasing deterioration over time with tenofovir. The finding of improvement in eGFR with telbivudine treatment was consistent with that reported in previous studies where telbivudine significantly improved while adefovir and lamivudine worsened renal function[25,26]. CHB patients with impaired renal function at baseline have also shown an eGFR improvement after 1 year[27] and 2 years of treatment with telbivudine[11,28]. Similar results for telbivudine have also been reported in patients with cirrhosis, patients with compensated cirrhosis, or patients with no cirrhosis[29,30]. These findings imply that telbivudine may offer benefit in patients with known or at risk of renal impairment. Although telbivudine improves renal function, the mechanism of this renal protective effect remains to be determined[31].

The main limitations of the study are related to its design (open-label) and the relatively small sample size.

In conclusion, this study was the first prospective, randomised, comparative study of telbivudine-roadmap *vs* tenofovir-roadmap concept in HBeAg-negative patients with CHB. Both treatments based on the roadmap concept were effective over the 156 wk treatment period. Moreover, telbivudine showed an improvement in eGFR from baseline while a deterioriaton was observed with tenofovir; this could be an important consideration for long term therapy in CHB patients especially in those with a high risk for renal impairment.

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## COMMENTS

### Background

Hepatitis B virus (HBV) infection is the major cause of chronic hepatitis worldwide. Emergence of resistance due to prolonged nucleos(t)ide analogue use or incomplete suppression of HBV still remains an important concern. Therefore, early virologic response at week 24 of therapy has been used to predict better outcomes and to reduce the risk of antiviral resistance.

### Research frontiers

This study used the response-guided add-on strategy (roadmap concept). For patients with HBV DNA ≥ 300 copies/mL (≥ 51 IU/mL) at week 24, tenofovir was added to telbivudine by week 26 in the telbivudine arm, and telbivudine was added to tenofovir by week 26 in the tenofovir arm. For patients with HBV DNA < 300 copies/mL at week 24, telbivudine and tenofovir monotherapies in the respective arms were continued.

### Innovations and breakthroughs

This was the first prospective, randomised, 2-arm, open-label, non-inferiority study in hepatitis B e antigen (HBeAg)-negative chronic hepatitis B (CHB) patients that compared telbivudine and tenofovir administered as per the roadmap concept. The safety of the combination of telbivudine and tenofovir, for which limited data are currently available, was also evaluated.

### Applications

Efficacy was shown for both telbivudine-roadmap and tenofovir-roadmap regimens in HBeAg-negative CHB patients over 156 wk. Both treaments showed acceptable safety profiles. In addition, the telbivudine arm was associated with renal improvement.

### Peer-review

This is an extensive randomised study to compare the roadmap treatment strategy between telbivudine and tenofovir in patients with HBeAg-negative CHB patients. As antiviral treatment may be life-long, renal protection becomes an important consideration. The current manuscript should be of benefit to the hepatologists and liver transplantation specialists worldwide.

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CASE REPORT

# Spontaneous liver rupture as first sign of polyarteritis nodosa

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## Abstract

Polyarteritis nodosa (PAN) is one of the systemic vasculitis that affects the media wall of arteries of small and medium diameter. Diagnosis proves difficult due to the unspecific symptoms that dominate the clinical profile. Liver involvement is very diverse, ranging from the development of cirrhotic liver disease to acute abdomen presentation that requires surgery because of liver rupture. The management of these patients requires an expert multidisciplinary team. There are several cases in the literature that describe a sudden liver rupture as the first manifestation of a PAN. In this paper we present the case of a 75 years old patient without any previous disease, who is subjected to major hepatic resection for spontaneous liver rupture.

**Key words:** Polyarteritis nodosa; Spontaneous liver rupture; Liver surgery; Vasculitis; Rheumatology

**Core tip:** Spontaneous liver rupture is a rare entity with very few cases in the literature reviewed; even when it has an autoimmune disease such etiology and with no previous trauma. We present our experience managing an urgent abdominal hemorrhage caused by a liver rupture as a first manifestation of Polyarteritis in a 75-year-old woman.

Gómez-Luque I, Alconchel F, Ciria R, Ayllón MD, Luque A, Sánchez M, López-Cillero P, Briceño J. Spontaneous liver rupture as first sign of polyarteritis nodosa. *World J Hepatol* 2016; 8(32): 1414-1418 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i32/1414.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i32.1414

## INTRODUCTION

The first International Chapel Hill Consensus Conference of Rheumatological Diseases defined polyarteritis nodosa (PAN) as a systemic necrotizing vasculitis of arterial tunica media of small and medium sized arteries without the presence of any glomerulonephritis or vasculitis in arterioles, venules and capillaries or without association with anti-cytoplasmic neutrophil antibodies positivity[1].

PAN is a common systemic vasculitis generally involving several organs such as kidneys, skin, central and peripheral nervous system and the gastrointestinal tract. The certain diagnosis is a complex task because of nonspecific laboratory tests and clinical features; therefore it must be based on histopathological analysis by biopsies.

The "American College of Rheumatology: Defined the criteria for the diagnosis of PAN in 1990[2]. To diagnose a PAN the patient must have 3 characteristics out of a list of 10 features, estimating a diagnostic sensitivity of 82.2% with a specificity of 86.6% (Table 1).

Liver involvement in this disease is uncommon and difficult to diagnose. Hepatomegaly (21%), jaundice (12%) and alteration of biochemical liver-function markers (6%) have been reported in the literature in different publications[3]. The development of this type of autoimmune disease has been associated to positive hepatitis B surface antigen (HBsAg), although the exact etiology is unclear. It is reported that HBsAg positive patients may have a better response to treatment and therefore better prognosis[4]. In addition, PAN may cause aneurysm development due to fibrotic arterial lumen occlusion and necrosis, including organs such as liver, spleen and kidneys. These may derive in chronic abdominal pain, gastrointestinal bleeding, stroke, intestinal perforation and even pancreatitis. In some cases infarction and hemorrhage may occur, causing hemoperitoneum bearing a poor prognosis.

Very few cases have been published to date in which spontaneous hepatic rupture would be the first clinical manifestation; even when it has an autoimmune disease such etiology and with no previous trauma.

We present our experience managing an urgent abdominal hemorrhage caused by a liver rupture as a first manifestation of PAN in a 75-year-old woman.

## CASE REPORT

A 75-year-old woman with no previous medical history except chronic anemia and well-controlled arterial hypertension with outpatient follow-up. She is referred for transfusion from another hospital because of a severe anemia. The patient reported feeling general malaise with unmeasured fever several days before. No nausea or gastrointestinal symptoms were noted.

On examination the patient's blood pressure was near the low end of normality without tachycardia. There was no neurological deficit, paraesthesia or loss of motor reflexes. The abdomen palpation proved pain predominantly in right quadrants, with some upper quadrant abdominal defense.

Blood tests found hemoglobin 6.7 g/dL with a hematocrit of 19.2% and white blood cell count of 18000 $mm^3$ (neutrophilia 70%). Coagulation tests showed an INR of 1.34 with a prothrombin activity of 54%. Liver function enzymes showed altered cytolysis enzymes (AST/ALT: 273/275 U/L) and cholestatic enzymes (GGT/FA: 90/159 U/L); bilirubin was within normal range. Other inflammatory parameters reflected reactive-C protein of 227.5 mg/dL.

With this scenario an abdominal computed tomography scan was performed (Figure 1) where liver damage was reported in the form of a right hepatic lobe lesion with poorly defined and confluent contours, heterogeneous density and hypodense predominance. There was also a heterogeneous subcapsular and subhepatic collection, with some hyperdense areas that could be a hematoma, along with a moderate amount of intraabdominal free fluid.

In this context an exploratory laparotomy was performed. There was a bleeding liver injury involving the inferior segments of the right hepatic lobe (segments Ⅴ-Ⅵ), the source of this bleeding was difficult to identify. Hemoperitoneum was present in all abdominal quadrants. An urgent right hepatectomy was executed. The patient received four red cell concentrates in the perioperative care and no vasoactive drugs were necessary.

The postoperative was otherwise uneventful with only a persistent leukocytosis outstanding, without any other signs of sepsis. White blood cell count was normal at the time of discharge.

The pathology report described the existence of an inflammatory and haemorrhagic abscess with a volume of 10 cm × 7 cm × 2 cm as the cause of the hemoperitoneum. The remaining liver parenchyma appeared normal.

In the microscopic study, the liver specimen was compatible with nongranulomatous acute necrotizing vasculitis (Figure 2). The gallbladder sample (Figures 3 and 4) showed acute vasculitis with fibrinoid necrosis in

**Table 1 For classification purposes, a patient shall be said to have polyarteritis nodosa if at least 3 of these 10 criteria are present**

| | |
|---|---|
| Criteria diagnosis of polyarteritis nodosa | |
| Weight loss | Loss of 4 kg or more of body weight since illness began, not due to dieting or other factors |
| Livedo reticularis | Mottled reticular pattern over the skin or portions of the extremities or torso |
| Testicular pain or tenderness | Pain or tenderness of the testicles, not due to infection, trauma, or other causes |
| Myalgias, weakness or leg tenderness | Diffuse myalgias (excluding shoulder and hip girdle) |
| Mononeuropathy or polyneuropathy | Development of mononeuropathy, multiple mononeuropathys, or polyneuropathy |
| Diastolic BP > 90 mmHg | Development of hypertension with diastolic BP higher than 90 mmHg |
| Elevated BUN or creatinine | Elevation of BUN > 40 mg/dL or creatinine > 1.5 mg/dL, not due to dehydration or obstruction |
| Hepatitis B virus | Presence of hepatitis B surface antigen or antibody in serum |
| Ateriographic abnormality | Arteriogrm showing aneurysms or occlusions of the visceral arteries, not due to arteriosclerosis, fibromuscular dysplasa, or other noninflammatory causes |
| Biopsy of small or medium-sized artery containing PMN | Histologic changes showing the presence of granulocytes or granulocytes and mononuclear leukocytes in the artery wall |

Avaialable from: Lightfoot RW Jr, Michel BA, Bloch DA, Hunder GG, Zvaifler NJ, McShane DJ, *et al*. The American College of Rheumatology: 1990 criteria for the classification of polyarteritis nodosa. *Arthritis Rheum* 1990; **33**: 1088-1093. BP: Blood pressure; BUN: Blood urea nitrogen; PMN: Polymorphonuclear neutrophils.

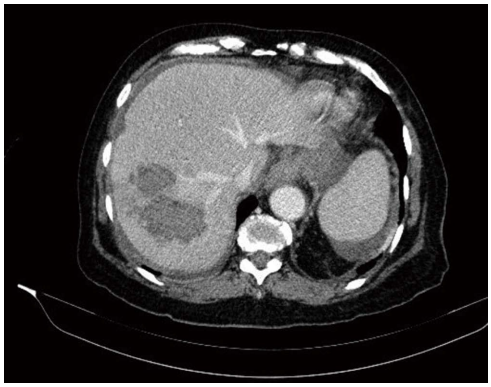

**Figure 1 Computed tomography scan of the liver.**

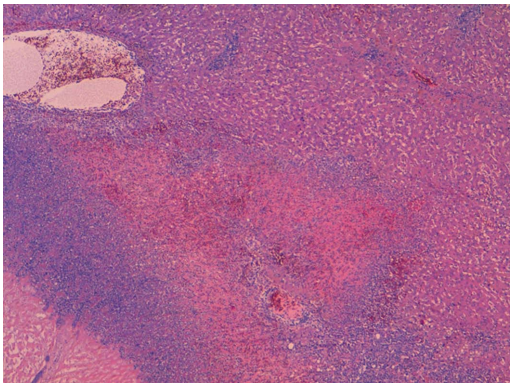

**Figure 2 Liver (hematoxylin and eosin; × 40 original magnification): Bleeding, abscesses and avascular necrosis.**

muscular arteries of parietal medium caliber. All these findings are compatible with PAN type vasculitis.

In the year of follow-up after the surgery, the patient has been treated with prednisone and cyclophosphamide with good results. Outpatient blood tests were negative for HBsAg and the autoimmunity study revealed positive antinuclear antibodies. After two months cyclophosphamide was discontinued because of pancytopenia, with the patient reaching a full recovery after drug suspension. Currently, one year after diagnosis, treatment consists of 10 mg of prednisone once a day, the patient is asymptomatic.

## DISCUSSION

The diagnosis of PAN is presented as a challenge for the clinical practice. This is because it has mainly nonspecific symptoms, which may involve more than one organ and the absence of specific serological tests for this disease. PAN usually appears as a chronic disease with periods of remission and deterioration[5].

Hepatic involvement may be of a PAN clinical profile. Different clinical entities have been described in the literature ranging from chronic liver failure and cirrhosis to acute hepatitis, hepatic regeneration nodules and vascular and bile-duct complications. In most cases the pathogenesis involves immune complex depositions leading to obstruction in hepatic blood flow and obliteration of small vasculature resulting in aneurysm formation (13%-60%)[6-8]. These may cause complications of difficult diagnosis. Most patients are asymptomatic at the moment of diagnosis and only 10%-15% of patients with hepatic artery aneurysms have presenting symptoms, such as abdominal pain, gastrointestinal bleeding or cholestasis[9].

Spontaneous intrahepatic hemorrhage caused by the rupture of a hepatic artery aneurysm is a rare complication of PAN, however it has a high mortality[10,11]. There are about fifteen reported cases in which the diagnosis of PAN was learned due to hemodynamic instability secondary to spontaneous hepatic rupture that required an urgent laparotomy, as in the case presented.

There are different options when deciding how to manage these patients. Some of the reported cases resolved the bleeding using vascular radiology techniques with selective artery embolization[12,13]. In other patients with hemodynamic stability expectant attitude was decided, administering blood transfusion after corticosteroid and immunosuppressive treatment[12,14-16].

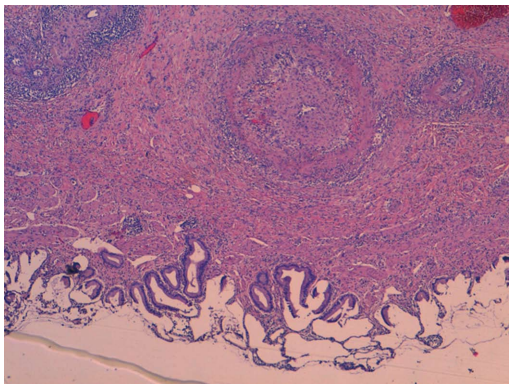

**Figure 3 Gallbladder (hematoxylin and eosin; × 40 original magnification): Acute vasculitis.**

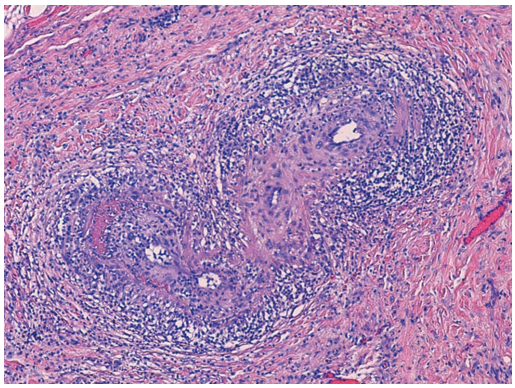

**Figure 4 Gallbladder (hematoxylin and eosin; × 100 original magnification): Acute vasculitis with fibrinoid necrosis in muscular arteries of parietal medium caliber.**

In those cases in which surgical management was decided, most did not do any type of liver resection, opting for hepatic parenchyma hemostasis and packing[4,5] with high mortality rate.

In one case reported[17] a right hepatectomy surgery was performed on a 20-year-old with severe bleeding. This patient died after 12 wk because of several complications after surgery.

This article presents the first case in which an urgent major hepatectomy for treatment of a liver rupture secondary to a previously unknown PAN is performed. In this case the patient is still alive after approximately one year has passed without any kind of complication.

Early and proper diagnosis is decisive in this disease when it is not presented acutely as in the case presented. For this reason, a whole and exhaustive history is required. To confirm the diagnosis of PAN a pathological study is crucial. The biopsy can be obtained from muscle tissue. In cases where the biopsy can not be performed or it is assumed that it would be negative, arteriography is mandatory to confirm the presence of aneurysms[18]. After that, treatment with steroids and immunosuppressive drugs has been found to eliminate all clinical manifestations of the disease. Decrease of the aneurysm size and its risk of rupture has been described with this treatment[12].

## ACKNOWLEDGMENTS

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## COMMENTS

### *Case characteristics*

The patient reported feeling general malaise with unmeasured fever and she feels pain predominantly in right quadrants on the abdomen.

### *Clinical diagnosis*

The most frequent at the beginning of disease symptoms are fever, weight loss, muscles pain, peripheral neuropathy, gastrointestinal disorders and skin lesions.

### *Differential diagnosis*

For diagnosis, the criteria established by the American College of Rheumatology are often used, a high clinical suspicion, a biopsy showing vasculitis or arteriography showing aneurysms.

### *Laboratory diagnosis*

The increased erythrocyte sedimentation rate and C-reactive protein is practically constant during the active phase of the disease. Other common findings are leukocytosis, eosinophilia, and normochromic anemia.

### *Imaging diagnosis*

Is useful to perform an arteriography or reconstructions high-quality computed tomography (CT)-scan imaging that showing the presence of aneurysms or occlusions of visceral arteries not display due to arteriosclerosis?

### *Pathological diagnosis*

Histological alterations show granulocytes or granulocyte and mononuclear leukocytes into the arterial wall of medium diameter.

### *Treatment*

The treatment has undergone major changes in recent years although cyclophosphamide remains the cornerstone despite its side effects, there are promising new therapies such as biologic therapies.

### *Related reports*

There are several cases in the literature related to liver rupture due to polyarteritis nodosa (PAN) them out various treatment are carried with different results. There is so far an established treatment for this type of clinical presentation. The decision is based on the clinical condition of the patient and therapeutics means available in the hospital.

### *Term explanation*

The patient had low suspicion of vasculitis, but had a history of hypertension, the presence of aneurysms in the CT-scan and a PAN conclusive biopsy. The hepatitis B surface antigen was negative and antinuclear antibodies were positive. It shows the difficult diagnosis of this disease and the need for a broad differential diagnosis.

### *Experiences and lessons*

In this case report, the authors show an uncommon PAN debuts with a spontaneous liver rupture as first symptom that requires urgent liver major resection in a patient without previous clinical manifestations.

### *Peer-review*

This case report describes spontaneous liver rupture due to polyarteritis nodosa

which was treated by surgical intervention. The paper is well written.

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