ISSN 1948-5182 (online)

# World Journal of *Hepatology*

*World J Hepatol* 2020 November 27; 12(11): 883-1135

Published by Baishideng Publishing Group Inc

World Journal of Hepatology

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## ABOUT COVER

Associate editor of *World Journal of Hepatology*, Dr. Yong-Ping Yang is a Distinguished Professor at Peking University Health Science Center in Beijing, China. Having received his Bachelor's degree from Yanbian University in 1985, Dr. Yang undertook his postgraduate training at PLA Medical College, receiving his Master's degree in 1992. He rose to Chief Physician in the Hepatology Division of the Fifth Medical Center of the Chinese PLA General Hospital in 2003 and has held the position since. His ongoing research interests involve liver fibrosis, cirrhosis and hepatocellular carcinoma, with a particular focus on cryoablation and cryo-immunotherapy for hepatocellular carcinoma. Currently, he serves as Chairman of the Department of Liver Disease of the Chinese PLA General Hospital and as President of the Chinese Research Hospital Association for the Study of the Liver Disease. (L-Editor: Filipodia)

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## RESPONSIBLE EDITORS FOR THIS ISSUE



**NAME OF JOURNAL**
*World Journal of Hepatology*

**ISSN**
ISSN 1948-5182 (online)

**LAUNCH DATE**
October 31, 2009

**FREQUENCY**
Monthly



**PUBLICATION DATE**
November 27, 2020

World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 883-896

DOI: 10.4254/wjh.v12.i11.883

ISSN 1948-5182 (online)

*REVIEW*

# Malnutrition in cirrhosis: More food for thought

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**Author contributions:** Chapman B, Sinclair M and Testro A conceived the idea and designed the literature review; Chapman B drafted the manuscript; all authors critically reviewed the manuscript and approved the final version of the manuscript for publication.

**Conflict-of-interest statement:** Nothing to disclose.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

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## Abstract

Malnutrition is highly prevalent in liver cirrhosis and its presence carries important prognostic implications. The clinical conditions and pathophysiological mechanisms that cause malnutrition in cirrhosis are multiple and interrelated. Anorexia and liver decompensation symptoms lead to poor dietary intake; metabolic changes characterised by elevated energy expenditure, reduced glycogen storage, an accelerated starvation response and protein catabolism result in muscle and fat wasting; and, malabsorption renders the cirrhotic patient unable to fully absorb or utilise food that has been consumed. Malnutrition is therefore a considerable challenge to manage effectively, particularly as liver disease progresses. A high energy, high protein diet is recognised as standard of care, yet patients struggle to follow this recommendation and there is limited evidence to guide malnutrition interventions in cirrhosis and liver transplantation. In this review, we seek to detail the factors which contribute to poor nutritional status in liver disease, and highlight complexities far greater than "poor appetite" or "reduced oral intake" leading to malnutrition. We also discuss management strategies to optimise nutritional status in this patient group, which target the inter-related mechanisms unique to advanced liver disease. Finally, future research requirements are suggested, to develop effective treatments for one of the most common and debilitating complications afflicting cirrhotic patients.

**Key Words:** Malnutrition; Cirrhosis; Liver transplantation; Chronic liver disease; Nutrition; Sarcopenia



**Core Tip:** Malnutrition is widespread in liver cirrhosis. This paper highlights the multifactorial aetiology of liver-related malnutrition, and details the complex

**Country/Territory of origin:** Australia

**Peer-review report's scientific quality classification**
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 16, 2020
**Peer-review started:** June 16, 2020
**First decision:** September 24, 2020
**Revised:** October 8, 2020
**Accepted:** October 20, 2020
**Article in press:** October 20, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Kaya M, Kim SE
**S-Editor:** Zhang L
**L-Editor:** A
**P-Editor:** Wang LL

challenges cirrhotic patients face in achieving nutritional targets. Although potentially modifiable, there are a scarcity of successful treatments hence the evidence base pertaining to nutritional interventions is surprisingly weak. Further research is required to bridge the gap between actual and ideal nutritional status in cirrhosis. If this goal can be realised, the potential impact on patient and clinical outcomes is immense.

**Citation:** Chapman B, Sinclair M, Gow PJ, Testro AG. Malnutrition in cirrhosis: More food for thought. *World J Hepatol* 2020; 12(11): 883-896
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/883.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.883

## INTRODUCTION

Malnutrition is common in chronic liver disease and increases as the severity of liver disease progresses. It affects up to 80% of patients with decompensated cirrhosis[1-3], and is more widespread than the traditionally recognised sequalae of advanced liver disease, namely hepatic encephalopathy (40%)[4], bleeding oesophageal varices (5%-15%)[5,6], refractory ascites (5%-10%)[7], spontaneous bacterial peritonitis (1.5%-3%)[8] and hepatocellular carcinoma (3%-5%)[9,10]. Compromised nutritional status occurs regardless of the cause of liver disease[11], though is reported most commonly in those with alcoholic cirrhosis and cholestatic liver disease[12,13].

The impact of malnutrition on patient outcomes is increasingly recognized. Malnutrition increases the incidence and severity of decompensation symptoms, contributes to compromised immune function, reduces muscle mass, decreases functional status and quality of life, delays wound healing and is associated with increased mortality[14-16]. Malnutrition is particularly associated with the development and severity of hepatic encephalopathy[17]. Malnourished patients also require prolonged mechanical ventilation and have longer length of stay in both the intensive care unit and hospital following liver transplant[18,19]; all of which translate to significantly increased healthcare costs. Optimising nutritional status in this patient population is therefore of critical importance.

Despite the known deleterious effects of malnutrition, successful strategies to counter its impact in cirrhosis are lacking. In theory, malnutrition is a modifiable element to target to improve the course of liver disease and patient outcomes, though is rarely achieved in practice. So why can't patients identified with malnutrition simply eat more to improve their nutritional state? And why has food failed to provide the therapy desperately needed to treat malnutrition in cirrhosis? In this review, we aim to describe the factors contributing to liver-related malnutrition to highlight the challenges cirrhotic patients face in attaining nutritional targets. We also emphasize areas where data to support clinical recommendations are limited, with the goal of encouraging further research in the area.

## MALNUTRITION IN CIRRHOSIS-ETIOLOGY

The development of malnutrition in cirrhosis is multifactorial, and primarily stems from inadequate dietary intake, altered metabolism and malabsorption (Figure 1). Patients may be afflicted with any or all of these etiological factors, which present unique barriers to effective nutritional support and management.

### *Inadequate dietary intake*

Reduced dietary intake plays a central role in the pathogenesis of malnutrition in cirrhosis. Whilst poor appetite may simply be ascribed to generalized ill-health; factors specific to liver disease play a significant role. Inflammation, early satiety from ascites, hepatic encephalopathy, adverse gastrointestinal symptoms, taste changes and unpalatable dietary restrictions all influence food consumption and their contribution to a negative energy balance needs to be appreciated to identify appropriate interventions to improve dietary intake.

**Inflammation:** Cirrhosis is a pro-inflammatory state primarily triggered by bacterial

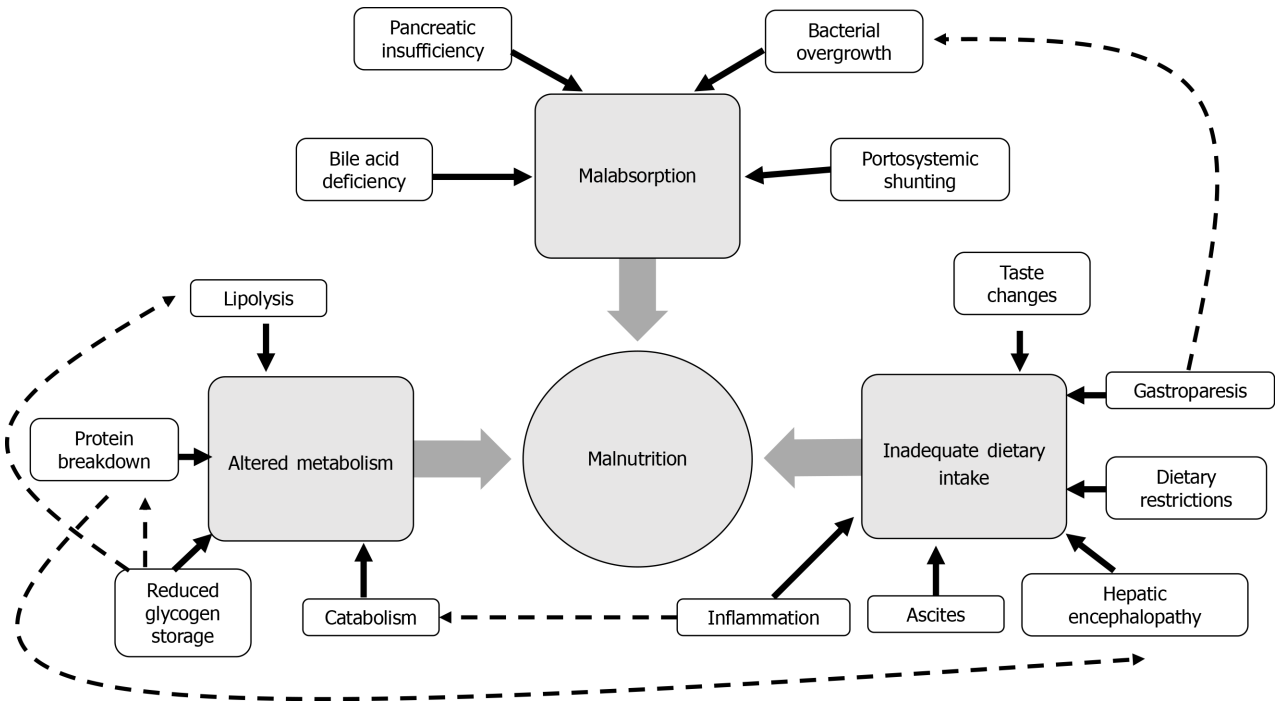


**Figure 1 Multifactorial aetiology of malnutrition in cirrhosis.** Direct contributing factors represented by solid black arrows, inter-related factors represented by dashed black arrows.

translocation from the gut to the circulation due to portal hypertension and increased intestinal permeability[20]. Systematic inflammation induces a range of brain-mediated responses including fever, anorexia and taste changes, with particular aversion to sweet flavours[21]. An increased production of cytokines ensues, which have demonstrated anorexigenic effects[20] and elevate energy expenditure[22].

Pro-inflammatory cytokines such as interleukin-1b, and stimulants that release cytokines (lipopolysaccharides), have been shown to reduce both the quantity and frequency of spontaneous food intake in humans and animals[23]. The precise mechanism by which pro-inflammatory factors act on the neural system to inhibit appetite is highly complex, involving different cell groups and neurotransmitters[24]. The function of inflammation-associated anorexia is to help sustain bodily functions in the face of infection and injury, and is observed during both acute and chronic inflammatory disease. Ultimately, energy is redistributed away from activities that are considered superfluous (*i.e.* food seeking and digestion), towards that required for mounting the immune response[24].

**Ascites:** Ascites is a common symptom of hepatic decompensation that directly impacts oral intake by limiting physical capacity of the stomach, and indirectly by contributing to post-prandial discomfort. Patients with refractory ascites have a high prevalence of malnutrition and characteristically demonstrate the lowest calorie intake of all patients with liver disease[25]. In addition to the effect of ascites on gastric reserve, depressed appetite and early satiety; repeated paracentesis causes significant nutrient losses. Not only does the body expend considerable energy to heat a large body of ascitic fluid, but ascitic fluid has considerable calorie content (in the form of proteins, carbohydrates and fats), and removal of this *via* large-volume paracentesis results in calorie debt[26]. Failure to replace this energy loss exacerbates the catabolic state already seen in advanced cirrhosis. Infusion of serum albumin after paracentesis is necessary to promote plasma volume expansion and prevent hyponatremia[1], though albumin replacement after ascitic drainage has no effect on nutritional status or repletion of protein stores[27].

Following paracentesis, patients generally report improvement in early satiety and an ability to consume larger meals and more calories[28,29]; changes which obviously correspond to increased gastric reserve. However, an increased calorie intake may be short-lived in refractory ascites as fluid reaccumulates, and translation to improved patient outcomes are not always realised[30,31]. Nutrition intervention trials in patients with refractory ascites that have successfully demonstrated enhancements in nutritional and clinical parameters have shared features of long duration of

intervention, intensive dietetic input, and the requirement of artificial nutrition support (enteral nutrition, parenteral nutrition) to meet nutritional requirements[26,32].

**Gastroparesis and autonomic dysfunction:** Abdominal pain, nausea and bloating symptoms are not exclusive to those with ascites and are a frequent complaint in many cirrhotic patients[33,34]. Symptoms can sometimes be explained by the presence of organic disorders, however in many cirrhotic patients, no clear cause is apparent[35].

Distorted metabolic, hormonal and neural function may account for this global gut dysfunction in the absence of a specific diagnosis. Insulin resistance and subsequent elevated postprandial glucose have been shown to delay gastric emptying and lower spontaneous dietary intake in cirrhotic patients compared to healthy controls[36-38]. In addition to a high prevalence of gastroparesis in cirrhosis, small bowel motility may also be abnormal, and has been shown to be worse in those with portal hypertension manifesting in symptoms of diarrhoea and abdominal pain[39,40].

Autonomic dysfunction is also implicated in gastrointestinal symptom development, in a similar fashion to long-term autonomic and peripheral neuropathies observed in diabetes mellitus. Autonomic neuropathy encompassing both sympathetic upregulation and parasympathetic downregulation has been reported in 30%-70% of patients with cirrhosis, leading to "an effective vagotomy" and possibly accounting for gastric and intestinal dysmotility in these patients[35,41-43].

Despite the known association of gastrointestinal dysfunction with cirrhosis, the role of adverse gastrointestinal symptoms in directly limiting energy intake and thus contributing to weight loss and malnutrition has not been explored extensively. In a single study comparing the oral intake of 40 cirrhotic patients to controls, patients with significant gastrointestinal symptoms reached satiation earlier compared to patients without symptoms and healthy controls, resulting in significantly lower energy intake[44]. Another study by the same researchers demonstrated that severity of gastrointestinal symptoms were associated with recent weight loss and impaired health-related quality of life, which correlated with severity of liver disease[45].

**Hunger hormones:** Disrupted glucose and insulin metabolism also contribute to abnormal hormone levels that help control appetite and food intake. Leptin and ghrelin influence energy intake and expenditure[46], with the normal role of leptin involved in suppression of energy intake and accelerating energy expenditure; whilst ghrelin (the "hunger hormone") increases before a meal to stimulate appetite and dietary intake.

In cirrhosis, levels of leptin and ghrelin are abnormal. Leptin is significantly elevated[36,47-49], translating to reduced food intake and increased resting energy expenditure. Although baseline ghrelin has not shown to be significantly different between cirrhotic subjects and controls[36,50], cirrhotic patients have an irregular pattern of ghrelin secretion compared to healthy controls[36]. In liver disease, ghrelin levels fail to rise pre-prandially, so the expected effect of ghrelin on increasing appetite and meal initiation is lost. This blunted ghrelin level is likely related to a combination of insulin resistance, elevated postprandial glucose, and overexpression of serum leptin[36,51,52].

**Hepatic encephalopathy:** Hepatic encephalopathy and sarcopenia are closely related to malnutrition, sharing common etiological factors and pathophysiological pathways all intrinsically linked to muscle health in liver disease. Skeletal muscle tissue plays a central role in removing ammonia from the circulation when its clearance by the liver is impaired. Thus in situations of muscle wasting, commonly precipitated by inadequate dietary intake and hyperammonemia itself in cirrhosis, the neuropsychiatric symptoms of encephalopathy are worsened[17,53].

The spectrum of neurocognitive impairment in cirrhosis ranges from minimal to overt hepatic encephalopathy, which manifest in variable degrees of impaired cognition, alertness and attentiveness[17]. This altered cognitive state, coupled with periods of increased (daytime) somnolence, limit the opportunity for cirrhotic patients with even low-grade encephalopathy to achieve an adequate dietary intake[54]. Forgetfulness, sleeping through meal and snack periods, and difficulty with meal preparation are significant barriers encountered in clinical nutrition practice with this patient group. Compliance with dietary therapies is also problematic[53] and patient management requires a multidisciplinary approach with reliance on patient supports and caregivers. The cycle of poor nutritional intake, leading to muscle loss and sarcopenia that worsens encephalopathy, which in turn exacerbates reduced dietary intake and malnutrition, is difficult to break.

Inappropriate dietary recommendations due to incorrect beliefs that protein restriction is necessary to improve encephalopathy have the potential to worsen malnutrition in this high-risk population. This strategy has no scientific merit though

remains broadly practiced[55]. In a randomized trial by Córdoba *et al*[56], patients hospitalised with encephalopathy demonstrated no benefit of protein restriction in resolution of encephalopathy when compared to normal protein diet[56]. The study also showed even short-term protein restriction to 0.5 g/kg/d resulted in elevated muscle tissue breakdown. Recommendations have since evolved to promote a higher protein intake of 1.2-1.5 g/kg/d to prevent muscle wasting and reverse muscle loss in those who are sarcopenic[57].

**Unpalatable diets:** The recommendation to implement a sodium restriction is often the first dietary advice provided to patients with liver disease, due to the effect of sodium on fluid retention and subsequent development of peripheral oedema and ascites. International consensus guidelines recommend a dietary sodium restriction of < 2000 mg/d for management of ascites[27]. Unfortunately, limiting dietary sodium can negatively impact nutritional status. Many low-sodium foods are unpalatable, and advice to "remove all salt and processed foods" from the diet, without providing appropriate dietary education regarding nutritional adequacy has the potential to worsen malnutrition due to additional dietary constraints.

Sodium restriction alone will only eliminate ascites in approximately 10%-15% of patients[58], and some authors have shown no benefit to a sodium restricted diet when compared to an unrestricted diet in reducing ascites when diuretics were also administered[59]. A recent systematic review concluded increased calorie intake in conjunction with a low-sodium diet resulted in significantly improved outcomes[31]. Thus, advice to follow a low-salt diet should be provided by a dietitian experienced in the management of liver disease, to ensure overly restrictive diets are avoided.

**Taste changes:** Taste changes are another common complaint in cirrhosis, and generally manifest as a reduction in taste acuity for detection and recognition of some or all of the basic tastes of bitter, salt, sweet, and sour[60-64]. Reduced calorie intake has been demonstrated in patients suffering dysgeusia both with and without liver disease[60]. In addition to exacerbation of protein energy malnutrition in cirrhosis, impaired gustatory function has shown to adversely affect general wellbeing and quality of life by reducing pleasure associated with food intake[65].

Zinc and vitamin A are important nutrients involved in maintaining taste integrity, and are commonly deficient in cirrhosis[65]. Zinc is involved in the synthesis and activity of the salivary protein gustin, which plays a role at taste bud receptor sites[66] as well as being critical to a number of enzymatic processes and formation of structural proteins related to taste[67]. Vitamin A is required for the production of mucopolysaccharides in the epithelial cells of taste buds[65]. The literature regarding the association between zinc and vitamin A status with taste acuity in liver disease is variable though. Several authors failed to show any association between low serum zinc[61,62,65] or vitamin A[65] concentration and taste acuity. In contrast, two intervention trials observing the effect of long term zinc supplementation in cirrhosis demonstrated improved taste perception after supplementation, with a subsequent increase in serum zinc concentration[68,69]; whilst another group studying deficient patients supplemented with vitamin A resulted in both enhanced taste function and repletion of serum vitamin A levels[64].

### *Altered metabolism in cirrhosis*

Increased energy expenditure, reduced synthesis of endogenous substrates, insulin resistance and low respiratory quotient characterize the metabolic disturbances common in patients with chronic liver disease.

**Increased energy expenditure:** Hypermetabolism, defined as measured resting energy expenditure (REE) > 20% above predicted REE, is often encountered in cirrhosis. Hypermetabolic patients are more often malnourished, have reduced lean body mass, and have reduced survival after liver transplant compared to those with normal metabolic rate[70,71]. Hypermetabolism in cirrhosis makes nutritional targets even more unattainable for this patient group, causing a negative impact on nutritional status. The prevalence of hypermetabolism ranges from 15% in a recent study of 268 New Zealand patients awaiting liver transplant[72], up to 34% in an early German study, which remains the largest to date with 473 cirrhotic patients considered for transplant[73].

Comorbidities typically associated with cirrhosis give rise to a theoretical explanation for elevated total energy expenditure in liver disease. These include hyperdynamic circulation (related to increased sympathetic nervous system activity), inflammation, frequent infections and ascites[74]. Pro-inflammatory cytokines induce lipolysis and protein breakdown to mobilize fatty acids and release amino acids,

respectively, which are used in production of glucose by gluconeogenic organs (liver, kidney and intestine). The conversion of fat and protein to provide an available energy source in the form of glucose is an inefficient process and known to be expensive in terms of energy utilisation[57]. Compromised gut barrier function also promotes inflammation and development of cirrhotic complications, in particular bacterial infections, with sepsis a known contributor to increased energy expenditure[39]. Extracellular fluid (including ascites), is generally considered metabolically inactive as it does not consume oxygen or produce carbon dioxide. However in liver cirrhosis, some researchers have demonstrated a small but significant increase in metabolic rate prior to removal of ascitic fluid, compared with post-paracentesis energy requirement, potentially attributable to hemodynamic changes and the energy required to heat ascites[75].

The precise reason for hypermetabolism in cirrhosis is uncertain though, with conflicting etiology proposed by researchers. The New Zealand group found no relationship between hypermetabolism and patient gender, disease origin, protein depletion, ascites or severity of liver disease[72]. Conversely, other authors purport an inverse association between the severity of liver disease and resting energy expenditure[76], and presence of ascites with metabolic rate[75]. Given the inconsistency in clinical factors correlating with hypermetabolism in cirrhosis, simple identification of such patients is not feasible, and estimation of nutritional requirements *via* traditional methods (predictive equations) is known to be inaccurate in advanced cirrhosis[77,78]. Current international guidelines recommend measurement of resting metabolic rate *via* indirect calorimetry wherever possible[57,79].

**Altered macronutrient metabolism:** Cirrhosis also affects macronutrient metabolism and is a key factor in contributing to malnutrition in liver disease. The liver's ability to store glycogen is markedly reduced. Hypoglycaemia occurs readily in the fasted state, and compromised glucose utilization due to insulin resistance is common. Impaired glycogen storage leads to early onset of gluconeogenesis; increasing the use of muscle glycogen, amino acid deamination, free fatty acid oxidation, and hepatic production of ketone bodies as an energy source[28,73,80,81].

This accelerated starvation response has been quantified by several authors. An early study by Schneeweiss *et al*[76] demonstrated the percentage of total calories derived from fat (86%), carbohydrate (2%) and protein (12%) were significantly different after an overnight 10-hour fast compared to healthy controls who metabolized 45%, 38% and 17% of fat, carbohydrates and protein, respectively[76]. This reduced storage capacity for glycogen means the fuel sources being used by cirrhotic patients after a 10-h fast are similar to that used after three days in an individual with a healthy liver[76,82]. A low respiratory quotient, indicating increased lipid and decreased glucose oxygenation, confirms this change in macronutrient utilisation[73]. The cascade effect of these metabolic alterations combined with poor dietary intake leads to loss of lean body mass and subcutaneous fat wasting. Thus, the prevention of long periods of fasting may reduce the muscle and fat loss commonly seen in cirrhosis. Intervention trials providing a late evening snack to individuals with liver disease support this rationale, with improved nitrogen balance and increased muscle mass demonstrated[82,83].

The metabolic switch of primary fuel source from glucose to amino acids and fatty acids is a prime characteristic of liver disease, and occurs even in the setting of only mild to moderate cholestasis. Increased protein requirements ensue, not only due to this increased rate of amino acid oxidation for gluconeogenesis, but also subsequent to decreased synthesis of protein, as well as protein losses in ascitic fluid and from the gastrointestinal tract[80,84,85]. The minimum protein required to maintain nitrogen homeostasis in healthy individuals is 0.8 g/kg/d[86]. Studies indicate that patients with cirrhosis only achieve positive nitrogen balance at a level of 1.23 g/kg/d, and are able to utilise protein up to 1.8 g/kg/d[87]. Normal to high protein intake does not precipitate hepatic encephalopathy, and thus a protein intake of 1.2-1.5 g/kg/d in cirrhosis is recommended by expert consensus[57,79] to prevent and/or reverse loss of muscle mass common in liver disease.

Lipid metabolism in cirrhosis is characterised by rapid oxidation in the fasted state, peripheral lipolysis and fat malabsorption. During fasting, lipids are oxidised as the preferred substrate as fat stores are mobilised, so plasma free fatty acids as well as glycerol and ketone bodies are increased. After a meal, suppression of lipid oxidation is not uniformly impaired, as would normally be the case with insulin release following a meal in healthy controls[88]. Lipolysis may be secondary to this insulin resistance but might also occur in an effort to provide cells with substrates for oxidation in the setting of dietary fat malabsorption.

### *Malabsorption in cirrhosis*

Malabsorption is the final key contributor to negative energy balance and malnutrition in cirrhosis. Reduced bile flow in cholestatic patients decreases intestinal luminal bile salt availability and micelle formation, with subsequent malabsorption of fat and fat-soluble vitamins[89]. Pancreatic insufficiency may also be present and cause macronutrient maldigestion. Medications that alter the intestinal microbiota (*e.g.*, antibiotics leading to decreased bacterial synthesis of short-chain fatty acids) or decrease bile acid availability (*e.g.*, cholestyramine for pruritis) will also cause malabsorption of luminal nutrition, decreasing the amount of calories available for the body to use.

Malabsorption is further exacerbated by portal hypertensive enteropathy and subsequent changes in the gut microbiota that impair absorption and utilisation of nutrients[90,91]. Increased intestinal permeability and dysbiosis are common features linking the liver to a number of nutritional and gastrointestinal diseases[92]. Mucosal changes are frequently encountered upon endoscopic examination of the GI tract[93,94], where the prevalence of portal hypertensive gastropathy has been reported in 20%-98% of cirrhotic patients[95]. Major predictors of portal hypertensive gastropathy are the presence of oesophageal varices and increased severity of cirrhosis[96,97]. Gastroparesis and delayed gut transit mean that small bowel bacterial overgrowth is also common[98], and appears to be related to liver disease severity[99]. This further compounds dysbiosis and associated malabsorption.

## NUTRITIONAL INTERVENTIONS IN CIRRHOSIS

Despite the high prevalence of malnutrition in decompensated liver disease and it's known deleterious effects, successful strategies to counter malnutrition in cirrhosis are lacking. In theory, malnutrition is a modifiable syndrome to target that may impact the course of liver disease and would theoretically be expected to improve patient outcomes, although evidence to support this remains limited. Indeed, recent international guidelines counter whether malnutrition can be reversed at all in the face of deteriorating liver function[57].

### *Oral supplementation*

A small number of studies have demonstrated dietary counselling and oral nutritional support can improve nutritional intake, nitrogen balance and selected patient outcomes[80,100-102]. Supplementation with high calorie and protein oral nutrition supplements in addition to dietary counselling improved anthropometric parameters and muscle function measured *via* handgrip strength[101,102], with reduction in hospitalisations also seen in one study[101]. No significant differences were observed with regards to liver function, clinical outcome (*e.g.*, decompensation symptoms, infection rate) or mortality between intervention and control groups in any of the aforementioned studies. Recent meta-analysis on nutrition therapy in liver cirrhosis also failed to show any survival benefit with intervention, though studies included were heterogeneous in terms of severity of liver disease and duration of intervention[103,104].

A possible reason for the lack of demonstrated benefit observed in the literature relates to the marked discrepancy in patients nutritional intake *vs* that recommended by current international guidelines[57,79], with up to 75% of patients not achieving calorie targets[105], generally agreed to be around 32-35 kcal/kg/d. Dietary protein intake in cirrhotic patients is also found lacking. In a Canadian study of 631 patients awaiting transplantation, only 24% achieved the recommended target of > 1.2 g/kg/d, with 26% consuming a very low protein intake of < 0.8 g/kg/d, which resulted in a 2-fold increase in wait-list mortality[106].

Fortunately, the literature regarding inclusion of a late evening snack in cirrhosis is more conclusive. Cirrhotic patients who consume a late evening snack are able to demonstrate changes in substrate utilisation *via* increased respiratory quotient, indicating increased use of glucose similar to that of healthy controls, as well as an improvement in nitrogen balance[87,106]. This improvement in the pattern of fuel utilisation from inclusion of a late evening snack has also shown to translate to improved clinical outcomes in longer-term studies. In a comprehensive study by Plank *et al*[82], which followed 103 patients for 12-mo and compared outcomes from daytime *vs* nighttime consumption of a high calorie dietary supplement (710 kcal), significant improvements in total body protein accretion equivalent to 2 kg of lean tissue sustained over 12 mo were demonstrated in the nighttime supplementation group[82].

Hence shortening periods without food by including a late evening snack is considered a useful strategy to reverse protein catabolism and sarcopenia of cirrhosis. In addition to improved body composition, a meta-analysis concluded a late evening snack in cirrhosis also confers benefits on quality of life and survival, and reduction in frequency and severity of hepatic encephalopathy[83]. Poor patient compliance is an obstacle that still plagues implementation of a late evening snack however, with only half of the patients in the Plank *et al*[82] study able to consume the supplement at the prescribed amount and time[82], thus strategies to enhance patient compliance in clinical practice need to be considered.

Several randomized controlled trials have demonstrated the positive effects of oral BCAA supplementation in not only improving hepatic encephalopathy, but also nutritional status, liver function, quality of life and survival in malnourished cirrhotic patients[107-109]. Each of these trials were conducted in the outpatient setting and demonstrated good patient compliance with supplementation ranging between 14-30 g BCAA per day. A prolonged intervention period and follow up (> 12 mo) were also shared features, and likely provided sufficient time for these patients to increase their muscle mass and improve nutritional status, leading to superior ammonia clearance. A recent Cochrane review of 16 randomized trials confirmed the beneficial effect of BCAAs on hepatic encephalopathy symptoms, though found no such gains for survival, quality of life, or nutritional parameters[110].

### Enteral nutrition

Consensus guidelines from international societies recommend enteral nutrition for such patients who are unable to achieve adequate dietary intake[57], though only a handful of investigations have actually evaluated enteral nutrition in patients with liver disease[111]. These studies are afflicted by either small sample size or limited duration of therapy. The study with the longest treatment duration was published over 30 years ago and involved a 60 d intervention with NG feeding[112] with subsequent gain of body weight as muscle, bone and fat stores (which only became evident after 30 d), though patient outcome data pertaining to liver disease was not described. The remaining studies all have an intervention period of 4 wk or less; two of which found those treated with EN had improved survival compared to oral diet[113,114], while another study of mean 2.8 wk NG feeding found no survival benefit[115]. However, given the relatively short duration of EN provision in all published literature, it is difficult to make any inferences about the effect of EN on short- to medium-term survival. It is currently unknown whether EN given over a longer period (*e.g.* > 8 wk) could reduce complications and improve clinical outcomes including mortality. It is also unknown if enteral nutrition support delivered in the pre-transplant period can affect post-liver transplant operative outcomes; as the aforementioned studies all exclude patients awaiting liver transplant.

### Parenteral nutrition

The indication for parenteral nutrition (PN) support in cirrhosis is consistent with the recommendation in non-cirrhotic patients; those who cannot be fed orally or where enteral nutrition is either contraindicated (*e.g.* intestinal ileus, obstruction) or not tolerated, should be considered for parenteral nutrition[79]. ESPEN guidelines recommend PN be initiated immediately in cirrhotic patients with moderate to severe malnutrition who cannot receive adequate nutrition *via* the enteral route, on the basis of higher rates of complications and reduced survival in malnourished cirrhotic patients[54]. In situations where fasting is prolonged greater than 72 h, PN is also endorsed. Strict adherence to aseptic central line management is paramount in these patients, to minimise risk of infection and sepsis in this high risk group[79] (Table 1).

### Need for future studies

Several meta-analyses have found no convincing evidence that either oral feeding, or enteral or parenteral nutrition improved outcomes in patients with liver disease[103,104,116]. The most recent[104] included 13 randomized trials including 663 patients, and although fixed-effects analysis demonstrated that nutrition intervention prevented hepatic encephalopathy (0.73; 95%CI: 0.55-0.96) and infection (0.66; 95%CI: 0.45-0.98) the results were not confirmed in random-effects analysis. Included studies were particularly heterogeneous with the difference in daily calorie and protein intake ranging 10-fold between studies, and duration of therapy ranging from 3 to 365 d. An earlier meta-analysis of six trials and 470 patients in which the primary outcome measure was survival, found no reduction in mortality [RR: 0.75 (0.42-1.32), $P$ = 0.31][116]. A Cochrane review from 2012 included 37 trials and most analyses failed to

**Table 1 Strategies to treat malnutrition in cirrhosis**

| Nutritional recommendations |
|---|
| Small, frequent meals and snacks (5-7 per day) |
| High calorie intake (≥ 32 kcal/kg/d) |
| High protein intake (1.2-1.5 g/kg/d) |
| Late evening snack containing protein and carbohydrate |
| Add oral nutrition supplements when unable to meet energy-protein requirements *via* ad-libitum dietary intake |
| Low sodium diet (≤ 2000 mg/d) if ascites or oedema present |
| Supplement with branched chain amino acids (25%-30% of total protein requirement) if hepatic encephalopathy or sarcopenia present, whilst ensuring overall protein intake meets requirements |
| Initiate enteral feeds (nasogastric) if unable to meet energy-protein needs *via* oral diet (polymeric, energy-dense formula). Consider nasojejunal tube if severe gastroparesis or intolerance of nasogastric feeds |
| Initiate parenteral nutrition if malnourished and enteral route either not accessible or unable to tolerate full energy-protein requirements |

find any significant differences, with the conclusion that "data do not compellingly justify the routine use of parenteral, enteral or oral nutrition support in patients with liver disease"[103]. All authors reported a low quality of trials with an urgent need for data from well-designed and implemented randomized studies to inform future clinical practice.

Despite the low grade of evidence supporting nutritional recommendations in cirrhosis, it is well accepted that malnutrition is associated with a host of poor outcomes, and efforts to prevent its occurrence and progression should be prioritised. Given the limitations of current studies, there is an urgent need for large, well-designed trials with appropriate intervention and duration. Most importantly, analysis of clinical outcomes is required to determine whether improving malnutrition can have a lasting impact on pre- and post-liver transplant morbidity and mortality.

## CONCLUSION

Malnutrition negatively impacts the course of patients with liver cirrhosis and has a complex, multifactorial etiology. It remains a potentially reversible prognostic marker in cirrhosis, but remains a challenging problem with little evidence to guide intervention. The presence of anorexia and other symptoms leading to poor oral intake, the catabolic nature of the disease process, and underlying metabolic changes leads to significant difficulties in adequately meeting the nutritional needs of cirrhotic patients. Based on the published literature, it appears these barriers are often insurmountable. Many patients cannot achieve a stable anabolic state with a dietary intake far distant from what is recommended. There is an urgent need for further large-scale research to provide evidence for many aspects of the appropriate nutrition management for patients with cirrhosis, and indeed determine whether artificial nutrition support is able to bridge the nutrition gap so often encountered in clinical practice. A focus on patient reported outcomes will also enhance compliance with dietary therapies and analysis of clinical endpoints is critical.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 897-907

DOI: 10.4254/wjh.v12.i11.897

ISSN 1948-5182 (online)

*MINIREVIEWS*

# Paraneoplastic syndromes in cholangiocarcinoma

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**Author contributions:** Shah PA and Tahir Z designed the study. Rahman SU and Sana MK did database screening and data extraction. Tahir Z, Shah PA, Rahman SU, Ali A, and Sana MK did the literature review, analyzed the data, and wrote the manuscript. Rahman SU, Sana MK, Ali A, Tahir Z, and Shah PA reviewed the paper for final submission.

**Conflict-of-interest statement:** The authors declare that they have no conflict of interest.



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## Abstract

Paraneoplastic syndromes are the symptoms or signs which result from damage to tissues that are distant from the site of malignancy, due to complex interactions between the body's immune system and malignant neoplasm. Cholangiocarcinoma (CCA) is an aggressive epithelial malignancy of hepatobiliary tree and it is found to be associated with various paraneoplastic syndromes. These syndromes can present as dermatological, neurological, renal, hematological, or multi-systemic manifestations. Clinical suspicion and timely recognition of these syndromes can lead to early diagnosis of covert malignancies like CCA. The management plan remains the removal of the underlying cause which in this case is CCA.

**Key Words:** Cholangiocarcinoma; Paraneoplastic syndrome; Malignancy; Immune system; Biliary tree; Multi-organ



**Core Tip:** Various case reports have been published focusing on single paraneoplastic syndromes associated with cholangiocarcinoma (CCA) but none of the studies has reported these manifestations collectively. This review summarizes different paraneoplastic syndromes which are associated with CCA to give a better idea about how it affects various organ systems.

**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 16, 2020
**Peer-review started:** June 16, 2020
**First decision:** July 30, 2020
**Revised:** August 13, 2020
**Accepted:** September 8, 2020
**Article in press:** September 8, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Coulouarn C, Dourakis SP
**S-Editor:** Zhang H
**L-Editor:** A
**P-Editor:** Wang LL

**Citation:** Rahman SU, Sana MK, Tahir Z, Ali A, Shah PA. Paraneoplastic syndromes in cholangiocarcinoma. *World J Hepatol* 2020; 12(11): 897-907
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/897.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.897

## INTRODUCTION

Cholangiocarcinoma (CCA) is an aggressive epithelial malignancy of hepatobiliary tree, accounting for 10%-20% of primary liver cancers[1,2]. It is strongly linked to chronic liver disease and is classified according to an anatomical location in the biliary tree as intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) subtypes[3]. The incidence of CCA, particularly iCCA has increased worldwide between 1993 and 2002. Highest rates were found among Asian countries with South Korea on the top having an age-standardized incidence rate (ASR) of 2.80, followed by Thailand (2.19) and Japan (0.95)[4]. In the USA, the incidence of iCCA has increased in the last 40 years between 1973 and 2012 from 0.44 to 1.18 cases per 100000 person-years[5]. iCCA accounts for about 20% of the deaths from hepatobiliary cancers, which cause 13% of the total cancer mortality worldwide[6]. Recent diagnostic techniques and early management have led to an improvement in 1-year mortality over time but the 5-year survival is still as low as 10% due to the appearance of clinical symptoms in the later course of the disease[7].

Paraneoplastic syndromes are the symptoms or signs which result from damage to tissues that are remote from the site of malignancy, due to complex interactions between the body's immune system and malignant neoplasm[8]. CCA has been reportedly found to be the source of various paraneoplastic manifestations including alopecia[9], sensory neuropathies[10], hypercalcemia, polycythemia, leukocytosis[11] and increased parathyroid hormone-related protein (PTHrP)[12-14].

Several individual cases of paraneoplastic syndrome with CCA have been reported in the literature. This mini-review summarizes all these cases with their clinical presentation and pathophysiology associated with CCA.

## PARANEOPLASTIC SYNDROMES

### *Dermatological manifestations*

**Acanthosis nigricans:** Acanthosis nigricans (AN) presents as a brown to black hyperpigmented velvety patch found on neck, groin, and axilla[15]. However, AN associated with internal organ malignancy mostly appears as a diffuse patch over palms and soles, and can also involve the oral cavity and/or esophagus[16]. Of all the reported cases, internal organ malignancies are found to be associated with AN of palms in 90% of the cases[17]. Paraneoplastic syndrome that occurs as a result of CCA is associated with the production of biologically active particles in the malignant tissue. These include growth factors like epidermal growth factor (EGF) or alpha-transforming growth factor (αTGF) which are associated with the malignant proliferation of skin resulting in AN[18]. AN resolves after effective treatment of the underlying malignancy and might progress if there is any recurrence or metastasis of the tumor as stated in the study of Ravnborg.

**Alopecia:** Alopecia is the most common hair disease reported in oncology patients[19]. It usually presents as a round patch of hair loss on the top of the skull with well-demarcated edges. The type of alopecia associated with cholangiocarcinoma is usually alopecia areata[20]. Alopecia in CCA is triggered by several neurological, hormonal, and emotional factors but in most cases, the actual cause remains undetermined. Alopecia caused by CCA has an increasing trend of incidence in the people of the United States as compared to those in Europe hinting towards the role of environmental factors as well[21]. Although it can occur as an independent condition, but remission of cancer resulting in the resolution of alopecia, and relapse leading to recurrence favors the paraneoplastic nature as mentioned in the study of Antoniou[20].

**Dermatomyositis:** It is relatively a rare paraneoplastic manifestation of CCA[22]. It presents as weakness of the proximal group of muscles with a bluish-purple skin lesion on upper eyelids (heliotrope rash) and erythematous papules on knuckles are noticed (Gottron papules) as evident from the study of Suh[23]. Muscle weakness in

dermatomyositis caused by CCA is attributed to increasing energy consumption of carcinoma cells than normal cells, resulting in excessive glucose breakdown[24]. Dermatomyositis also results from an autoimmune response against highly active cancer cells that cross-react and attack the affected muscles[25]. Myositis specific auto-antigens are also expressed in tumor cells that cross-react and cause this disease. Successful treatment of CCA results in the improvement of dermatomyositis[26].

**Disseminated superficial porokeratosis:** Disseminated superficial porokeratosis (DSP) is a benign proliferation of keratinocytes resulting in a hyperkeratotic skin tumor[27]. DSP is linked to internal organ malignancies such as hepatocellular carcinoma and CCA suggesting a paraneoplastic nature[28]. DSP presents as well-demarcated reddish-brown papules, ranging from 0.5 cm to 1.5 cm, and become noticeable a few months before the onset of cancer-related symptoms. These are visible on the extensor surface of all limbs and trunk, consisting of multiple annular and itchy lesions with elevated borders sparing mucosa, palms, and soles as explained by Cannavo in his study[29]. Overexpression of the *p53* gene product is associated with the appearance of widespread DSP as a manifestation of internal organ malignancy like CCA[30]. All of these abnormalities in the *p53* pathway result in a lack of keratinocyte differentiation and dysregulation of loricrin expression. Loricrin is a precursor protein formed in the last stage of keratinization and its dysregulation results in the formation of a cornified envelope[31].

**Necrolytic Migratory Erythema:** Necrolytic migratory erythema (NME) is an erythematous erosive patch with an advancing scaly border[32]. NME in the setting of CCA, presents as annular erythematous lesions of almost 1-2 cm in size with a central glassy surface, surrounded by scaling that occurs on the face, trunk, and extremities as mentioned by Chiyomaru in his case report. NME mostly presents before CCA is clinically diagnosed[33]. Several conditions like primary sclerosing cholangitis, liver fluke infection, and biliary malformation are associated with increased risk of CCA which results in paraneoplastic NME[34]. Moreover, low nutritious conditions are also aiding the development of NME. Thus patients with NME, without a known cause, should always be checked and screened for malignancies periodically[33].

**Persistent erythema multiforme:** Erythema multiforme (EM) is one of the cutaneous disorders that occur in the setting of a pre-existing internal organ malignancy and are collectively known as paraneoplastic dermatoses. EM is typically self-limited and benign. It usually occurs early in the disease course of CCA. Persistent erythema multiforme (PEM) is a rare form of erythema multiforme that comprises of both typical and atypical cutaneous/mucosal lesions and doesn't resolve on its own[35]. On examination, EM appears as a painful erythematous rash with scaling that occurs in patches over the chest, upper back, and both thighs. After a few days, it changes to hemorrhagic bullae with violaceous edges. Vital signs are normal and there is no mucosal involvement as shown by the study of Tzovaras[36]. EM is sudden in onset and usually resolves in 1- 6 d. Skin paraneoplastic syndromes are commonly associated with internal organ malignancies but EM is a rare skin manifestation in these cases. It is believed that PEM occurs in the setting of CCA as a result of continuous stimulation by antigenic tumor material[37]. There is no direct involvement of tumor cells and no other acquired factors are present marking REM as a paraneoplastic manifestation of CCA. Moreover, the treatment of CCA results in regression of PEM, and relapse of the tumor results in its reappearance[36].

**Sweet syndrome:** Sweet syndrome (SS) is an acute febrile neutrophilic dermatosis. Malignancy associated Sweet syndrome occurs in approximately 15% of the population suffering from solid tumors[38]. It presents as rapidly growing painful erythematous plaques over the face, neck, and legs and is associated with fever, generalized malaise, cough, and arthralgia according to the case report presented by Shinojima[39]. According to recent studies, it is observed that granulocyte-colony stimulating factor (G-CSF) has a major role in the pathogenesis of SS[40]. Increased G-CSF results in production, activation, and chemotaxis of the neutrophils[41]. Malignant tumors like CCA result in excessive production of G-CSF that further stimulates neutrophils resulting in paraneoplastic SS[42].

**Bazex syndrome:** Bazex syndrome is characterized by the appearance of hyperkeratotic lesions on various parts of the body in association with an underlying malignancy. It clinically presents as pruritic scaly dusky red eruptions covered with adherent scales on face, ear, buttocks, palms, and soles. They are commonly associated with fatigue, recurrent abdominal pain, nausea, vomiting, constipation, weight loss,

and liver enlargement as mentioned by Karabulut in his study[43]. Pathophysiology of bazex syndromes is cross-reaction of antigens from tumor to skin. This cellular immune system alteration results in increase release of various growth factors like epidermal growth factor-alpha[44]. Treatment of bazex syndrome shows how strongly this manifestation is paraneoplastic. Regular dermatological treatment doesn't show any response but the removal of the underlying tumor results in complete resolution of the disease[45].

**Erythema gyratum:** Erythema gyratum is rapidly moving erythema and is a marker of underlying malignancy. It is most commonly associated with lung, esophageal, and breast carcinoma[46]. Clinically it presents as a 3-wk history of rash on the lower limbs. The eruption of the rash began as small erythematous macules which gradually enlarge resulting in concentric raised scales on the lateral aspect of the right thigh as Liau mentioned in his study[47].

**Pityriasis rubra pilaris:** Pityriasis rubra pylaris (PRP) is a papulosquamous dermatosis of skin. It clinically presents as a 10-d history of widespread pruritic rash that begins appearing on thighs and progresses gradually over shins, lower back, trunk, face, and forearms as mentioned in the case report presented by Bar-Ilan. It is associated with mild fever as well[48]. Pathophysiology involved in the appearance of PRP is increased secretion of peptides and hormones from the tumor due to cross-reactivity of the antigens[49].

**Sign of leser-trelat:** Leser-trelat sign is the appearance of multiple pigmented seborrheic keratosis mostly associated with underlying malignancy. It is mostly associated with GI adenocarcinoma and rarely with CCA[50]. It clinically presents as a 1-week history of worsening jaundice, pale stools, and recent onset abdominal pain as evident by the study of Morgenthau[51]. The pathophysiology behind this sign is a sudden increase in cytokines and various growth factors like epidermal growth factor-alpha resulting in hyperpigmentation of the skin[52].

**Subacute cutaneous lupus erythematosus:** Subacute cutaneous lupus erythematosus (SCLE) is an inflammatory skin disorder mimicking skin manifestations of systemic lupus erythematous. It is one of the rare paraneoplastic manifestations linked to CCA. It clinically presents as explosive onset of new pruritic rash along with arthralgia and lower extremity edema in a patient with previous history of CCA as explained by Opneja in his study[53]. The pathophysiology behind SCLE lies in self-activation of the body's immune system resulting in photosensitive rash as it is in the usual SLE[54].

A summary of all the dermatological paraneoplastic syndromes is explained in Table 1 at the bottom of the review

### *Neurological manifestations*

**Limbic encephalopathy:** Limbic encephalopathy (LE) is the sub-acute onset of memory impairment and confusion[55]. LE is reported in CCA but is a rare finding. In the starting phase, it presents as a polyneuropathy quite similar to diabetic polyneuropathy, with gradual progression to focal seizures. As the disease progresses, temporal lobe association is noted resulting in pilomotor erection (autonomic seizures) and eventually symptoms of rapidly progressive dementia appear[56]. Thus, autonomic seizures, delusion, and rapidly progressive dementia are the hallmarks of LE[57]. Different mechanisms are linked to LE in the setting of CCA. The formation of new anti-neuronal antibodies is associated with limbic encephalitis. In some cases, autoantibodies are formed against intracellular antigens in the mesiotemporal region while others suggest that they are formed against surface antigens[58]. Both hypermetabolism and hypometabolism of mesiotemporal lobe has also been reported after the onset of LE symptoms[59].

**Paraneoplastic cerebellar degeneration:** Paraneoplastic cerebellar degeneration (PCD) typically presents in women with a sudden onset of ataxia progressively involving limbs and trunks, dysarthria, diplopia, and dysphagia that occurs in the background of a malignancy. It is rarely reported with CCA. In a case report by Bruhnding *et al*. it began with lower limbs, but gradually involved upper limbs as well. Sensation in legs was affected as well. Eventually, the patient was unable to stand anymore. Dysmetria ensued and interfered with self-sufficiency in feeding leading to a weight loss of 60 pounds over six months. Imaging revealed a 2 cm liver mass. Biopsy proved intrahepatic cholangiocarcinoma. Anti-Yo antibodies were also found positive in association with PCD[60]. The underlying mechanism is not clearly understood. The

**Table 1 Summary of literature on dermatological paraneoplastic syndromes in cholangiocarcinoma**

| Ref. | Year | Paraneoplastic syndrome | Mediator |
|---|---|---|---|
| Dermatologic | | | |
| Ravnborg *et al*[15] | 1993 | Acanthosis nigricans | TGF-alpha |
| Suchonwanit *et al*[19] | 2018 | Alopecia | T-lymphocytes |
| Antoniou *et al*[20] | 2012 | Alopecia | T-lymphocytes |
| Suh *et al*[23] | 2016 | Dermatomyositis | |
| Yasuda *et al*[26] | 2018 | Dermatomyositis | |
| Sotoodian *et al*[27] | 2018 | Disseminated superficial porokeratosis | p53 |
| Cannavó *et al*[29] | 2008 | Disseminated superficial porokeratosis | p53 |
| Chiyomaru *et al*[33] | 2010 | Necrolytic migratory erythema | |
| Tzovaras *et al*[36] | 2007 | Persistent erythema multiforme | |
| Shinojima *et al*[39] | 2006 | Sweet syndrome | G-CSF, IL-1, IL-6 |
| Karabulut *et al*[43] | 2006 | Bazex syndrome | |
| Liau *et al*[47] | 2016 | Erythema gyratum | |
| Bar-Ilan *et al*[48] | 2017 | Pityriasis rubra pilaris | |
| Morgenthau *et al*[51] | 2019 | Sign of leser- trelat | EGF-alpha |
| Opneja *et al*[53] | 2015 | Subacute cutaneous lupus erythematosus | |

TGF-alpha: Tissue growth factor-alpha; G-CSF: Granulocyte-colony stimulating factor; IL: Interleukin; EGF-alpha: Epidermal growth factor-alpha.

autoimmune nature of PCD is thought to be due to malignant cells expressing onconeural antigens that are otherwise found on neurons[61]. Thus cross-reactivity leads to the development of PCD. However, no direct link between these antibodies and PCD has been developed yet.

### *Renal manifestations*

**Glomerulonephritis:** Glomerulonephritis is defined as inflammation of small blood vessels inside the kidneys. Fibrillary glomerulonephritis (FGN) is a rare type of glomerulonephritis. A rare case of FGN is recently reported showing its association with iCCA. It presents as edema of lower limbs and face with uncontrolled hypertension. Nephrotic range proteinuria is evident (3 g/d) with 24 h-proteinuria of 0.74g/d mentioned by Normand in his study. Microscopic hematuria is also present. Complete remission of glomerulonephritis indicates the paraneoplastic nature of this disease[62].

### *Hematological manifestations*

**Paraneoplastic vasculitis:** Vasculitis is an inflammation of the wall of a blood vessel. Malignant diseases are both associated with vasculitis of arteries and veins. Paraneoplastic vasculitis constitutes less than 5% of all the forms of vasculitis[63]. Vasculitis is more commonly associated with hematological malignancies than solid tumors[64]. Small vessels are frequently linked to the paraneoplastic nature of vasculitis. The type of vasculitis associated with CCA is giant cell arteritis. It presents with one-month history of headache, scalp tenderness, pain, and stiffness in the neck, shoulder, and pelvic girdles. The resolution of symptoms right after removal of the tumor indicated the paraneoplastic nature of this vasculitis[65].

**Trousseau syndrome:** Trousseau syndrome, also known as migratory thrombophlebitis, is an acquired abnormality of blood clotting. According to several reports, it is concluded that several clotting disorders are closely linked to internal organ malignancy[66]. There have been 2 cases of trousseau syndrome with underlying isolated CCA, while others had either a hepatocellular CA or a lung adenocarcinoma along with CCA[67]. Trousseau syndrome, in the setting of CCA, presents as weight loss, mild shortness of breath, right upper quadrant tenderness, and abnormal liver

function tests with raised alkaline phosphatase as evident from the studies of Jang and Blum[68]. It is believed that tissue hypoxia leads to activation of the coagulation pathway and endothelial adhesion molecules[69]. Low molecular weight heparin (LMWH) along with removal of the primary tumor has been found effective in its treatment[70].

**Anti-phospholipid antibody syndrome:** Antiphospholipid antibody syndrome (APAS) is an autoimmune disorder that results in the formation of antibodies against phospholipids on platelets resulting in hypercoagulability. APAS has been linked to various solid organ malignancies but a few cases are found in association with CCA[71]. APAS, in the setting of CCA, presents as unilateral leg pain, swelling, and tenderness. Moreover, lupus anticoagulant is raised with normal antinuclear antibodies as mentioned by Samadian in his case report[72].

**Paraneoplastic leukemoid reaction:** Leukemoid reaction means an increase in the number of leukocytes (WBC's > 50000) particularly neutrophils, in reaction to any infection or carcinoma. Leukemoid reaction in the setting of CCA mimics a pyogenic liver abscess and clinically presents as pyrexia and leukocytosis[73]. The fever is intermittent and remains there for at least a month along with weight loss, progressive generalized weakness, and a leukocyte count above 20000 (> 78% neutrophils) mentioned by Ham in his study related to leukemoid reaction[74]. Only 2 cases of paraneoplastic leukemoid reaction have been reported with the primary cause of CCA[75]. Treating CCA resulted in the resolution of leukemoid reaction.

### *Multisystemic manifestations*

**Polyarteritis nodosa:** Polyarteritis nodosa (PAN) is a rare form of systemic necrotizing vasculitis that has heterogeneous forms of presentations[76]. PAN that occurs due to underlying neoplasia is termed as paraneoplastic vasculitis. It may precede or follow the onset of neoplasia or it may also be evident on the recurrence of many malignant diseases, showing a strong link with carcinomas[77]. PAN occurring in the setting of CCA is a rare finding but a study reports an association of CCA with PAN[78]. Paraneoplastic vasculitis is mostly cutaneous but it can also affect internal organs[79]. The earliest sign experienced in PAN is bilateral numbness in lower limbs followed by gradually increasing fever. After approximately 2 wk, an arthritis-like pathology is noticed with severe pain in bilateral lower limbs, ankles, metatarsal, and phalangeal joints. After a few months, skin manifestations appear which are necrosis and gangrene evident on distal phalanges. Lastly, gastrointestinal symptoms appear comprising of severe abdominal pain, nausea, and vomiting. The gradual sequence of these clinical symptoms has been mentioned by Hatzis in his case report. Digital ischemia is also a complication of paraneoplastic vasculitis[80]. Paraneoplastic vasculitis such as PAN is highly associated with raise in titers of anti-neutrophilic cytoplasmic antibodies (ANCA)[81]. Moreover, patients with raised ANCA and PAN also have raised CA 19-9, a tumor marker that rises in CCA and pancreatic carcinomas[82]. Recent studies suggest immune dysregulation as the primary cause of paraneoplastic vasculitis. Another study states that it might be due to cross-reaction of tumor antigens, directly causing vascular damage or indirectly by releasing humoral agents like chemotactic factors. PAN is poorly responsive to steroids or other treatment modalities but the removal of the tumor or chemotherapy results in its resolution[83].

**Adult-onset still disease:** Adult-onset Still disease (AOSD) is an adult version of juvenile idiopathic arthritis. It is caused by an altered immune response of the body against any foreign body or carcinoma. Previously, AOSD has been linked to various carcinomas like breast, esophageal, thyroid, and lung[84]. This is the first case, reporting link of AOSD with CCA. Symptoms of AOSD occur before malignancy is diagnosed[85]. AOSD linked to CCA clinically presents as high-grade fever and chills for 1 week along with other symptoms like sore throat, myalgia, pleuritic chest pain, cough, and pain in various joints as mentioned by the study of Raza[85].

### *Humoral manifestations*

**Humoral hypercalcemia of malignancy:** Parathyroid hormone-like hormone (PTHLH) is formed by proliferating bile duct epithelial cells in CCA that further interacts with various growth factors resulting in loops of uncontrolled proliferation[86]. PTHLH derived from CCA cells is involved in causing humoral hypercalcemia of malignancy (HHM). HHM is evident in approximately 10%-20% of the patients with underlying malignancies[87]. Almost 80%-90% of them are due to PTHLH. Clinical signs evident in a patient with HHM are changes in mental status, constipation, nausea, abdominal

discomfort, polydipsia, polyuria, and weakness. These are due to the increased concentration of calcium in the body[88]. There is good evidence that proves hypercalcemia as a paraneoplastic manifestation of CCA. Patients showing symptoms of hypercalcemia with normal PTH levels and an increased PTHLH in the setting of CCA supports that it is paraneoplastic. PTHLH works with tumor growth factor-alpha and tumor necrosis factor-alpha to cause hypercalcemia that is HHM.

A summary of all the paraneoplastic syndromes associated with other systems is explained in Table 2 at the bottom of the review.

### *Association with other neoplasms*

Recent diagnostic studies have indicated the presence of certain serological markers in CCA which are raised in other malignancies too suggesting that CCA might be having a concomitant neoplasm that shares the same paraneoplastic syndrome. For example, erythematous skin rash, associated with CCA, has raised CA 19-9. This serological marker is also linked to certain malignancies like colorectal and pancreatic carcinomas[53]. Another example is hypercalcemia. It is associated with CCA and also found to be raised in 10%-20% of malignancies like squamous cell carcinoma of the lung, head and neck, esophagus, and skin cancers[89]. Further detailed studies might reveal the hidden facts of this involvement.

### *Early detection of occult malignancy*

CCA has poor prognosis which has led to equalization of incidence and mortality rates[90]. Although CCA has a poor prognosis, it has been reported that early screening and diagnosis can lead to decreased mortality rates. All the manifestations explained above showed how treating CCA resulted in resolution of symptoms. Thus any patient with the above paraneoplastic manifestation and clinical symptoms should always be screened for cholangiocarcinoma as this could lead to a better chance of survival.

## CONCLUSION

CCA can cause a wide range of paraneoplastic syndromes. The exact mechanism linking them to CCA in most of these syndromes remains undetermined. Hence, no particular treatment modality can be recommended. The best management plan remains the removal of the underlying cause which in this case is CCA. The timely recognition of these syndromes and clinical suspicion can lead to early diagnosis of covert malignancies like CCA. The presence of symptoms can also predict the efficacy of treatment, relapse, or recurrence of the disease. Further studies are pertinent to understand the underlying mechanisms and targeted therapies for these paraneoplastic syndromes.

**Table 2 Summary of literature on paraneoplastic syndromes in cholangiocarcinoma associated with other systems**

| Ref. | Year | Paraneoplastic syndrome | Mediator |
|---|---|---|---|
| Neurological | | | |
| Schmidt *et al*[57] | 2016 | Limbic encephalopathy | ANNA-1, Anti-Ma2, Anti-PCA-2/anti-Tr, Anti-VGKC |
| Bruhnding *et al*[60] | 2016 | Paraneoplastic cerebellar degeneration | Anti-Yo antibodies |
| Renal | | | |
| Normand *et al*[62] | 2017 | Glomerulonephritis | |
| Hematological | | | |
| Solans-Laque *et al*[65] | 2008 | Vasculitis | |
| Blum *et al*[67] | 2016 | Trousseau syndrome | |
| Jang *et al*[68] | 2006 | Trousseau syndrome | |
| Samadian *et al*[72] | 1999 | Anti-phospholipid Antibody Syndrome | |
| Ham *et al*[74] | 2015 | Paraneoplastic leukemoid reaction | |
| Multisystem | | | |
| Hatzis *et al*[78] | 1998 | Polyarteritis nodosa | p-ANCA |
| Raza *et al*[85] | 2013 | Adult-onset still disease | |
| Humoral | | | |
| Erdinc *et al*[88] | 2019 | Humoral hypercalcemia of malignancy | PTHrP |

TGF-alpha: Tissue growth factor-alpha; G-CSF: Granulocyte-colony stimulating factor; IL: Interleukin; EGF-alpha: Epidermal growth factor-alpha; ANNA: Antineuronal nuclear antibody; PCA: Purkinje cell antibody; VGKC: Voltage-gated potassium channel; ANCA: Antineutrophil cytoplasmic antibody; PTHrP: Parathyroid hormone-related protein.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 908-918

DOI: 10.4254/wjh.v12.i11.908

ISSN 1948-5182 (online)

*MINIREVIEWS*

# Noninvasive scores for the prediction of esophageal varices and risk stratification in patients with cirrhosis

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**Author contributions:** Bangaru S drafted the manuscript; Benhammou JN provided critical revision of the manuscript; Tabibian JH provided supervision and critical revisions; all authors approved the submitted manuscript.

**Conflict-of-interest statement:** No potential conflicts of interest, no financial support.



**Manuscript source:** Invited manuscript

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## Abstract

The primary purpose of variceal screening in patients with cirrhosis is to detect gastroesophageal varices at high risk of hemorrhage and implement preventative intervention(s). It was previously recommended that all patients with cirrhosis undergo initial and periodic longitudinal variceal screening *via* upper endoscopy. However, there has been growing interest and methods to identify patients with cirrhosis who may not have clinically significant portal hypertension and therefore be unlikely to have varices requiring intervention or benefit from upper endoscopy. Because the population of patients with compensated advanced chronic liver disease continues to grow, it is neither beneficial nor cost-effective to perform endoscopic variceal screening in all patients. Therefore, there is ongoing research into the development of methods to non-invasively risk stratify patients with cirrhosis for the presence of high-risk esophageal varices and effectively limit the population that undergoes endoscopic variceal screening. This is particularly important and timely in light of increasing healthcare reform and barriers to healthcare. In this review, we discuss and compare, with respect to test characteristics and clinical applicability, the available methods used to non-invasively predict the presence of esophageal varices.

**Key Words:** Gastroesophageal varices; Variceal screening; Advanced chronic liver disease; Cirrhosis; Non-invasive screening; Upper endoscopy

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 24, 2020
**Peer-review started:** June 24, 2020
**First decision:** July 30, 2020
**Revised:** August 13, 2020
**Accepted:** September 10, 2020
**Article in press:** September 10, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Gencdal G
**S-Editor:** Zhang L
**L-Editor:** Webster JR
**P-Editor:** Ma YJ



**Core Tip:** Because the population of patients with compensated advanced chronic liver disease continues to grow, it is neither beneficial nor cost-effective to perform endoscopic variceal screening in all patients. Therefore, there is ongoing research into the development of methods to non-invasively risk stratify patients with cirrhosis for the presence of high risk esophageal varices and effectively limit the population that undergoes endoscopic variceal screening. These topics are reviewed in this article.

**Citation:** Bangaru S, Benhammou JN, Tabibian JH. Noninvasive scores for the prediction of esophageal varices and risk stratification in patients with cirrhosis. *World J Hepatol* 2020; 12(11): 908-918
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/908.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.908

## INTRODUCTION

Variceal screening and surveillance is an important part of the management of patients with cirrhosis. The primary goal of upper endoscopy (EGD) in this context is to identify patients with gastroesophageal varices (GEV) at high risk of hemorrhage so that strategies to minimize this risk, including potential endoscopic treatments, can be implemented[1]. The previous American Association for the Study of Liver Diseases (AASLD) guidelines on the management of GEV and the Baveno consensus conference in its first five editions recommended variceal screening and periodic surveillance with EGD in all patients with cirrhosis. However, the introduction of transient elastography (TE) in clinical practice has allowed the identification of patients with early chronic liver disease manifested by advanced fibrosis, an entity that was subsequently termed compensated advanced chronic liver disease (cACLD)[2]. This population comprises a heterogeneous group of patients with varying degrees of portal hypertension (PH), ranging from no PH (hepatic venous portal gradient (HVPG) of 1-5 mm Hg) to mild or "subclinical" PH (HVPG of 5-9 mmHg) to clinically significant portal hypertension (CSPH) (defined as an HVPG of ≥ 10 mmHg)[2-4]. Above this threshold of 10 mmHg, all complications of PH, including the development of GEV and variceal hemorrhage, are more likely to occur[4-6]. Reflecting this, the prevalence of GEV ranges from 20%-40% in patients with cACLD to as high as 85% in patients with decompensated cirrhosis (who have CSPH)[3]. GEV also have a variable risk of hemorrhage: The overall rate of variceal hemorrhage is around 10%-15% per year, but this varies with both the severity of liver disease (Child class B or C) and with endoscopic features of the varices including size and the presence of high risk stigmata[3,7]. Furthermore, there are small but notable risks associated with EGD, and the costs incurred on both the patient and the healthcare system in the context of a growing chronic liver disease population is substantial[7,8].

In light of this heterogeneity, the most recent AASLD guidance statement and the 2015 Baveno VI consensus statement recommend the use of non-invasive tests to stratify patients and rule out high risk esophageal varices (HREV) in patients with cACLD[2]. The AASLD practice guidance states that patients with a liver stiffness of < 20 kPa as measured by TE and a platelet count (PC) of > 150000/mm$^3$ can avoid EGD but that those who do not meet these criteria, known as the Baveno VI criteria, should receive a screening EGD[3]. There are ongoing efforts to develop alternative non-invasive models using clinical, biochemical, and radiographic parameters to stratify patients for variceal screening[9]. The goal is to balance good test characteristics (< 5% of patients with HREV are missed) with ease of administration and widespread availability of testing in clinical practice[2]. This review will discuss the non-invasive methods for esophageal variceal (EV) prediction in patients with cACLD.

## PLATELET COUNT TO SPLEEN DIAMETER RATIO

Because low PC and enlarged spleen size are independently suggestive of PH, their

combination into the PC to spleen diameter ratio (PC/SD) was evaluated for the prediction of EV. In the initial proof-of-concept retrospective study of 137 adult patients with confirmed EV by EGD, a PC/SD cutoff value of 909 (n/mm$^3$)/mm offered a net present value (NPV) of 73% and a positive predictive value (PPV) of 74%[10]. A 2012 systematic review and meta-analysis of PC/SD including 1275 adult patients with cirrhosis yielded a pooled sensitivity of 89% [95% confidence interval (CI): 87%-92%] and pooled specificity of 74% (95%CI: 70%-78%), but the pooled positive and negative likelihood ratios were only moderately helpful[11]. The largest study was a 2017 Cochrane meta-analysis including 2637 patients across 17 studies evaluating the PC/SD at a cut-off of 909 (n/mm$^3$)/mm demonstrated an even better sensitivity of 0.93 (95%CI: 0.83-0.97) and specificity of 0.84 (95%CI: 0.75-0.91) for the detection of varices of any size. However, it was noted that 7% of adults with any EV would be missed[12]. They therefore further evaluated the ability of the PC/SD to predict the presence of HREV [also known as varices needing treatment (VNT)], which refers to medium or large varices, varices with high risk stigmata, or small varices in Child C cirrhosis. Interestingly, the PC/SD performed worse in the prediction of HREV at a cut-off value around 909 (n/mm$^3$)/mm (between 897 and 921), with a sensitivity of 0.85 (95%CI: 0.72-0.93) and specificity of 0.66 (95%CI: 0.52-0.77).

While the PC/SD is advantageous in that it is easy to calculate and relies on only two data points, its test characteristics are not adequate for the prediction of EV or HREV. The authors considered that it could potentially be incorporated into a more comprehensive prediction rule[11]; however, an additional challenge with widespread use is that spleen diameter is not consistently included in ultrasound reports.

## TRANSIENT ELASTOGRAPHY

Liver stiffness (LS) as measured by transient elastography (TE) performs well in the diagnosis of cirrhosis with an area under the receiver operating characteristic (AUROC) of 0.96, and at a cut-off of 17.6 kPa, the NPV and PPV for the diagnosis of cirrhosis are 92% and 91%, respectively[13]. A meta-analysis of 11 studies evaluating LS and HVPG demonstrated a significant correlation ($r$ = 0.783, 95%CI: 0.737-0.823) and that LS also had good diagnostic performance for the assessment of CSPH, with a sensitivity of 87.5% and specificity of 85.3%[14]. A 2013 meta-analysis including 5 studies and 420 patients demonstrated that LS by TE is an accurate means of diagnosing CSPH, with an AUROC of 0.93 (95%CI: 0.90-0.95), sensitivity of 0.90 (95%CI: 0.81-0.95), and specificity of 0.79 (95%CI: 0.58-0.91)[15].

Several studies have subsequently been conducted to evaluate the accuracy of TE in the diagnosis of EV with variable findings. In a prospective study including patients with cirrhosis of multiple etiologies, a cut-off value of 27.5 kPa provided a NPV of 95% in diagnosing HREV[13]. However, subsequent meta-analyses demonstrated that LS alone is not sufficiently accurate to diagnose either EV or HREV. Based on these studies, the AUROC for TE in the diagnosis of HREV ranged from 0.78 to 0.83[15,16], and the AUROC for TE in the diagnosis of EV ranged from 0.82 (95%CI: 0.79-0.86) to 0.84 (95%CI: 0.80–0.87)[17].

It is important to note that these studies included patients with multiple and varied etiologies of chronic liver disease which contributed substantial heterogeneity[15,16] although the majority of patients across these studies had untreated viral or alcoholic cirrhosis[15]. In addition, the TE-LS cutoffs evaluated varied significantly across studies, ranging from 12.0 to 29.7 kPa for the detection of any EV and from 14.6 to 38.2 for the detection of HREV[16,17]. The optimal cutoffs for TE-LS used to stage fibrosis and diagnosis cirrhosis vary with etiology of liver disease and may be disease-specific. Therefore, this may be the case for TE in the diagnosis of EV and HREV and perhaps establishing disease-specific cut-offs would improve test characteristics. However, the sensitivity of TE in the diagnosis of EV or HREV is good but the specificity is only moderate. Therefore, it was concluded that although TE has a role in the assessment of PH, it should not be used alone in selecting patients for variceal screening[18].

## COMBINATION OF LIVER STIFFNESS, SPLEEN DIAMETER, AND PLATELET COUNT

The role of LS in combination with other parameters has been evaluated. LS, SD and PC have been evaluated in various combinations for the prediction of EV. One such

score is called the liver stiffness – spleen diameter to platelet ratio (LSPS) and is calculated as follows: LS × SD/PC. LSPS is accurate in the diagnosis of CSPH with an AUROC of 0.918 (95%CI: 0.872-0.965, $P < 0.0001$)[19]. In a prospective study of patients with cirrhosis due to hepatitis B, it was found that LSPS < 3.5 has a 94.0% NPV for the prediction of HREV while LSPS > 5.5 has a PPV of 94.2. LSPS had excellent accuracy with AUROC of 0.953 and performed better in the prediction of HREV than any of the components individually and PC/SD[20]. However, a second study including patients with diverse etiologies of cirrhosis showed that LSPS < 3.21 offered a better NPV in the prediction of EV, again demonstrating heterogeneity in optimal cutoffs[19]. Furthermore, these studies suggested better performance of LSPS in Child A than Child B + C cirrhosis[20].

Another study developed an EV prediction score using multivariable analysis of the individual parameters of the LSPS which is calculated accordingly: - 4.364 + 0.538 (spleen diameter) – 0.049 (PC) – 0.044 (LS) + 0.001 (LS × PC). This score had an AUROC of 0.909 (95%CI: 0.841-0.954, $P < 0.0001$) and it performed similarly when evaluated by etiology of liver disease[19]. A third score, calculated simply by PC/$\log_{10}$ LS, was evaluated in a prospective study of 107 patients. It was found that values ≤ 122,000/μL × kPa predicted high-risk varices with 100% sensitivity and 100% NPV, which would prevent 20.6% of patients from receiving unnecessary screening endoscopy ($p = 0.003$)[21].

These studies together demonstrate that combinations of LS, SD, and PC can perform well in the diagnosis of CSPH and EV/HREV. However, despite their excellent test characteristics, these scores have not gained momentum, and one important reason for this is that calculating a score is cumbersome when applied to busy clinical practice because it requires an additional step. The Baveno VI consensus acknowledged this in favoring a method that combines data points sequentially rather than *via* a calculation.

## LIVER STIFFNESS AND PLATELET COUNT: THE BAVENO VI CRITERIA

The combination of LS and PC has demonstrated high performance in the prediction of CSPH and EV, and the use of sequential clinical parameters is quick and simple to apply in clinical practice. A 2014 prospective, proof-of-concept study of 49 patients with TE-LS ≥ 13.6 kPa and EGD noted that 90% of patients with EV had a PC < 150000/mm$^3$ and an abnormal ultrasound suggesting a simple sequential strategy could be used to avoid EGD in low-risk patients[22]. A subsequent 2015 retrospective study of 271 patients (71 training, 200 validation) with Child Pugh A cirrhosis and LS > 13.6 kPa found that the optimal threshold for excluding HREV was the combination of LS ≤ 25 kPa and PC ≥ 100000/mm$^3$. This combined model had a NPV of 100% for the prediction of HREV in both the training and validation cohorts[23]. Of note, the majority of patients had hepatitis C cirrhosis and in addition, the frequency of GEV was low (10% overall) which is good in that it reflects real-life practice in compensated cirrhosis but worth noting because it does affect the model development and test characteristics[23].

Based on these findings that HREV could be excluded with a very low miss rate[22,23], the 2015 Baveno VI consensus conference recommended that surveillance endoscopy is not necessary for patients with compensated cirrhosis who have normal platelets > 150000/mm$^3$ and LS < 20 kPa[2]. Many studies including high-volume single center retrospective studies and meta-analyses have validated the Baveno VI recommendation in patients with different etiologies of cACLD (including hepatitis B, hepatitis C, alcohol, and non-alcoholic steatohepatitis) and variable prevalence of EV, ranging from 23% to 65%[21,24-32]. Across all of these studies, the overall missed HREV rate has been 2% or less, in keeping with the proposed < 5% threshold defined by Baveno VI. In these studies, 20% of EGDs could have been saved by applying the criteria. As is most frequently the case, the most common etiologies across these multiple studies were viral and alcohol-related cirrhosis. However, a 2018 large multi-center cross-sectional study of 790 patients with cirrhosis due to nonalcoholic fatty liver disease (NAFLD) demonstrated a HREV miss rate of 0.9% using the Baveno VI criteria[33]. A subsequent 2019 retrospective cross-sectional study evaluated Baveno VI in 227 patients with cACLD due to cholestatic liver diseases including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), which are mechanistically distinct in that they may have a pre-sinusoidal component of PH. Baveno VI had a 0% false negative rate in the prediction of HREV in PBC and PSC[34]. The robustness of the Baveno VI criteria in ruling out HREV led to its adoption in the AASLD practice

guidance statement[3].

## EXPANDING ON THE BAVENO VI CRITERIA

Noting that the total number of EGDs avoided using the Baveno VI criteria is low relative to the prevalence of HREV, several studies have attempted to expand the Baveno VI and improve its discriminatory accuracy by adjusting the LS and PC cutoff values. Based on 2 large-scale retrospective studies, a PC > 110000/mm$^3$ and LS < 25 kPa was shown to potentially spare up to 40% of EGDs where the Baveno VI criteria would spare only 20% at an acceptable missed VNT rate of 1.6% (95%CI: 0.7% -3.5%)[32,35]. This came to be known as the "Expanded Baveno VI criteria" and was initially shown to maintain a similar missed VNT rate of < 5% across different subgroups including hepatitis C, alcohol, non-alcoholic steatohepatitis, and PSC/PBC[32,34,35]. However, a large-scale retrospective study in an Asian population showed that while the Expanded Baveno VI criteria spared more EGDs compared to the Baveno VI criteria (51.7% *vs* 27.6%), it missed an unacceptable number of HREV in comparison (6.8% *vs* 3.8%)[30].

Subsequently, a large meta-analysis including 30 studies and 8469 patients reproduced a similar finding, that although the Expanded Baveno VI criteria could reduce the proportion of unnecessary EGDs, it would do so at a higher rate of missed HREVs[31]. Thus, the Expanded Baveno VI criteria are not recommended. LS < 25 kPa with a PC > 125000/mm$^3$ was evaluated as an alternate expansion of the Baveno VI criteria and was shown to spare an additional 15% of endoscopies above the Baveno VI criteria with an acceptable missed HREV rate in a large retrospective study of 442 patients[32] but this was not subsequently validated. This same study looked at PC > 150000/mm$^3$ and model for end stage liver disease 6 as a method of ruling out HREV but misclassified 10% of patients[32].

Some studies have examined disease-specific cut-offs. A large-scale NAFLD patient cohort was also used to identify a NAFLD-specific LS and PC cutoff to be applied in a similar fashion and found that the best thresholds to rule out HREV were PC > 110000/mm$^3$ and either LS < 30 kPa with the medium-sized probe or LS < 25 kPa using the extra-large probe[33]. They demonstrated that applying these criteria in the NAFLD population would reduce the number of screening EGDs by almost half with an acceptable HREV miss rate of < 5%[33]. However, this has not subsequently been validated and an additional challenge is that LS measurements are less accurate in obese patients, in fact, TE is not technically feasible in approximately 20% of patients[36]. One retrospective study of hepatitis B-related compensated cirrhosis showed that after removing patients meeting Baveno VI criteria, the remaining patients could be further selected for absence of HREV using LS, PC, or the Lok index cutoff [- 5.56 – 0.0089 × PC ($10^3$/mm$^3$) + 1.26 × (Aspartate Transaminase/Alanine Aminotransferase) + 5.27 × International Normalized Ratio Lok] = [exp (logodds)]/[1 + exp (logodds)][27] stratified by alanine aminotransferase and total bilirubin[29]. This study is specific to hepatitis B and does not put forth a single recommendation but rather suggests that Baveno VI can be optimized further.

## SPLEEN STIFFNESS MEASUREMENT

Portal hypertension leads to splenic congestion which leads to architectural changes in the splenic arteries and veins, resulting in fibrosis of the spleen and therefore, a rise in spleen stiffness. Methods for measuring spleen stiffness include shear wave elastography, TE, and acoustic radiation force impulse imaging. Of these methods, acoustic radiation force impulse imaging has been studied most frequently because this method is not limited by the presence of ascites or obesity[37]. Spleen stiffness measurement (SSM) appears to perform well in the prediction of CSPH: In a prospective study of 78 patients, SSM was able to diagnose HVPG ≥ 10 mmHg and HVPG ≥ 12 mmHg with AUROCs of 0.97 and 0.95, respectively[37]. Some studies have indicated that SSM is superior to LS in diagnosing CSPH[38-40]; however, other studies provide contrary views[41-43]. According to present literature, it is difficult to determine which metric is superior.

Several studies have explored SSM in the prediction of EV[40,44-47]. A prospective study of 135 patients demonstrated that patients with any EV had higher SSM than those with no EV (3.37 m/s *vs* 2.79 m/s, $P$ < 0.001); and patients with HREV had an even greater difference in SSM (3.96 m/s *vs* 2.93 m/s, $P$ < 0.001)[44]. In addition, at a

cutoff value of < 3.20 m/s, NPV for excluding HREV was 99%[44]. SSM was therefore evaluated in 2 prospective studies and demonstrated good diagnostic accuracy for prediction of any EV, with AUROC of 0.872 to 0.933 at a cutoff of 2.89-3.18 m/s, and good diagnostic accuracy for the prediction of HREV, with AUROC of 0.930–0.969 at cutoffs of 3.30 m/s[45,47]. One study demonstrated that the combination of SSM by TE at a cutoff of ≤ 46 kPa and Baveno VI criteria would have safely spared (0 HREV missed) 37.4% of EGDs compared with only 16.5% when using the Baveno VI criteria alone[24]. In these studies, SSM has demonstrated good performance across different subgroups including viral, non-viral, and Child B cirrhosis, but these subgroups all used different SSM cutoffs which complicates translation to clinical practice[45,47]. In subsequent meta-analyses, heterogeneity in the technique of obtaining SSM and in cutoffs used was a problem and as a result, diagnostic accuracy was not as high[43,46]. Furthermore, SSM is not widely available at this time and therefore this cannot be recommended on a large scale.

## VIDEO CAPSULE ENDOSCOPY

Video capsule endoscopy (VCE) has been evaluated for the diagnosis of HREV. However, a Cochrane systematic review of 6 studies could not substantiate VCE as a non-invasive method of assessing for EV. The pooled sensitivity was 73.7% (95%CI: 52.4%-87.7%) and the pooled specificity was 90.5% (95%CI: 84.1%-94.4%)[48]. It was concluded that the sensitivity of VCE is not sufficient to replace EGD as a method of variceal screening in these patients. Given its higher specificity, it was recommended that it could be considered in patients who refuse or have a contraindication to EGD[49]. However, this is not likely cost-saving, not widely available, and is still a procedure requiring endoscopy staff and specialized equipment and with a certain level of procedural risk (*e.g.* capsule retention)[48,49].

## EVENDO SCORE

Despite the excellent performance characteristics of LS and PC, TE is far from widely available and therefore there is interest in developing prediction scores independent of LS. With this in mind, the EVendo score was recently developed and validated in a multi-center study of 238 patients with cirrhosis. The score was developed using a machine learning algorithm to identify factors significantly associated with the presence of EVs and HREVs. The investigators then developed the EVendo score, which is calculated as follows: [(9.5 × international normalized ratio + aspartate transaminase/35)/(platelets/150 + blood urea nitrogen/20 + hemoglobin 15)] + 1 point for ascites. This score identified patients with EVs in the training set with an AUROC of 0.84 and was then validated in an independent prospective cohort with good performance (AUROC of 0.82 for EV in all patients, AUROC of 0.81 in subgroup of patients with Child-Pugh A cirrhosis). The score identified patients with HREV in the training set with an AUROC of 0.74, in the validation set with an AUROC of 0.75, and in patients with Child-Pugh A cirrhosis with an AUROC of 0.75. An EVendo score below 3.90 would have spared 30.5% patients from EGDs, missing only 2.8% of VNT and 40.0% patients with Child-Pugh A cirrhosis from EGDs, missing only 1.1% of VNT[50].

The EVendo score is advantageous in that it relies on routinely collected laboratory values, has robust performance characteristics across a broad array of liver disease etiologies, and can be readily calculated using a published on-line calculator (https://www.mdcalc.com/evendo-score-esophageal-varices). As such, it is convenient for clinical use to risk stratify and triage patients with cirrhosis who are being considered for EV screening (Figure 1). However, further validation in larger cohorts will be useful to better define its clinical utility and suitability for broader use (Tables 1 and 2).

## CONCLUSION

In summary, the use of non-invasive testing to stratify cACLD patients for screening endoscopy and individualize care for PH shows promise and will continue to become more important as the cACLD population grows. However, several important caveats

**Table 1 Test characteristics for noninvasive detection of esophageal varices**

| Non-invasive test | Sensitivity | Specificity | PPV | NPV | LR (+) | LR (-) | AUROC |
|---|---|---|---|---|---|---|---|
| PC/SD | 89%-93% | 74%-84% | 73% | 74% | 3.5 | 0.12 | |
| TE | 84% | 62%-68% | | | 2.3-2.58 | 0.24-0.26 | 0.82-0.84 |
| LSPS | | | 94% (LSPS > 5.5) | 94% (LSPS < 3.5) | | | 0.882-0.953 |
| EV prediction score | | | | | | | 0.909 |
| SSM | 78%-94% | 76%-78% | | 99% | 3.4 | 0.2 | 0.872-0.933 |
| EVendo | 92.3% | 65.9% | | | | | 0.82 |
| Capsule endoscopy | 73.7%-83% | 84%- 90.5% | | | | | 0.90 |

Other noninvasive scores exist and may be used, as shown in Table 1; EVendo score selected based on it having the highest sensitivity, negative predictive value, and endoscopies saved, though it has not yet been validated outside of the United States. EV: Esophageal varices; PPV: Positive Predictive Value; NPV: Net present value; AUROC: Area under the receiver operating characteristic; PC/SD: Platelet count to spleen diameter ratio; TE: Transient elastography; LSPS: Liver stiffness–spleen diameter to platelet ratio; SSM: Spleen stiffness measurement.

**Table 2 Test characteristics for noninvasive detection of high risk esophageal varices**

| Non-invasive test | Sensitivity | Specificity | PPV | NPV | LR (+) | LR (-) | AUROC | HREV missed | EGDs saved |
|---|---|---|---|---|---|---|---|---|---|
| PC/SD | 85% | 66% | | | 3.03 | 0.30 | 0.83 | 7% | |
| TE | 78%-82% | 76%-77% | | | | | 0.78-0.83 | | |
| PLT/$\log_{10}$LS | 100% (< 122000); 86% (< 92000) | | | 100% (< 122k); 94% (< 92k) | | | | 0 | 20.6%; 6.3% |
| Baveno VI | 87%-97% | 32%-41% | 6% | 98%-100% | 1.31 | 0.39 | 0.746-0.96 | < 2% | 20%-27% |
| Expanded Baveno VI | 90% | 51% | | 92%-96% | | | | 6.8% | 51% |
| SSM | 81%-98% | 52%-66% | | 99.4% | 2.5 | 0.2 | 0.807 | 2% | 35.8% |
| EVendo | 100% | 49.3% | | 100% | | | 0.75 | 2.8% | 30.5% |
| Capsule endoscopy | 72%-73.7% | 90.5%-91% | | | | | 0.92 | | |

Other noninvasive scores exist and may be used, as shown in Table 2; EVendo score selected based on it having the highest sensitivity, negative predictive value, and endoscopies saved, though it has not yet been validated outside of the United States. HREV: High risk esophageal varices; PPV: Positive Predictive Value; NPV: Net present value; AUROC: Area under the receiver operating characteristic; EGD: Endoscopy; PC/SD: Platelet count to spleen diameter ratio; TE: Transient elastography; PLT: Platelets; LS: Liver stiffness; SSM: Spleen stiffness measurement.

need to be kept in mind.

Non-invasive prediction of EV cannot be applied to patients with decompensated cirrhosis given the paucity of applicable data and the much higher pre-test probability of HREV. Although the AASLD guidance statement recommends that patients meeting Baveno VI criteria can safely avoid screening EGD, there is still uncertainty regarding follow-up of patients who have been ruled out for HREV. It has been suggested that these patients can be followed with annual TE and PC and undergo screening when they no longer meet the Baveno VI criteria. However, long-term follow-up studies are needed to determine whether this strategy is sufficiently accurate to identify the development of HREV in someone who was previously at low risk. There is a lack of randomized controlled trial data to inform the selection of higher-risk patients by non-invasive methods for variceal screening EGD; while prospective data exist in this regard, *e.g.* with the EVendo score[50], further clinical validation is encouraged. Finally, despite the high performance of TE, there is considerable interest in developing scores that do not require TE given that it is not widely available; moreover, its measurement can be affected by several factors including obesity, ascites, and alcohol use, which may limit its application in advanced

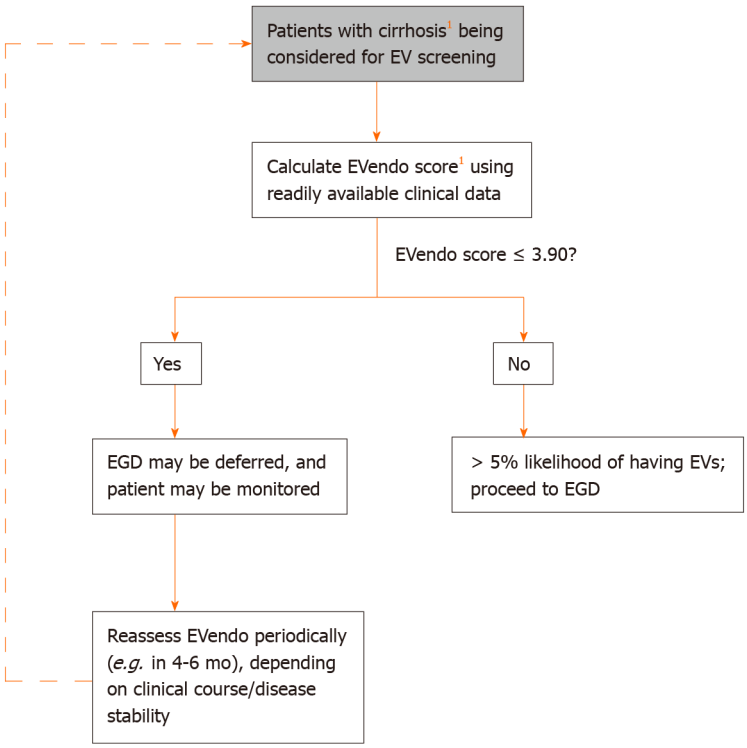


**Figure 1 Proposed algorithm for noninvasive esophageal variceal assessment to risk stratify patients using the EVendo score[1].** Patients with known (biopsy-proven) or suspected cirrhosis. Excluded from the original study were patients who: (1) Had a prior upper endoscopy (EGD) for esophageal variceal screening, surveillance, or treatment; (2) Had a prior EGD that incidentally revealed esophageal varices; (3) Had noncirrhotic etiologies for portal hypertension; (4) Were on dialysis; or (5) Were on anticoagulants that would affect international normalized ratio. Online calculator and additional guidelines available here: https://www.mdcalc.com/evendo-score-esophageal-varices. [1]Other noninvasive scores exist and may be used, as shown in Tables 1 and 2; EVendo score selected and shown here based on it having the highest sensitivity, negative predictive value, and EGDs saved, though it has not yet been validated outside of the United States. EGD: Endoscopy; EV: Esophageal varices.

liver diseases[36], and it is highly operator dependent, requiring completion of 100 examinations for sufficient experience[51]. Lastly, there is uncertainty regarding when surveillance can be stopped if there is improvement in fibrosis and PH with the removal of the source of ongoing liver injury (*i.e.*, post-SVR, after abstinence from alcohol, after weight loss and metabolic improvements). Future research should be directed at these efforts and areas of uncertainty.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 919-930

DOI: 10.4254/wjh.v12.i11.919

ISSN 1948-5182 (online)

*MINIREVIEWS*

# Natremia and liver transplantation: The right amount of salt for a good recipe

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**Author contributions:** Lenci I collected, analyzed and interpreted the data, and drafted the manuscript and revised it critically; Milana M, Grassi G, Signorello A, and Aglitti A collected the data and revised the manuscript critically; Baiocchi L made the study proposal, conceived of the study, corrected the manuscript and revised it critically.

**Conflict-of-interest statement:** There are no conflicts of interest to disclose for any of the authors.



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## Abstract

An adequate balance between electrolytes and clear water is of paramount importance to maintaining physiologic homeostasis. Natremia imbalance and, in particular, hyponatremia is the most frequent electrolyte abnormality observed in hospitalized subjects, involving approximately one-fourth of them. Pathological changes occurring during liver cirrhosis predispose patients to an increased risk of sodium imbalance, and hypervolemic hyponatremia has been reported in nearly 50% of subjects with severe liver disease and ascites. Splanchnic vasodilatation, portal-systemic collaterals' opening and increased excretion of vasoactive modulators are all factors impairing clear water handling during liver cirrhosis. Of concern, sodium imbalance has been consistently reported to be associated with increased risk of complications and reduced survival in liver disease patients. In the last decades clinical interest in sodium levels has been also extended in the field of liver transplantation. Evidence that [$Na^+$] in blood is an independent risk factor for in-list mortality led to the incorporation of sodium value in prognostic scores employed for transplant priority, such as model for end-stage liver disease-Na and UKELD. On the other hand, severe hyponatremic cirrhotic patients are frequently delisted by transplant centers due to the elevated risk of mortality after grafting. In this review, we describe in detail the relationship between sodium imbalance and liver cirrhosis, focusing on its impact on peritransplant phases. The possible therapeutic approaches, in order to improve transplant outcome, are also discussed.

**Key Words:** Sodium imbalance; Liver transplant; Cirrhosis; Vaptan; Transplant list; Hypervolemic hyponatremia

**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Italy

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** August 6, 2020
**Peer-review started:** August 6, 2020
**First decision:** September 17, 2020
**Revised:** September 19, 2020
**Accepted:** September 27, 2020
**Article in press:** September 27, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Dabbous H, Singh SA
**S-Editor:** Zhang L
**L-Editor:** Filipodia
**P-Editor:** Wang LL

**Core Tip:** Sodium imbalance represents an important issue in cirrhotic patients. In the last decades, the impact of altered sodium levels in the peritransplant phases has also gained a relevant clinical interest. In this review, we examined: (1) The determinants of an impaired sodium balance in the course of severe liver diseases; (2) The consequences of sodium imbalance on liver transplant; and (3) The possible corrective measures for this condition.

**Citation:** Lenci I, Milana M, Grassi G, Signorello A, Aglitti A, Baiocchi L. Natremia and liver transplantation: The right amount of salt for a good recipe. *World J Hepatol* 2020; 12(11): 919-930
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/919.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.919

## INTRODUCTION

We are all well aware that the human body is composed of a high percentage of water (approximately 60% by weight), with significant changes among different tissues and according to sex and age[1-3]. However, when we examine the human physio-pathological mechanisms, it would be better to keep in mind that this large fluid mass is actually composed of "salt" water[4]. The accurate proportion/relationship between electrolytes and water is of paramount importance to preserve cellular and tissue homeostasis. Moreover, specific transporters, such as the sodium pump or aquaporin channels, exist to keep definite gradients of electrolytes and water between the inside and outside of the cell[5]. Failure to maintain adequate solute concentration (osmolality) between the intra- and extracellular compartments may affect tonicity, determining cellular damage for shrinkage or swelling. The accumulation in the cell of sodium ($Na^+$), the most concentrated cation in extracellular fluid, is prevented by the sodium pump. For this reason, $Na^+$ is not only the main determinant of plasma osmolality but it also plays an important role in maintaining isotonicity between the intra- and extracellular environment[6].

Central regulation of the sodium-water balance and tonicity are obtained by brain/kidney crosstalk[7,8]. In brief, during physiologic conditions, neurohypophysis detects hypertonic signals recorded by osmoreceptors and activates vasopressin [*i.e.* antidiuretic hormone; antidiuretic hormone (ADH)] release by the pituitary gland. ADH, in turn, stimulates kidney reabsorption of water, thus reducing hyperosmolarity. Thirst is also stimulated by osmoreceptors as long as homeostasis is not reacquired. In hypotonic conditions, the mechanism is the reverse, with decreased ADH secretion and inhibition of thirst. Despite this fine-tuned regulation of osmolality, altered sodium serum levels are frequently encountered in clinical practice; in this case, our attention is usually recalled by the $Na^+$ reported value; however, changes in plasmatic sodium are more frequently the expression of an impaired water balance rather than electrolyte increase or loss.

In this review, we examined natremia changes in pathological conditions focusing on the aspects observed during liver cirrhosis. The consequences of altered sodium levels on the outcome of patients undergoing liver transplantation (LT) and the possible corrective measures will also be discussed.

## NATREMIA IMBALANCE IN GENERAL PRACTICE

While natremia imbalance should be considered a symptom rather than a disease, evidence in clinical practice of a plasma sodium concentration lower than 135 or higher than 145 mEq/L, respectively, defines hypo or hypernatremia. Hyponatremia is by far the more frequently encountered electrolyte abnormality in hospitalized patients[9-11]. In a large retrospective study, prevalence of $Na^+ < 135$ mEq/L was 22.1% and 14.7% in hospitalized and ambulatory patients, respectively[12]. Despite the finding of hyponatremia, a hypo, hyper or isotonic plasma/cell condition should not be ruled out since other solutes not able to cross plasma membrane (such as glucose) may impact tonicity[13,14]. However, hypotonic or dilutional hyponatremia caused by excess

water intake or impaired kidney water disposal is the most frequent form[14].

Hyponatremia should also be discriminated in the clinic according to the volume of extracellular fluid in hypo, normo, or hypervolemic forms[15]. In chronic diseases such as cardiac insufficiency, nephrotic syndrome, or liver cirrhosis, a hypervolemic hyponatremia is usually present, characterized by edema. Concern exists in hospitalized patients because of the strict relationship between morbidity/mortality and hyponatremia[16]. Moreover, an increased risk of mortality, in sodium-deficient hospitalized patients, seems to be present also for mild reduction of blood [$Na^+$] (130-134 mEq/L) and persists also in the 5-year follow-up after discharge[17,18].

With regard to mortality, the most dramatic complications of acute hyponatremia (also when a too rapid correction of sodium depletion occurs) are considered the neurologic ones[19,20]. Symptoms of onset are represented by lethargy, vomiting, headache and confusion, among others, reflecting impairment of the central nervous system as a consequence of a rapid change of extracellular tonicity exiting in brain edema and possible demyelination[14]. This condition is characterized by a relevant morbidity in more than one-third of subjects[21]. However, (1) since this important complication requires rapid modification of plasma sodium concentration and (2) considering that an adaptive response to osmotic changes exists in the brain (constituted mainly by the shift of fluid in the subarachnoid space[22] and loss of solutes by brain cells[23]), increased overall mortality observed in patients with hyponatremia does not seem completely explained by neurological involvement. In this perspective, besides patients in which sodium depletion is the main cause of death, there are others in whom hyponatremia is only the innocent expression of a chronic severe disease, thus not eliciting a direct impact (or with just a partial impact) on mortality[24].

Hypernatremia, recognized by a plasma [$Na^+$] > 145 mEq/L (as also stated before), is a condition with a lower prevalence in comparison with hyponatremia, but it is associated, like the latter, with significant mortality. While hyponatremia may be present in different conditions of extracellular fluid tonicity, the hypernatremia is constantly characterized by a hypertonic plasma[14]. In the pathogenesis of the increased plasma sodium concentration, an imbalance between clear water and $Na^+$ loss/intake occurs, with concomitant failure of the compensatory systems, including the brain-kidney axis and the thirst triggering mechanism[25]. Given the important defense operated by thirst stimulation against hypernatremia, increased plasma sodium levels usually occur when the sensitivity to central stimuli is absent or greatly reduced. Therefore, in clinical practice, patients with reduced consciousness, such as those in an intensive care unit are at major risk for hypernatremia[26-28]. In outpatient settings increased sodium levels are rarely observed and the main causes are represented by diarrhea (in elderly and small children), excessive use of diuretic drugs, diabetes insipidus, and others[25,26].

From all the above, it is clear that complex diagnostic flow charts are sometimes needed to achieve a correct diagnosis in patients with sodium imbalance. In other clinical situations, such as in liver cirrhosis, changes in sodium blood levels are instead characteristic of the disease.

## NATREMIA IMBALANCE IN LIVER CIRRHOSIS: IT ALL STARTS HERE

Impaired water handling has long been demonstrated in liver cirrhosis[29]; this is well documented by the frequent finding of ascites and edema in patients with relevant liver impairment[30]. Ascites and dilutional hyponatremia are, in liver cirrhosis, the ending evolution of the same pathological sequence of events. In fact, as an important step, a hyperdynamic circulation with increased cardiac output and decreased blood pressure for reduced peripheral vascular resistance develops[31]. Fall of peripheral resistance, which is not justified by increased requirement of oxygen by tissues, is clearly evident also by the subject examination usually exhibiting warm extremities, palmar erythema, spider naevi, and other typical signs. Rearrangement of the systemic circulation is thought to occur as a response to portal hypertension, stimulating splanchnic vasodilatation and portal-systemic collaterals' opening[32]. Vasodilation in this setting seems to be maintained by the excretion of several modulators[33]. Among these, nitric oxide has long been identified as of major importance[32,34-36].

Finally, arterial underfilling activates baroreceptors stimulating chronic ADH release. Increased reabsorption of clear water is then the determinant of fluid accumulation and hypervolemic hyponatremia in decompensated cirrhosis[37]. A simplified scheme of events leading to dilutional hyponatremia during cirrhosis is reported in Figure 1. Considering (1) the constant occurrence of altered clear water

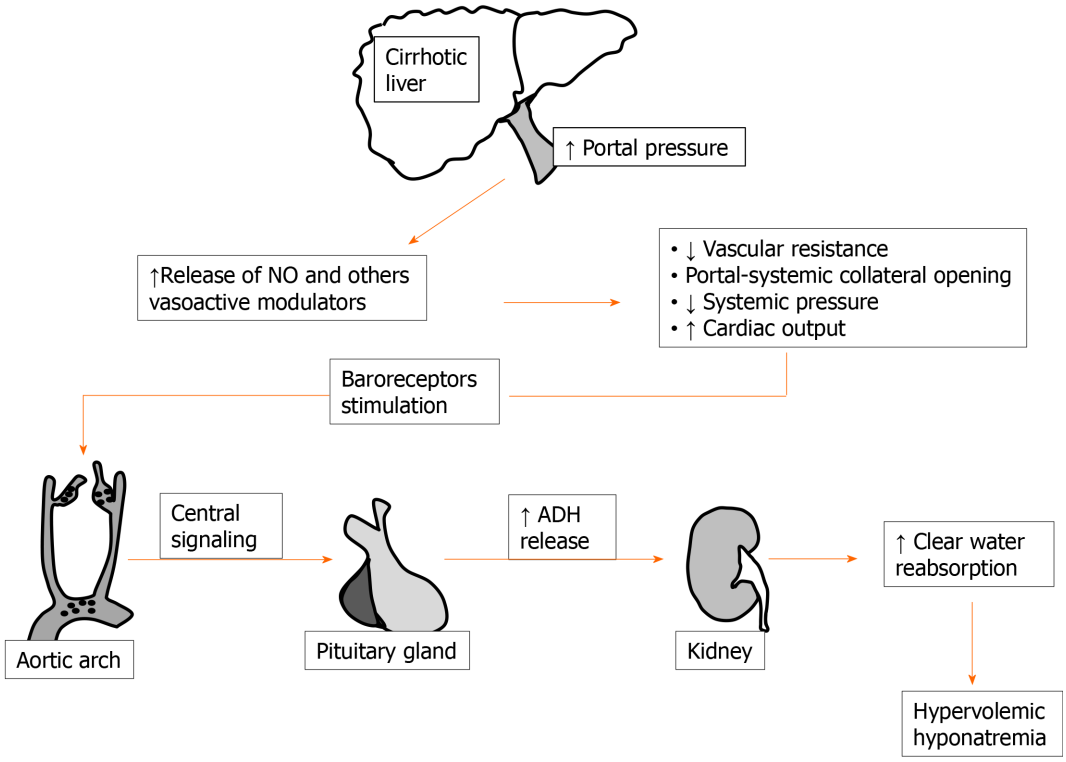


**Figure 1 A simplified scheme including the sequential factors and the organs involved in hypervolemic hyponatremia onset, during cirrhosis, is depicted.** ADH: Antidiuretic hormone; NO: Nitric oxide. ↑: Increase; ↓: Decrease.

handling in the majority of patients with advanced liver disease and (2) in order to have a more tailored indication on pathological sodium levels in these patients, the beginning of the threshold limit for hyponatremia in cirrhotic patients was reduced to [$Na^+$] < 130 mEq/L[38,39].

Detection of persistent hyponatremia in cirrhotic patients represents a worrisome event because of its relationship with complications and mortality[40,41]. In 2006, a prospective survey on 997 cirrhotic patients with ascites, coming from 28 worldwide centers, was performed in order to assess the impact of natremia on the onset of complications[42]. This study demonstrated a prevalence of hyponatremia ($Na^+$ < 135 mEq/L) of nearly 50%, while 21.6% exhibited a $Na^+$ < 130 mEq/L. Moreover, low sodium levels were associated with a more severe grade of liver disease (Child-Pugh C) and an increased risk of major complications, such as hepatic encephalopathy, hepatorenal syndrome (HRS), and spontaneous bacterial peritonitis, excluding only gastrointestinal bleeding. Of particular concern is the association between natremia and HRS. The latter, that is currently diagnosed on the basis of acute kidney injury criteria (baseline increase of serum creatinine ≥ 0.3 mg/DL and/or ≥ 50% within 48 h), has, in fact, a recorded mortality higher than 50%[43,44]. HRS is still in search of adequate medical treatment, and the complex interplay between hyponatremia and HRS are discussed in detail in a 2015 review by Mohanty *et al*[45].

Finally, hyponatremia has been demonstrated to impact the health-related quality of life (HRQL) of cirrhotic patients. This is also observed in the absence of hepatic encephalopathy[46]. In a study, sodium correction in 21 patients with cirrhosis determined a statistical improvement in HRQL and cognitive performance in comparison with the corresponding baseline values[47]. This finding, as also suggested in an editorial of the same journal, underscored the fact that correction of hyponatremia in cirrhosis did not correspond to just "treating a number" but to a cure for several clinical complications, including a reduced HRQL[48].

Hypernatremia is more seldom observed and less studied in cirrhotic patients, accounting for 2%-5% of cases only[42,49]. It has been described in severely ill patients after the lactulose-induced loss of fluid or in concomitance with decompensated diabetes; however, a definite picture of hypernatremia prevalence, morbidity, and mortality in cirrhosis is not available at present[50,51].

Given the importance of sodium imbalance in liver diseases, with regard to its relationship with complications and mortality, the attention to sodium was also

translated in the field of liver transplantation in the last decades. Natremia levels were, in fact, examined for their possible role as a prognostic factor in the waiting list, as a predictor of outcome after grafting, and as possible effectors of complications in the peritransplant phases.

## NATREMIA IMBALANCE AND LIVER TRANSPLANTATION

Starting from the new millennium, it was evident in the United States that liver transplantation should be offered on a "sickest first basis" rather than on-list waiting time[52]. In this perspective, prognostic scores to forecast a short term decrease in cirrhotic patients acquired paramount importance in order to select subjects more in need of a transplant with a consequent reduction of in-list mortality. The model for end-stage liver disease (MELD) score (calculated on the base of bilirubin, creatinine, and international normalized ratio values)[53], originally proposed to evaluate 3-mo liver-related mortality in cirrhotic patients undergoing trans-jugular-portal-systemic shunt, seemed at that time adequate also for the selection of best liver transplant (LT) candidates[54,55]. However, assessment of the MELD score in the real LT world suggested that dynamic evaluation of these parameters or adoption of adjunctive parameters should determine a better selection of patients for LT[56].

A study on 507 patients evaluated for LT in the United States aimed to find additional parameters (not included in MELD) indicating short-term mortality[41]. This research evidenced that persistent ascites and sodium serum levels < 135 mEq/L represented important predictors of mortality that improved the performance of low (< 21) MELD score. Since the impact on survival of water imbalance was not captured by the MELD score and in need to find a possible objective parameter paralleling the severity of edema, a study was conducted to evaluate the predictive value of MELD and sodium serum levels in the waiting list for LT[57]. In 554 cirrhotic patients in a single United States center: (1) A natremia < 126 mEq/L at listing was associated with an increased (nearly 8 times) risk of death; (2) The estimation of risk based on sodium levels was independent by MELD score; and (3) The inclusion of Na with MELD seemed to improve performance in estimating mortality at 3 mo and 6 mo. The role of sodium levels as an independent factor predicting in-list mortality was then definitively demonstrated in a very large study with data retrieved from the Organ Procurement and Transplantation Network database[58]. This research demonstrated a better graft allocation employing the MELD score and $Na^+$ value together (more evident for low MELD score), with a possible 7% reduction of mortality in the list when this scheme was applied to retrospective results.

Further evidence-based studies led to the incorporation of natremia in the MELD score, giving origin to MELD-Na in the United States and UKELD in the United Kingdom, respectively[59,60]. Both scores were demonstrated to perform better than the original MELD at 3 mo and 6 mo[61], even if their superiority was not demonstrated when evaluating patients with acute liver failure[62]. At present, the importance of sodium serum level inclusion in the MELD score has also been confirmed in an updated model undergoing optimization of coefficient bounds[63]. However, some authors advanced concern in including natremia for LT allocation since this parameter could be artificially altered in the clinical setting, and its contribution in the MELD model seems to be limited as well as restricted to low score[64].

Interest in sodium serum levels and LT then rose also with regard to surgical outcome. In an early retrospective European study on 241 cirrhotic patients undergoing LT, a prevalence of hyponatremia (< 130 mEq/L) of 8% was found[65]. Hyponatremic patients had ascites in 100% of cases and more severe liver disease before LT. In the first month after LT, the incidence of neurologic, infectious, or kidney complications was statistically more frequent in patients transplanted with a $[Na^+]$ < 130 mEq/L in comparison with others. This translated into a significant reduction of 3 mo survival (84% *vs* 95%; $P < 0.05$). A subsequent United Kingdom multicenter study reassessed this issue on 5152 patients undergoing LT and in whom pre-transplant sodium data were available[66]. Patients were stratified according to blood $[Na^+]$ in severely hyponatremic (< 130 mEq/L), hyponatremic (130-134 mEq/L), normal (135-145 mEq/L), and hypernatremic > 145 mEq/L. The 3-mo mortality was increased in patients with sodium < 130 mEq/L, accounting for approximately 15% of cases, while the impact on mortality of hypernatremia was even more evident, accounting for 25% of cases. However, the finding of increased sodium levels was 20-times less frequent than hyponatremia in the study. Finally, patients with sodium serum levels falling between 130-134 mEq/L did not exhibit a difference in mortality in comparison with

eunatremic subjects.

Despite the fact that the main cause of death in all groups in the study was represented by infections, thus evolving in multi-organ failure, the authors suggested that the occurrence of central nervous system complications was the first trigger increasing mortality in groups with natremia imbalance. In fact, a previous study demonstrated that rapid corrective osmotic changes occurring during transplant and in early postoperative phases might be responsible in patients with deranged sodium serum levels of pontine and extrapontine myelinolysis[67,68]. Unfortunately, the occurrence of this complication was not assessed in the study. Prevalence of central pontine myelinolysis after LT and according to pretransplant natremia levels was then evaluated in a large United States study[69]. Central pontine myelinolysis was evidenced in 0.5% of the entire cohort (2175 patients) and was associated with the presence of hyponatremia. Interestingly in this American study, differently from previous European data, even if $Na^+$ levels were associated with longer intensive care unit and in-hospital stay, an increased 90 d mortality after LT was not found.

The possible role of hyponatremia on LT short-term survival was again challenged by a following United States large study[70]. In this cohort of nearly 20.000 patients, there was no difference in short-term (90 d) survival after LT between hyponatremic and normonatremic patients. On the other hand, an important (statistically significant) reduced survival was observed in hypernatremic ($Na^+$ > 145 mEq/L) subjects. The interesting discrepancy between the European and American studies does not have a clear explanation so far. However, it is possible that in European studies: (1) Hyponatremia was the expression of more severe liver disease; (2) The use of marginal graft was more largely applied; and (3) Different etiologies of liver diseases (with worse outcomes) were more represented[70]. More recently, a monocentric study with a limited number of patients ($n$ = 306) reassessed the issue of natremia and short-term neurological complications[71]. In this research, while either hypo (< 130 mEq/L) or hypernatremia (< 145 mEq/L) did not have an effect on short-term survival after LT, a relationship between the magnitude of sodium levels correction (> 10 mEq/L), neurological complication, and reduced outcome was observed.

## THERAPEUTIC STRATEGIES FOR SODIUM IMBALANCE IN PATIENTS UNDERGOING LT

As reported above, the general issue of sodium imbalance in cirrhosis acquires particular importance with regard to patients proceeding toward LT. The management of hyponatremia in liver disease patients (the most frequent electrolyte alterations observed) changes widely according to the clinical picture. Acute hypovolemic hyponatremia (observed for extended diuretic therapy or fluid loss) may be managed with success by employing sodium and fluid replacement therapy[37]. On the other hand, treatment of chronic hypervolemic (dilutional) hyponatremia, that represents the expression of a more general impairment of clear water handling, is complex and overall results remain unsatisfactory. The heterogeneous management of this condition by different transplant centers, in the lack of a shared guideline, reflects the complexity of this pathological alteration and the physicians' concern. The United States data show that the majority of LT centers delist patients with $Na^+$ < 120 mEq/L and that a specific protocol to manage low natremic levels before surgery is adopted in less than one-third of transplant centers[72]. These findings suggest that the establishment of an optimal therapy of dilutional hyponatremia in cirrhotic patients may increment their access to transplants, also reducing in-list mortality and possible peritransplant complications.

In clinical practice and in this setting, treatment for decreased natremia is a multistep route to be tailored according to therapy response and target[73]. Low-level hyponatremia ($Na^+$ < 135; ≥ 130 mEq/L) is not deemed worthy of treating since it is considered a frequent and harmless feature of severe liver disease. Sodium values lower than 130 mEq/L may be considered for corrective measures, even if symptoms are seldom observed above 125 mEq/L. Less aggressive, first-line therapy is represented by the withholding of diuretic drugs and fluid restriction. An adequate reduction of fluid intake (1-1.5 L/d) is frequently difficult to obtain because of compliance issues. When this measure is effective, blood sodium level rises within 24-48 h[72,74]. Correction of hypokalemia and administration of albumin may also improve sodium levels[72-74]. Potassium, when it is administered to restore the normal serum range, migrates in the cell, shifting sodium in the extracellular space to equilibrate the net charge concentration. The use of albumin, for sodium correction in cirrhotic

patients, despite the efficacy, remains more controversial due to the cost and its transient effect[75]; however, in hyponatremic patients awaiting LT, it seems a reasonable second-line treatment to reduce possible perisurgical complications after the failure of fluid restriction.

In a large United States study on cirrhotic hyponatremic (< 130 mEq/L) patients, albumin administration was statistically associated with normalization of sodium levels; this, in turn, had a positive effect on 30-d survival[76]. The effect of albumin on natremia may be related to both its oncotic and non-oncotic (inhibition of vasodilators release) properties, as underlined in a commentary in the same journal[77].

The possibility to administer hypertonic saline (generally contraindicated in cirrhotic patients since it increases ascites and edema) may be considered as a short–term treatment, in the few days preceding LT, in severely hyponatremic patients. However, drastic changes in sodium serum levels (> 8 mEq/L daily) should be carefully avoided for the possible onset of significant adverse events in these fragile patients[73]. In this perspective, it seems reasonable to postpone hypertonic saline infusion after attempting to correct sodium levels with fluid restriction or albumin infusion. In fact, the latter strategies are not flawed by significant complications.

Finally, a more complex therapeutic approach includes the use of vaptans; the latter are specific inhibitors of vasopressin (V)-receptors[78]. Among different V receptors (V1a, V1b, and V2), the one involved in kidney clear water reabsorption is the V2. Great interest was raised on V2-vaptans inhibitors (lixivaptan, stavaptan, and tolvaptam) for their possible beneficial effects in cirrhosis since they seemed to target the specific physio-pathological mechanisms leading to dilutional hyponatremia and edema during end-stage liver diseases. Three meta-analyses, focusing on cirrhotic patients, during the last decade consistently demonstrated correction of natremia and reduction of ascites and related complications, such as spontaneous bacterial peritonitis. However, a clear effect on survival was never observed also after a long-term (> 26 wk) follow-up[79-81]. Moreover, a safety warning was released by the Food and Drug Administration in 2013 to avoid tolvaptan in patients with underlying liver disease, since increased liver enzymes were observed in 4.4% of patients (significant alteration in 1%) in a trial on autosomal dominant polycystic kidney disease[82]. On the other hand, despite the fact that early data on autosomal dominant polycystic kidney disease evidenced changes in liver biochemistry during vaptans (tolvaptan) therapy[83], a significant increase in adverse events in cirrhotic patients was never observed. A more worrisome complication related to V2-specific vaptans use might be considered the too rapid (> 8 mEq/L day) correction of sodium levels, with possible neurologic deleterious effect. For this reason, their administration should be decided, managed, and monitored by expert centers. However, the utility of long-term routine use of tolvaptan (the only V2-specific oral vaptan available) for chronic hyponatremia in cirrhotic patients, remains undefined and uncertain since the quick reversal of the therapeutic effect when treatment is withdrawn and the possible adverse events. For these reasons, hyponatremia in cirrhosis remains an "off label" indication for this drug in the majority of countries.

The clinical setting of LT is nevertheless different from the general management of liver cirrhosis. Sometimes, a patient needing an accelerated LT because of a poor prognosis may present with severe hyponatremia. This condition exposes the candidate to a well-recognized risk of neurological complications in the perisurgical phase and maybe to an "a priori" exclusion from transplant. In this complex and specific clinical situation, in which possible drug-induced liver toxicity appears negligible as LT is already required, it seems correct to consider vaptans treatment[72,84]. In this perspective, tolvaptan was experimented for the first time by our group in two LT candidates presenting with severely reduced sodium levels[85]. These subjects were in need of an expedited LT (MELD > 30), both with a natremia approaching 120 mEq/L. After the failure of fluid restriction and hypertonic saline administration, they underwent a short-term (5 d) low-dose (15 mg daily) administration of tolvaptan, with a rise of sodium levels > 130 mEq/L. The drug administration (after acquisition of an informed consent due to the risk of increased liver damage) was carefully followed–up, with frequent testing of liver function parameters and sodium levels, as the patients were hospitalized in our liver sub-intensive unit. We did not observe any major changes in patient biochemical tests. One patient was transplanted and the outcome was uneventful, while the other died of multi-organ failure since an appropriate graft was not retrievable despite the urgency. Our data demonstrated that this short-term low-dose administration was effective, safe, and feasible also in the presence of relevant cholestasis and coagulopathy (both patients had bilirubin > 10 mg/dL and international normalized ratio > 2.5). Our experience was also replicated in a case report of an LT candidate with a less severe liver impairment (MELD17)[86].

Finally, a study was undertaken with tolvaptan in LT candidates in order to improve refractory ascites[87]. Ten patients were treated with a very low dose of tolvaptan (starting from 3.75 mg/d) together with standard diuretic therapy. Six of them had a reduction > 1.5 Kg of body weight within 1 wk of treatment. No major changes were observed with regard to liver function tests or natremic levels.

Also in the lack of unequivocal data and clear guidelines, a possible multi-step approach to severe hypervolemic hyponatremia, for candidates to expedite LT, is proposed in Figure 2.

## CONCLUSION

Sodium imbalance, and in particular dilutional hyponatremia, is frequently encountered in patients with severe liver disease. Low sodium levels in cirrhosis should be considered as the expression of a more complex impairment of water handling rather than a mere electrolyte deficiency. In this perspective, the scarce effects of standard maneuvers for natremia correction are not surprising since the ideal treatment should be targeted to reverse the mechanisms at the base of hyperdynamic circulation or the increased excretion of nitric oxide and ADH. While waiting for appropriate medical treatment, sodium imbalance continues to affect survival and to increase complications in cirrhotic patients. It also sometimes prevents their transplantability, increasing their list mortality and the complexity of post-transplant outcomes. A definitive solution to these aspects would be an important achievement in the future. For the time being, we have to consider that LT represents the most effective therapy for chronic natremia unbalance in cirrhosis and that hyponatremic patients are those experiencing the major survival benefit from this procedure[88].

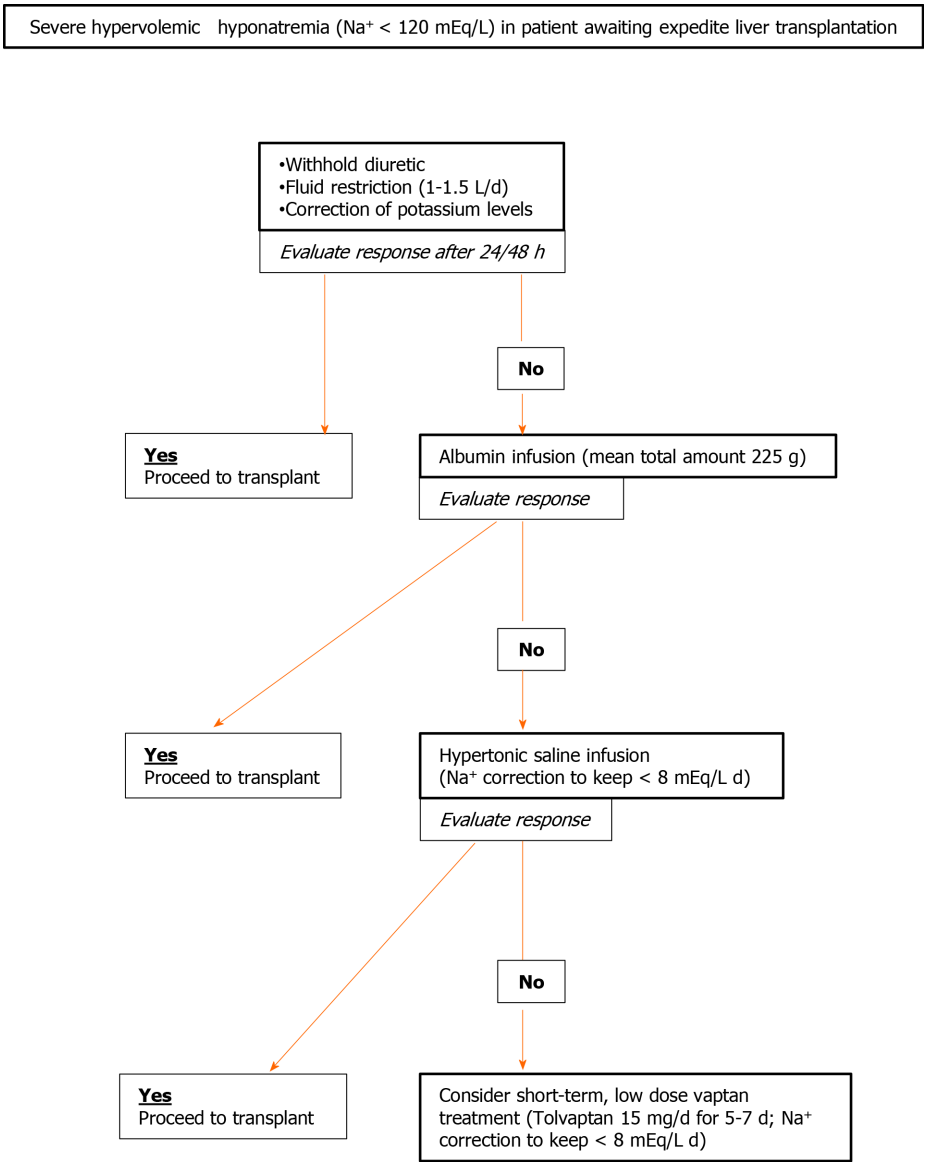


**Figure 2 Proposed algorithm for correction of severe hypervolemic hypernatremia in cirrhotic patients in wait of expedited liver transplantation.** Indications are mainly desumed by small studies or on the base of expert opinion[74-78,86-89]. For vaptan, consider (1) the "off-label" indication and (2) the warning on tolvaptan liver toxicity limiting its use in patients with liver injury, in some countries. Acquisition of patient informed consent is suggested before treatment.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 931-948

DOI: 10.4254/wjh.v12.i11.931

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Basic Study

# Inhibition of vascular adhesion protein-1 modifies hepatic steatosis *in vitro* and *in vivo*

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**Author contributions:** Shepherd EL, Karim S, Newsome PN and Lalor PF contributed to the writing and revising of the manuscript.

**Institutional review board statement:** The study was reviewed and approved by The Black Country Research Ethics Committee (06/Q702/61).

**Institutional animal care and use committee statement:** All mice were maintained and housed under conventional conditions in the Biomedical Services Unit at the University of Birmingham, United Kingdom. All animal experiments were performed under a Home Office project license in accordance with United Kingdom legislation and welfare guidelines, and studies were approved by the local ethical review board.

**Conflict-of-interest statement:** This study includes independent research supported by the Birmingham National Institute for Health Research (NIHR) Birmingham Biomedical Research Centre based at the University of

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## Abstract

### *BACKGROUND*

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance and dyslipidaemia and currently is estimated to affect up to a third of all individuals in developed countries. Current standard of care for patients varies according to disease stage, but includes lifestyle interventions common insulin sensitizers, antioxidants and lipid modifiers. However, to date specific therapies have shown little histological or fibrosis stage improvement in large clinical trials, and there is still no licensed therapy for NAFLD. Given the high prevalence, limited treatment options and significant screening costs for the general population, new treatments are urgently required.

### *AIM*

To assess the potential for inhibition of the amine oxidase enzyme vascular adhesion protein-1 (VAP-1) to modify hepatic lipid accumulation in NAFLD.

### *METHODS*

We have used immunochemical and qPCR analysis to document expression of VAP-1 and key functional proteins and transporters across the NAFLD spectrum. We then utilised hepatocytes in culture and human precision cut liver slices in concert with selective enzyme activity inhibitors to test the effects of activating the semicarbazide-sensitive amine oxidase activity of VAP-1 on hepatic lipid uptake and triglyceride export. A murine model of NAFLD was also used to determine the consequences of VAP-1 knockout and gene expression arrays were used to

Birmingham. The views expressed are those of the authors and not necessarily those of the NHS, the National Institute of Health Research or the Department of Health and Social Science. These studies were in part funded by a Biotechnology and Biosciences Research Council case studentship (BB/G529824/1) to P. F. Lalor for S. Karim. No other conflict of interest.

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

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United Kingdom

**Peer-review report's scientific quality classification**

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

**Received:** June 15, 2020
**Peer-review started:** June 15, 2020
**First decision:** June 20, 2020
**Revised:** July 23, 2020

quantify the effects of VAP-1 activity on key lipid modifying and proinflammatory gene expression.

*RESULTS*

We confirmed that increasing severity of NAFLD and progression to cirrhosis was associated with a significant increase in hepatocellular VAP-1 expression. Hepatocytes *in vitro* exposed to recombinant VAP-1 and its substrate methylamine showed increased lipid accumulation as determined by quantification of Oil Red O uptake. This was recapitulated using hydrogen peroxide, and lipid accumulation was accompanied by changes in expression of the lipid transporter molecules FABP3, FATP6, insulin receptor subunits and PPARα. Human liver tissue exposed to recombinant VAP-1 or substrates for endo/exogenous VAP-1 produced less triglyceride than untreated tissue and demonstrated an increase in steatosis. This response could be inhibited by using bromoethylamine to inhibit the SSAO activity of VAP-1, and mice deficient in VAP-1/AOC3 also demonstrated reduced steatosis on high fat diet. Exposure of human liver tissue to methylamine to activate VAP-1 resulted in increased expression of FABP2 and 4, FATP3-5, caveolin-1, VLDLR, PPARGC1 and genes associated with the inflammatory response.

*CONCLUSION*

Our data confirm that the elevations in hepatic VAP-1 expression reported in nonalcoholic steatohepatitis can contribute to steatosis, metabolic disturbance and inflammation. This suggests that targeting the semicarbazide sensitive amine oxidase capacity of VAP-1 may represent a useful adjunct to other therapeutic strategies in NAFLD.

**Key Words:** Non-alcoholic fatty liver disease; Hepatocyte; Lipid; Cell biology; Vascular adhesion protein-1; Steatosis



**Core Tip:** Incidence of non-alcoholic fatty liver disease (NAFLD) is dramatically increasing worldwide but to date there are no licenced therapies. The challenge remains management of the diverse pathophysiology from simple steatosis, through inflammation and fibrosis and the systemic complications of the metabolic syndrome. Vascular adhesion protein-1 (VAP-1) is an enzyme with proven contributions to systemic and hepatic glucose handling, inflammation and fibrosis. We now show an additional role in hepatic steatosis. Thus our important data suggests that targeting the semicarbazide sensitive amine oxidase capacity of VAP-1 may represent a useful adjunct to other therapeutic strategies in NAFLD.

**Citation:** Shepherd EL, Karim S, Newsome PN, Lalor PF. Inhibition of vascular adhesion protein-1 modifies hepatic steatosis *in vitro* and *in vivo*. *World J Hepatol* 2020; 12(11): 931-948
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/931.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.931

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance and dyslipidaemia[1] and currently is estimated to affect up to a third of all individuals in developed countries[2]. Although a systemic disease, within the liver NAFLD occurs as a disease spectrum ranging from steatosis alone, to steatohepatitis (NASH) which ultimately drives the development of significant fibrosis and cirrhosis. Patients with NAFLD have high mortality and in particular are at increased risk of suffering adverse cardiovascular events[3]. The current standard of care for management of patients is stratified according to disease stage, with lifestyle interventions common in simple steatosis but more extensive clinical intervention using insulin sensitizers, antioxidants and lipid modifiers in NASH[4]. However, to date specific therapies for NASH such as

**Accepted:** September 15, 2020
**Article in press:** September 15, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Mocchegiani F, Tan N
**S-Editor:** Gong ZM
**L-Editor:** A
**P-Editor:** Wang LL

obeticholic acid[5] and apoptosis signal regulating kinase 1 (ASK1) inhibition have shown little histological or fibrosis stage[6] improvement and there is still no licenced therapy for NASH. Transplantation is an option in some centres but as NASH will soon overtake hepatitis C infection as the major indication for liver transplantation, the demand far outstrips clinical capacity. Given the high prevalence, limited treatment options and significant cost associated with screening at risk patients, new treatments are urgently required.

VAP-1 (Semicarbazide Sensitive Amine Oxidase, SSAO, AOC3, EC 1.4.3.6) may present a novel avenue for therapeutic intervention. This molecule is a transmembrane protein and member of the copper containing amine oxidase enzyme family that is sensitive to inhibition by the urea derivative semicarbazide. It is structurally similar to other copper containing amine oxidases[7] such as lysyl oxidase (LOX). It is expressed on diverse cells including adipocytes, smooth muscle cells[8] and some endothelial cells[9], and can be secreted as a soluble protein into serum[10]. Serum concentrations of VAP-1 increase in obesity[11], diabetes[12,13] and inflammatory liver disease[14] and the molecule has promise as a serological indicator of disease severity in inflammatory liver disease[15]. Of note in patients with NASH, serum VAP-1 Levels correlate with severity of obesity and NASH, and more importantly fibrosis stage[16]. VAP-1 and other amine oxidase enzymes catalyse the deamination of amines to yield the corresponding aldehyde and hydrogen peroxide. Thus VAP-1 catalyses the oxidative deamination of both endogenous (methylamine and aminoacetone)[17] and exogenous (benzylamine)[18] amines. This enzymatic capacity is key in the context of diabetes and inflammation since the products of the reaction have been shown to alter glucose uptake[19], and administration of substrate modifies the effects of insulin[20] and activates NFκB to drive hepatic inflammation[21]. There is also evidence suggesting that serological lipid profiles[22] and atheroma risk[23] are linked to VAP-1 activity, since the insulinomimetic effects of the molecule alter lipid metabolism and storage[24] and prime adipocyte differentiation and lipolysis[25]. Since transgenic mice overexpressing VAP-1/SSAO show increased BMI and abdominal fat pad weight if exposed to methylamine[26] it is likely that VAP-1 contributes to the storage and distribution of lipids in NAFLD. In support of this recent studies from our group have demonstrated that wild type mice given an anti-VAP-1 therapeutic antibody, show reduced steatosis on MCD diet[16]. However, to date the mechanisms underlying this response have not been characterised, particularly in a human context. Therefore, in the current investigation we aimed to assess the potential for VAP-1 inhibition to modify hepatic lipid accumulation.

## MATERIALS AND METHODS

### *Human and murine tissue samples*

All human tissue used was collected at the Liver and Hepatobiliary Unit, Queen Elizabeth Hospital, Birmingham, with prior written informed patient consent and local research ethics committee approval (06/Q702/61). Normal and steatotic donor tissue was surplus to requirement for transplantation, whilst NASH and ALD tissue was collected from end-stage fibrotic explanted livers upon transplantation. Tissue was immediately snap frozen and stored at -80 °C or formalin-fixed and paraffin-embedded. For functional assays and generation of tissue slices, tissue samples of approximately 30 g were cut from the periphery of freshly collected livers and immediately placed into (DMEM) prior to use.

All mice were maintained and housed under conventional conditions in the Biomedical Services Unit at the University of Birmingham, United Kingdom. All animal experiments were performed under a Home Office project license in accordance with United Kingdom legislation and welfare guidelines, and studies were approved by the local ethical review board. Male 8-10 wk old WT (Charles Rivers Laboratories Margate, United Kingdom) or *VAP-1*$^{-/-}$ mice (*AOC3* constitutive KO, Taconic, Denmark) were fed a high fat diet (HFD, Special Diets Services, Essex, United Kingdom) for 12 wk. Mice were sacrificed and the liver was removed and immediately snap frozen and stored at -80 °C for subsequent analysis.

### *Immunohistochemistry and histological analysis*

Haematoxylin and eosin staining was performed on Formalin fixed, paraffin embedded sections from human or mouse liver using standard protocols. To quantify lipid content in hepatocytes, fresh frozen tissue sections were incubated in 60% isopropanol for 5 min followed by Oil Red O reagent for 15 min at room temperature.

This was tipped off and 60% isopropanol was added for another 5 min. Slides were washed twice with water and finally mounted using aqueous mountant (Thermoscientific, Shandon). Sections were imaged using brightfield microscopy and % staining area was determined using morphometric analysis *via* Image J software version 1.42 (NIH), using 5 non-overlapping fields selected at random from each mouse (at × 20 magnification). 7 mm sections of formalin-fixed or snap frozen human or murine liver tissue were stained with haematoxylin and eosin or Van Geison's stain[27] according to standard protocols. For analysis of VAP-1 expression fixed tissue sections were stained using standard indirect immunohistochemical methods as described previously[16].

### Generation of precision-cut liver slices

To generate precision-cut liver slices (PCLS), 8 mm tissue cores from fresh tissue samples were aseptically obtained and placed in DMEM (Invitrogen, United Kingdom) at 4 °C prior to slicing[28]. A Krumdieck tissue slicer (Alabama Research and Development, United States) was set up aseptically in a class II microflow tissue safety hood according to the manufacturers instructions. Tissue cores were placed into the Krumdieck tissue slicer assembly and aseptic 240 μm thick PCLS were cut with a blade cycle speed ranging from 20-70/min depending on the type of tissue used (fatty or normal). PCLS were then immediately transferred to tissue culture media consisting of Williams E media (Sigma, United Kingdom) supplemented with 2% FCS (Invitrogen, United Kingdom), 0.1 μmol/L dexamethasone (Sigma, United Kingdom), and 0.5 μmol/L insulin (Novo-Nordisk) unless otherwise noted for specific assays. PCLS were cultured for up to 48 h *ex vivo* in static culture at 37 °C in 5% $CO_2$ in a humidified atmosphere.

### Maintenance of Huh7.5 cells

The human hepatoma-derived hepatocyte cell line Huh7.5 was also used in this study. The cells were seeded in to a T75 $cm^2$ flask (Corning, United Kingdom) cultured in complete Dulbecco's modified eagle medium (DMEM, GIBCO®, Invitrogen) containing 2 mmol/L L-Glutamine, 100 U/mL Penicillin and 100 μg/mL Streptomycin (Sigma, Dorset, United Kingdom). The medium was also supplemented with 10% foetal calf serum (FCS, Invitrogen, Paisley, United Kingdom) and 1 mL of non-essential amino acids (GIBCO®, Invitrogen). The cells were maintained in a 5% $CO_2$ humidified atmosphere at 37 °C.

### Assessment of lipid uptake in PCLS or cultured cells after VAP-1 treatment

In order to study the effects of rVAP-1 activity on lipid uptake in an intact organ culture system, PCLS were cultured in 24 well plates in Williams E media (Sigma, United Kingdom) supplemented with 2% FCS (Invitrogen, United Kingdom) and 0.1 μmol/L dexamethasone (Sigma, United Kingdom). Insulin was removed from the culture media unless specifically being studied. Similarly Huh7.5 cells subcultured into 24 well plates were used to quantify effects of VAP-1 activity on lipid uptake and retention. PCLS or cells were then treated with SSAO substrates (methylamine 200 μm, benzylamine 200 μm), recombinant VAP-1 (rVAP-1 500 ng/mL Biotie Therapeutics, Finland), insulin (0.10 IU), hydrogen peroxide (10 μm) or specific enzyme activity inhibitors (VAP-1 activity inhibitor 2-bromoethylamine hydrobromide (BEA) 400 μm, or MOA inhibitor clorgyline 200 μm, MAOB inhibitor pargyline 200 μm, or the lysyl oxidase inhibitor β-aminopropionitrile BAPN, 250 μm) alone or in combination for 18 h. Media alone was used as the control condition. This was followed by a 6-h incubation with 0.25 μm palmitic acid. When using cultured Huh7.5 cells, duplicate plates were treated identically and fixed and stained with Hoechst dye upon termination of experiment for signal normalisation after treatment.

PCLS or Huh7.5 cells were then stained with Oil Red O to permit lipid quantification. Here cells or PCLS were fixed briefly in 60% isopropanol for 5 min followed by a 45 min incubation with Oil Red O solution. After a brief rinse in isopropanol and water, the Oil Red O reagent was solubilized out of the treated cells in order for spectrophotometric quantification. Here 300 μL of isopropanol was incubated in each well on a plate shaker for 5 min. 100 μL of solubilised solution from each well or isopropanol control was added to triplicate wells in a 96 well falcon plate, and the plate was read at an absorbance of 500-520 nm. For PCLS signal was expressed per 500 mg of tissue. For cell enumeration, data from Huh7.5 treated with Oil red O and Hoechst dye was manipulated to express the amount of Oil red O per 100000 cells.

### Assessment of triglyceride secretion from PCLS

To quantify triglyceride secreted into the culture supernatant of PCLS under different conditions we used a chromogenic assay (Cayman Chemical Company: Colorimetric TG assay CAT10010303) according to manufacturer's instructions.

### RNA extraction from human liver tissue

Human liver tissue samples, roughly 2 cm/2 cm, were preserved in RNA later and stored at -80 °C. To isolate total RNA, blocks were removed from -80 °C storage and placed on ice, approximately 30 mg of tissue was excised. RNA was also extracted from treated PCLS and here the PCLS were submerged in buffer RLT + β-mercaptoethanol immediately after treatment. Total RNA was isolated from 30 mg liver tissue using RNeasy kit (Qiagen). Tissue was dissociated in RLT lysis buffer, placed in gentleMACS™ M Tubes (Miltenyi Biotech) and homogenized by gentleMACS ™Dissociator using program RNA_01.01. RNA concentration and purity were measured using a Nanophotometer™ (IMPLEN).

### Quantitative real-time PCR

To investigate the relative expression of the major lipid transporters in primary cells and human liver tissue the Fluidigm® 96.96 Dynamic Array™ was used. Taqman fluorogenic 5′ nuclease assays using gene-specific 5′FAM labeled probes were used in this array. All RNA samples were diluted to 125 ng/μL and approximately 1 μL of total RNA was transcribed to cDNA using the superscript TM III first strand synthesis supermix kit (Invitrogen) according to the manufacturer's instructions. All cDNA samples were then preamplified with the TaqMan PreAmp Master Mix (Applied Biosystems) according to manufacturers instructions. The Preamp thermocycling parameters were as follows: 95 °C for 10 min, 14 cycles at 95 °C for 15 s followed by 60 °C for 4 min. The PreAmplified template was diluted (at least 1:5 dilution) with 20 μL of 1 × Tris-EDTA buffer 100 × (Sigma) and stored at -20 °C until further use.

### Fluidigm® 96.96 Dynamic Array™ Integrated Fluidic Circuit chip preparation

The 96.96 syringes containing 150 μL of control line fluid were gently inserted into the dynamic array. The plate was then primed for 20 min on the IFC loader (136 × script), before 5 μL of each sample and probe were dispensed on to the 96.96 dynamic array on respective inlets on the plate. The plate was loaded on the IFC for 1 h and 30 min on the load mix (136 ×) script. The plate was then run in the fluidigm for two hours and 10 min. All conditions were run in triplicate for each tissue or cell sample ( Supplementary Table 1).

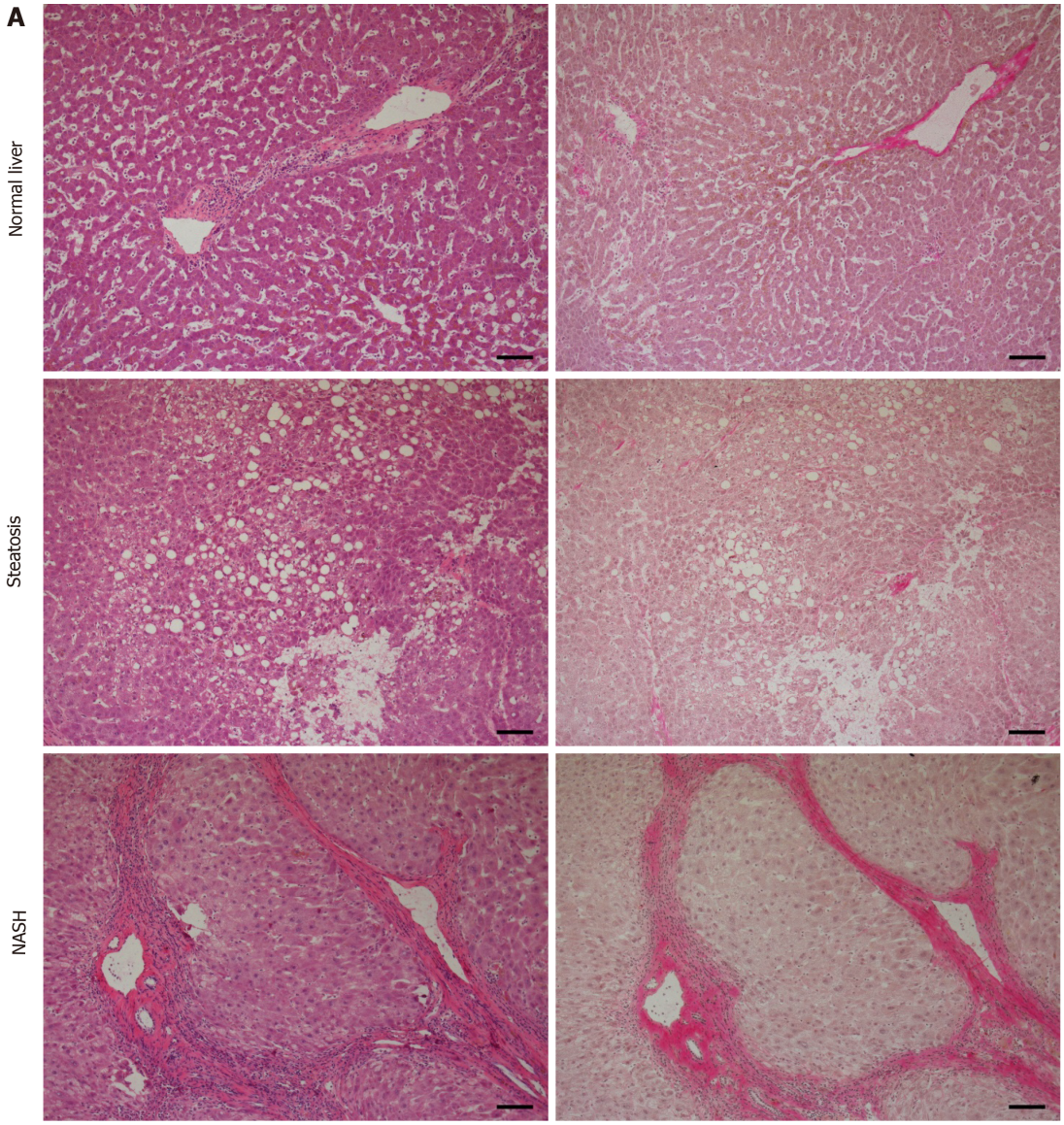
### Statistical analysis

Independent student's *t* tests were used to compare means of two samples unless otherwise stated. Analysis was performed using Graphpad Prizm. For gene array data results are expressed as mean of five replicate samples per tissue ± SEM run on triplicate arrays and normalized to pooled endogenous controls (B-actin and GAPDH). Data was log transformed using 2-delta CT. The relative expression values were used to calculate fold changes within each matched tissue sample. Thus for each patient/liver, the mean of the replicates were calculated and expressed as fold change *vs* the control condition.

## RESULTS

### Expression of VAP-1 increases in chronic liver disease

Several studies have previously demonstrated that VAP-1 is expressed on hepatic cells[29,30] and contributes to inflammation and fibrogenesis[16] in the context of disease. We wished to confirm its presence in our tissue samples and thus selected livers across the spectrum of disease from normal tissue, through steatosis to end stage cirrhosis in NASH. Figure 1A shows example of the typical histological appearance of selected livers within these categories. Fatty donor material demonstrated predominantly macrovesicular steatosis with little evidence of inflammation or fibrosis (Figure 1A). In contrast material from end-stage NASH cirrhosis contained significant lobular and scar associated inflammation. Van Geison's staining confirmed the presence of significant bridging fibrosis and presence of regenerative nodules in the cirrhotic samples as expected. Histochemical staining with antibody directed against VAP-1 (Figure 1B) confirmed staining was localised predominantly to perivascular structures


A
Normal liver
Steatosis
NASH
B
Normal liver
NASH

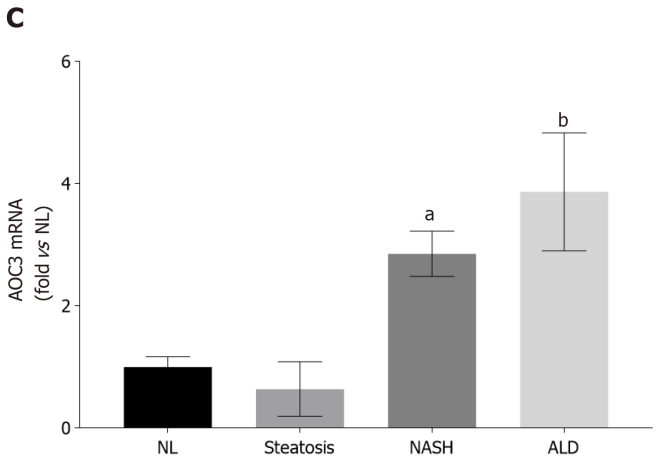



**Figure 1 Hepatocellular expression of vascular adhesion protein-1 increases in nonalcoholic steatohepatitis.** A: Representative brightfield images of sections from indicated disease types stained with Hematoxylin and Eosin (left panels) and Van Geison's Stain (right panels). Original magnification 10 ×, images representative of multiple fields of view from *n* = 3 Livers of each disease type. Scale bar is 100 μmol/L; B Immunohistochemical staining for vascular adhesion protein-1 (VAP-1) in representative acetone fixed frozen sections from normal and nonalcoholic steatohepatitis (NASH) livers. Isotype matched control antibody was negative (not shown). Fields were captured at 10 × original magnification with inset pictures captured at 40 × original magnification; C: Analysis of VAP-1 (AOC3) expression by quantitative qPCR analysis. mRNA expression of AOC3 in whole liver RNA from normal, steatotic, NASH, and alcohol-related cirrhosis (ALD) livers using fluidigm qPCR array®, run on triplicate arrays. Results are expressed as the mean fold change in gene expression normalized to pooled endogenous controls β-actin and GAPDH relative to normal livers defined as 1 ± SEM with means from five normal l, four steatotic, three NASH, and four ALD livers. [a]*P* < 0.05 or [b]*P* < 0.01 using a one way ANOVA with Bonferroni correction. NASH: Nonalcoholic steatohepatitis; ALD: Alcohol-related cirrhosis.

in normal liver and that expression increased dramatically in fibrotic tissue and was localised within fibrotic tissue and sinusoidal areas. QPCR analysis of AOC3 gene expression confirmed these findings (Figure 1C) with VAP-1 mRNA increasing significantly in the context of NASH. This was similar to the picture seen in alcohol-related cirrhosis (ALD, Figure 1C).

### *Uptake of fatty acids by hepatocytes is increased in the context of VAP-1 activity*

Next we used HuH7.5 cells to determine whether the amine oxidase activity of VAP-1 impacts upon hepatocyte lipid handling. Figure 2 shows that exposure of Huh7.5 cells to Oleic acid for 18 h, leads to accumulation of lipid within the cells that can be extracted and quantified or visualised using Oil red O (Figure 2A). Co-incubation of cells with recombinant Vap-1 and its substrate methylamine, increased uptake whilst methylamine alone had no effect. Importantly addition of exogenous $H_2O_2$ the recreate the generation *via* the SSAO activity of VAP-1 recapitulated the response (Figure 2B). We also used qPCR arrays to determine the consequences of oleic acid uptake on gene expression by Huh7.5 cells. Figure 2C shows that we observed notable changes in expression of genes linked to lipid transport and partitioning (FABP3, FATP6), and lipid metabolism (PPARA and PPARg) after treatment. To test if the same response would occur in primary hepatocytes we utilised precision cut liver tissue. Slices of approximately 250um thickness were generated from donor liver tissue, and reproducibility of cutting and viability in culture were confirmed (Supplementary Figure 1). PCLS exposed to OA for 24 h maintained morphological integrity and accumulated lipid within hepatocytes (Supplementary Figure 2B). As there was a gradual decline in viability over time, all experiments were performed on PCLS cultured for a maximum of 24 h. Figure 3A shows quantification of triglyceride content in supernatant from treated slices and reveals that exposure of liver tissue to methylamine, VAP-1 or $H_2O_2$ led to a reduction in triglyceride secretion (Figure 3A) Exposure of cultured normal human liver tissue to VAP-1 also induced a modest but significant lipid accumulation (Figure 3B). In agreement with our data with HuH7.5 cells, exposure to substrate for the SSAO activity of VAP-1 in the form of methylamine also increased lipid accumulation in tissue. However, addition of exogenous recombinant VAP-1 and methylamine resulted in the most dramatic increase. Bromoethylamine (VAP-1 inhibitor) reduced accumulation to control levels, whilst inhibitors of other monoamine oxidases A and B (MAOA and MAOB) did not reduce the uptake seen in the presence of VAP-1 plus substrate. We did note however that use of the lysyl oxidase inhibitor β-aminopropionitrile also reduced lipid accumulation.

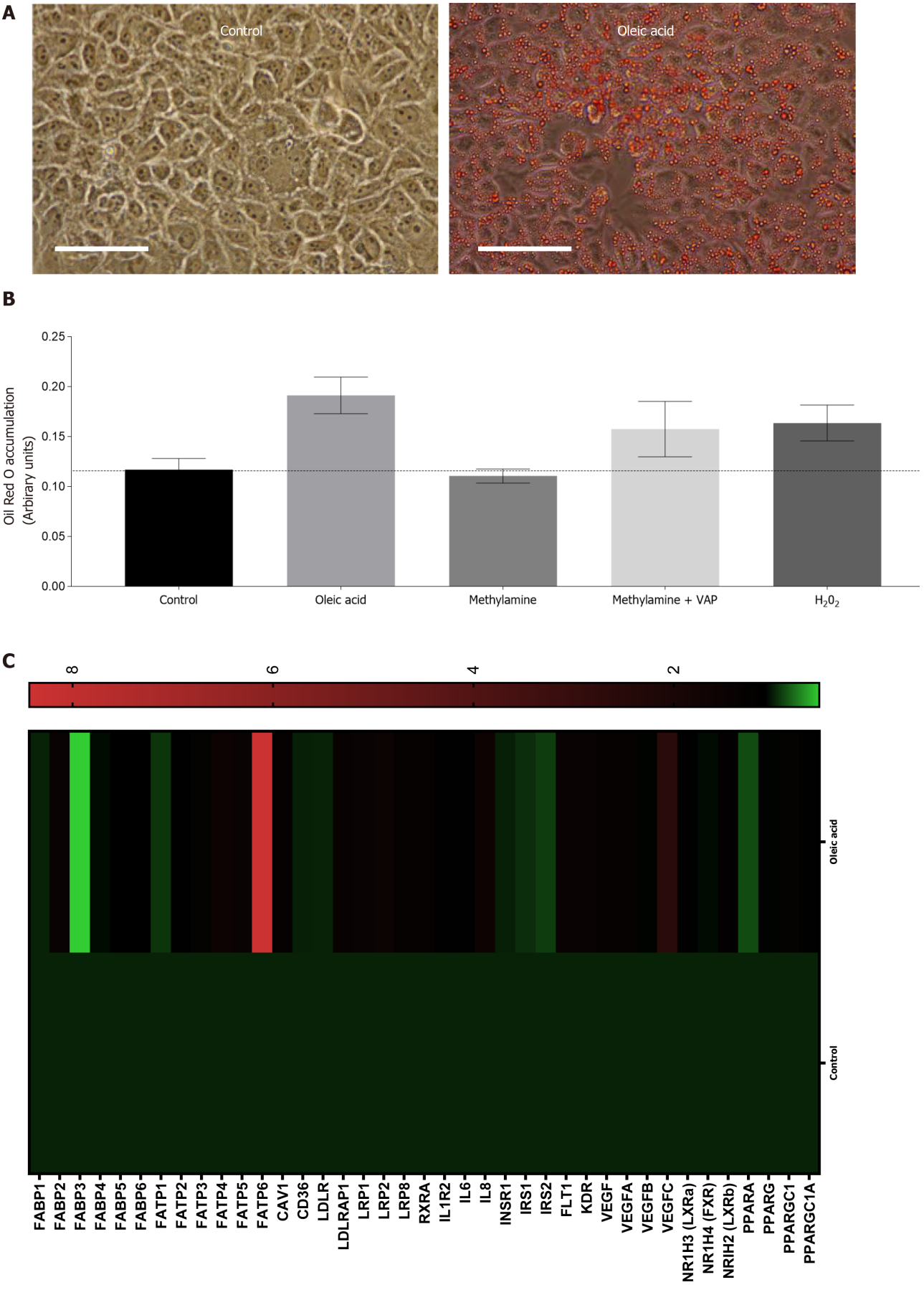



**Figure 2 Exposure of Huh7.5 cells to products of vascular adhesion protein-1 enzyme activity leads to lipid accumulation and gene expression changes.** A: Representative phase contrast images of confluent control Huh7.5 (left) or cells pretreated with 250 μm Oleic Acid for 6 h. All wells were

fixed and stained with Oil Red O, and images were captured at 40 × original magnification (representative of $n$ = 3 samples per condition). Bar is 50 mm; B: Quantification of oleic acid accumulation after vascular adhesion protein-1 (VAP-1) stimulation. Huh7.5 were pretreated with either methylamine (200 μm) alone, or in combination with recombinant VAP-1 (500 ng/mL) or $H_2O_2$ (10 μmol/L), for approximately 18 h. This was followed by incubation for 6 h with 250 μm OA. Cells were fixed and stained with Oil Red O and solubilized. Signal is expressed in arbitrary units. Data are mean ± SEM of triplicate experiments; C: Analysis of mRNA expression in HuH7.5 cells after exposure to oleic acid by quantitative qPCR analysis. mRNA expression for indicated genes was assessed using fluidigm qPCR array® according to manufacturer's instructions. Data is expressed as fold changes in relative gene expression compared to pooled housekeeping genes in control (untreated cells). Data are representative of triplicate conditions run on triplicate gene array plates.

### *Mice deficient in VAP-1 show reduced hepatic steatosis on high fat diet.*

To confirm our observation that VAP-1 contributes to hepatic lipid accumulation in a more physiological context we utilised mice deficient in SSAO activity and exposed them to a high fat diet for 12 wk. Figure 3C shows that whilst wild type livers demonstrated extensive macrovesicular hepatic steatosis, this was significantly reduced in SSAO knockout animals. Thus Oil Red O staining quantification showed a significant decrease in knockout animals after 12 wk on diet.

### *SSAO activity alters expression of hepatic lipid transporters*

Finally, in order to determine the possible molecular mechanism of our response we assessed changes in lipid transporter and key metabolic response gene expression following exposure of liver tissue to SSAO substrate. We documented the baseline expression of transporter molecule RNA in normal, steatotic and NASH cirrhotic livers (Supplementary Figure 2). Simple steatosis was associated with modest changes in members of the FABP, and FATP families. In contrast the development of NASH or alcohol related cirrhosis was associated with more profound increases in transporter expression along with changes in proinflammatory mediators and signalling molecules (Supplementary Figure 2). We next used precision cut liver slices used to assess the impact of SSAO activity on transporter molecule gene expression and noted that there were indeed selective changes in expression. Figure 4 shows that in general activation of the endogenous enzymatic capacity of VAP-1within tissue resulted in a modest but significant upregulation of lipid transporter molecules with the notable examples of FABP2, and 4 in addition to FATP4 and 5. We also report increased expression of caveolin 1, VLDLR, IRS1, VEGFc and PPARGC1 (Figure 4 and Supplementary Tables 2 and 3).

## DISCUSSION

Recent evidence has implicated the semicarbazide sensitive amine oxidase VAP-1 as a potential therapeutic target in metabolic[23] and liver disease[16] and indicate that levels of soluble VAP-1 in serum[15,31-33] have value as a prognostic marker. We have previously demonstrated that VAP-1 function supports key pathophysiological processes in the progression from NAFLD to NASH through its contribution to glucose homeostasis[34] hepatic inflammation[18,29,30] and fibrosis[16]. In this study we suggest that VAP-1 expression in the human liver is not significantly changed by steatosis, which is similar to the reported similarity in SSAO activity in adipose tissue in obese *vs* lean individuals[35]. However we note a profound increase in hepatic expression of VAP-1 in NASH and other cirrhotic disease in agreement with previous evidence[16,19,33]. We have also previously demonstrated that induction of hepatic steatohepatitis in murine models, leads to increased hepatic VAP-1 expression[16]. There are well described roles of VAP-1 in supporting leukocyte[30,36] and particularly monocyte[23,29] recruitment into tissue, and studies suggesting that inhibition of hepatic monocyte recruitment improves steatohepatitis and fibrosis[37]. Thus targeting VAP-1 may have effects on multiple contributing pathways in the pathogenesis of NAFLD.

One of the hallmarks of NAFLD is the presence of hepatic steatosis which is driven by increased abundance of free fatty acids from diet and adipocytes[38] and as a consequence of hepatic de novo lipogenesis[39]. In particular accumulation of the saturated free fatty acid palmitic acid (PA) is associated with disease progression[40] and hepatocyte lipotoxicity, which are a precursor to development of inflammation and fibrosis in NASH. We saw evidence of steatosis in Huh7.5 cells after exposure to OA (Figure 2), but no significant effect of exposure to methylamine, a substrate for VAP-1. Whilst this amine has been widely demonstrated to drive lipid accumulation and differentiation in adipocytes[41,42], the lack of response here likely reflects the absence of VAP-1 expression in this cell line. However, the combination of

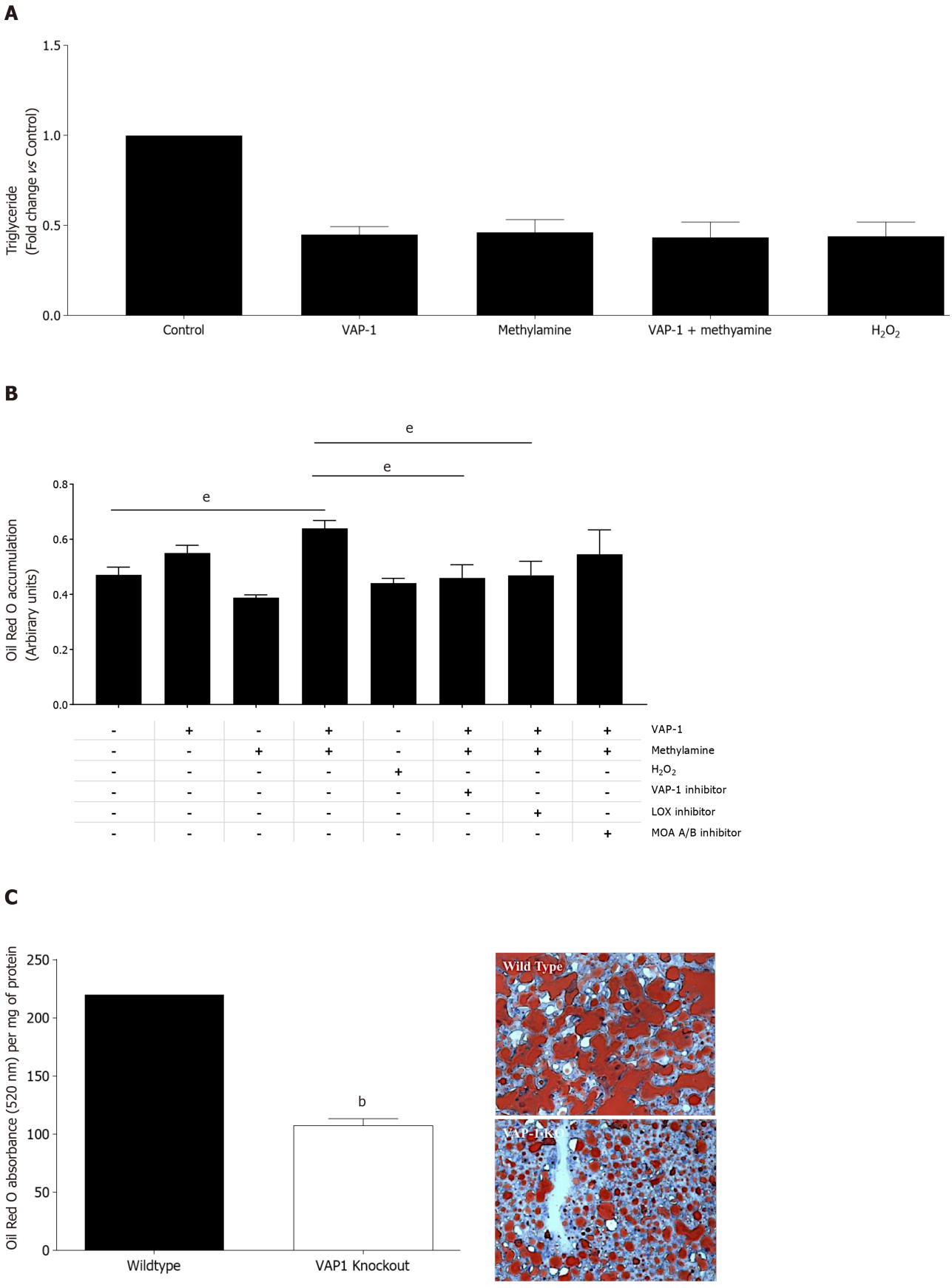



**Figure 3 Activation of vascular adhesion protein-1 enzyme activity results in reduced triglyceride export and increased steatosis in human liver tissue and VAP-1/AOC3 knockout protects against high fat diet induced steatosis in mice.** A: PCLS were pretreated with either, methylamine 200 μm, vascular adhesion protein-1 (VAP-1) 500 ng, $H_2O_2$ 10 μmol/L, or a combination of methylamine + VAP-1, benzylamine+VAP-1 for approximately 18 h and then 6 h with 250 μm fatty acid. Supernatants were collected after treatments and triglyceride secretion was quantified using a commercial assay (Cayman

Chemical Company) according to manufacturer's instructions. Data are triplicate samples from $n$ = 2 normal livers ± SEM. $P$ < 0.01 for all using a one way ANOVA; B: Lipid uptake in PCLS from normal liver tissue pretreated with either methylamine 200 μm, and rVAP1 500 ng/mL alone or in combination, or, $H_2O_2$ 10 μmol/L. Some slices were exposed to the combination of methylamine and VAP-1 plus selective enzyme inhibitors: Bromoethylamine (VAP-1 inhibitor 400 μm), β-aminopropionitrile (lysyl oxidase inhibitor, BAPN 250 μm) or the Monoamine oxidase A and B inhibitors Clorgylline and Pargylline (both at 200 μm). After approximately 18 h incubation, slices were exposed to 250 μm oleic acid for 6 h. PCLS were fixed and stained with Oil RedO, which was solubilized and signal normalized to per 500 mg of tissue. Data are mean of triplicate samples from $n$ = 2 normal livers ± SEM. Significance expressed as $^{e}P$ < 0.001 in one way ANOVA with Tukeys correction for multiple comparisons; C: Left - Accumulation of lipid in WT and VAP-1 KO mice fed on a high fat diet for 12 wk. 7um cryosections from WT and VAP-1 KO mouse livers were stained with ORO, which was then solubilized and signal expressed relative to protein concentration for each group of mice, Data are mean ± SEM of three mice per group. Significance expressed as $^{b}P$ < 0.01 one way ANOVA. Right – representative brightfield microscopy images of Oil red O stained cryosections from WT and VAP-1 KO mice.

recombinant VAP-1 and methylamine induced an accumulation of lipid in these cells. This is likely linked to peroxide generation as a consequence of amine oxidase activity, since addition of exogenous hydrogen peroxide recapitulated the response (Figure 2) and the effect was reduced in the presence of bromoethylamine but not MAOA or B inhibitor (clorgylline and pargyline). Hydrogen peroxide is a known adipocyte lipolysis inhibitor[43], and insulin mimic which increases hepatic glucose uptake[19]. Thus enhanced lipid accumulation within our cells in culture may reflect increased free fatty acid and glucose uptake and reduced lipolysis and export. In support of this concept we performed additional experiments using precision cut liver tissue slices. Here an endogenous supply of both VAP-1 and physiological amines would be present. Once again the administration of oleic acid induced a modest hepatocyte-specific lipid accumulation, but we also confirmed that methylamine induced lipid accumulation within the tissue, most likely due to activation of endogenous VAP-1. Similar results have been reported *in vivo* where benzylamine increases adipose tissue fat deposition in diabetic rats[44], and transgenic mice overexpressing VAP-1/SSAO supplemented with methylamine have increased BMI and abdominal fat pad weight[26] however this is the first observation in human liver tissue. Interestingly addition of exogenous VAP-1 caused a profound steatosis, suggesting the presence of exogenous substrates within tissue such as tyramine, histamine or dopamine[45]. When both VAP-1 + MA were added, the stimulatory effect was less marked which may suggest preferential or competitive use of methylamine or benzylamine over endogenous substrates. This also suggests the endogenous substrates may have multiple or more potent effects. In agreement Salmi *et al*[46], have shown that addition of BA reduces VAP-1-dependent lymphocyte binding to endothelial cells. Thus they suggested BA was a competitor for an endogenous substrate for VAP-1[46]. Regardless of substrate specificity, the observation that BEA specifically inhibited the MA/BA + VAP-1 effect, and that $H_2O_2$ recapitulates the response confirms that VAP-1/SSAO alters lipid accumulation in PCLS. We also observed reduced triglyceride export from treated slices in the context of VAP-1 activation which suggests that net accumulation of lipid is linked to both increased FFA uptake and reduced triglyceride export.

Our studies with mice deficient in VAP-1 suggested that this protected the animals from hepatic steatosis induced by high-fat diet exposure. To explain this, and our reductions in TG export and increased steatosis when SSAO activity was primed, we performed PCR arrays on cultured cells and liver tissue slices to quantify changes in expression of key lipid transporters and proinflammatory molecules. This also shed light on mechanisms which underpin the characteristic insulin resistance and altered sugar handling that are also a feature of metabolic syndrome and have previously been attributed at least in part to VAP-1 activity[8,47,48]. Our baseline analysis of liver tissue across the spectrum of NAFLD (Supplementary Figure 2) confirmed increasing dysregulation of insulin responses, PPAR activity and lipid transport and metabolism[49] as disease progresses, in keeping with altered fatty acid partitioning and binding, and lipolysis. For example FATP6 is known to play a role in the uptake of long chain fatty acids such as oleic and palmitic acid[50] and as such our increased expression in oleic acid exposed hepatocytes, or methylamine treated PCLS makes sense. Similarly, CD36 increases on hepatocytes during diet induced obesity in rodent models, and correlates with extent of hepatic triglyceride storage and secretion[51]. We observed increased RNA expression in liver samples from patients with steatosis in NASH and ALD. In conditions of lipid excess, this transmembrane scavenger receptor can operate to transport long chain fatty acids into the cell for transition into lipid droplets or use as an energy source. Mice with CD36-deficient hepatocytes are protected from high fat diet induced hepatic steatosis and have improved insulin sensitivity[52]. Thus our altered CD36 expression in diseased liver tissue may reflect a compensatory response to nutrient excess.

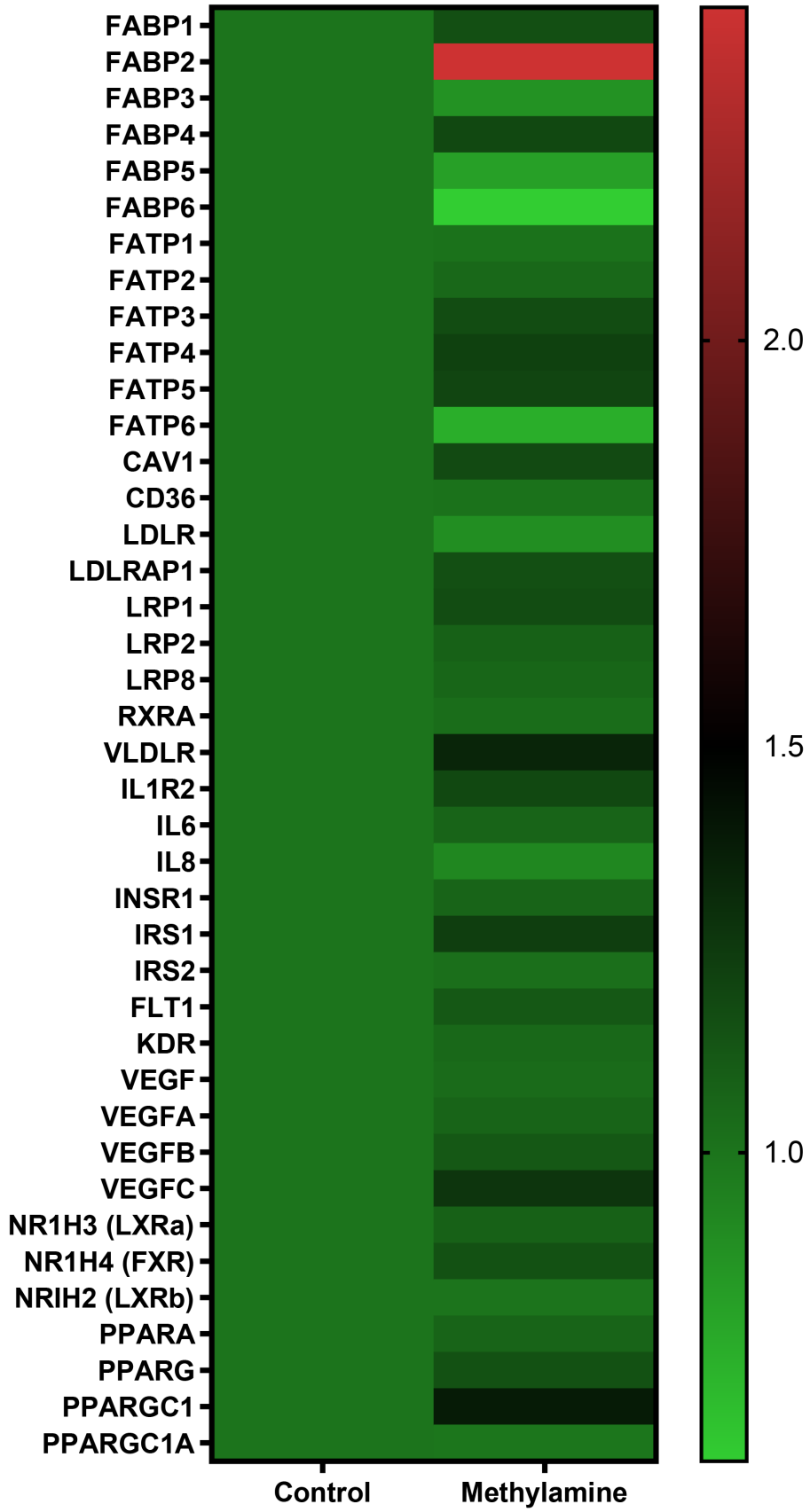



**Figure 4 Exposure of precision-cut liver slices from human donor liver tissue cells to substrate for vascular adhesion protein-1 enzyme activity leads to gene expression changes.** Precision-cut liver slices were treated with methylamine 200 μm for approximately 4.5 h. RNA was extracted and mRNA expression was carried out using a fluidigm qPCR array® run on triplicate arrays. Results are expressed as the mean fold change in gene expression normalized to pooled endogenous controls β-actin and GAPDH relative to untreated control livers. Data are indicative of triplicate arrays prepared from 2 donor livers.

The dramatic upregulation of FABP2 on steatotic tissue slices after even a short exposure to methylamine is in agreement with studies suggesting that FABP2 upregulation on cell lines is associated with reduced lipoprotein export. FABP4 was also increased in both treated cells and slices and was particularly elevated in NASH[49] and ALD liver tissue. FABP4 is shed from adipose tissue and plasma FABP4 Levels are considered an early indicator in the development of the metabolic syndrome[53]. Given the reported regulation of adipocyte FABP4 expression by PPARα[54], and the acquisition of an adipocyte-like phenotype by hepatocytes during steatotic liver injury this may also relate to PPARα activation[55] following VAP-1 engagement. Thus it is possible that similar regulatory mechanisms govern expression of FABP4 in hepatocytes. Increasing clinical evidence for targeting hepatic fat metabolism *via* PPAR blockade[56] is in keeping with our elevated expression in diseased tissue and after priming of VAP-1 activity *in vitro*.

We also noted early changes in expression of mRNA for Caveolin-1, an integral membrane protein found in caveoli that has been linked to formation and function of these intracellular structures[57]. In particular the molecule has been linked to insulin signalling and translocation of GLUT4 to the cell membrane[19], and is increased during adipogenesis[58]. Our reported increases in treated PCLS and diseased liver fit with the ability of hydrogen peroxide generated as a consequence of the enzyme activity of VAP-1 to prime uptake of glucose as a fuel for de novo lipogenesis. Our reported alterations in insulin receptor subunit expression are also suggestive of altered insulin-dependent responses in NAFLD and fit with the changes in insulin receptor expression when tissue slices are exposed to methylamine. Importantly the changes in gene expression reported in our tissue slice studies, occurred after a relatively short period of *in vitro* VAP-1 activation (4.5 h). Thus it is perhaps not surprising that we see only small but significant magnitude changes in expression of genes involved in the early stages of nutrient handling such as FABP2, VLDLR, caveolin and Insulin receptor subunits at this timepoint. We also note that it would be important to utilize specific VAP-1 inhibitors such as semicarbazide or bromoethylamine to confirm that the gene expression changes we report when cells and tissue slices are exposed to methylamine do indeed relate to its specific metabolism by VAP-1.

The contribution of other amine oxidase enzymes was tested through addition of BAPN, Clorgylline or Pargylline in combination with MA/BA + VAP-1. These inhibitors did not lead to inhibition of lipid accumulation, and if anything caused an increase. We observed the same effect in Huh7.5 exposed to methylamine (Figure 2) suggesting substrate, and possibly cell-specific effects of inhibitors. Interestingly when clorgylline and pargyline were added in combination with exogenouse VAP-1 we observed an increase in lipid accumulation compared to OA alone. This may suggest that monoamine oxidase blockade leads to upregulation of VAP-1 activity, or these inhibitors may be causing allosteric effects in VAP-1 thus increasing enzyme activity and lipid accumulation. Of note, presence of the LOX inhibitor BAPN did alter liver lipid accumulation when VAP-1 and methylamine were also present. Since administration of BAPN does not reduce weight gain in atherogenic rat models[59] it is unlikely that LOX has a significant role in systemic lipid handling. There are also reports suggesting that this agent is not specific for LOX and may also have a moderate inhibitory effect on VAP-1 in some cells[41,60]. Thus our response may reflect SSAO inhibition by BAPN. However oxidation of lysine by LOX leads to collagen and elastin crosslinking and ECM remodelling, and thus increased expression in fibrotic NASH livers is in keeping with previous reports of increases on hepatic stellate cells and myofibroblasts in disease[61], and antifibrotic benefit of lysyl oxidase blockade[62].

## CONCLUSION

Thus, in conclusion we have used human and murine model systems to demonstrate that metabolic features of NAFLD, linked to altered glucose and insulin responses, steatosis and lipid uptake and altered triglyceride export are all influenced by the amine oxidase activity of VAP-1. These findings are summarized in Figure 5. In light of previous evidence showing that VAP-1 also plays a role in M2 macrophage infiltration[63] and IL-1b function in steatosis, has roles in atheroma development[23] and influences hepatic inflammation and fibrogenesis[16] we would argue that the increased pharmaceutical interest in amine oxidase inhibitors is well placed[64].

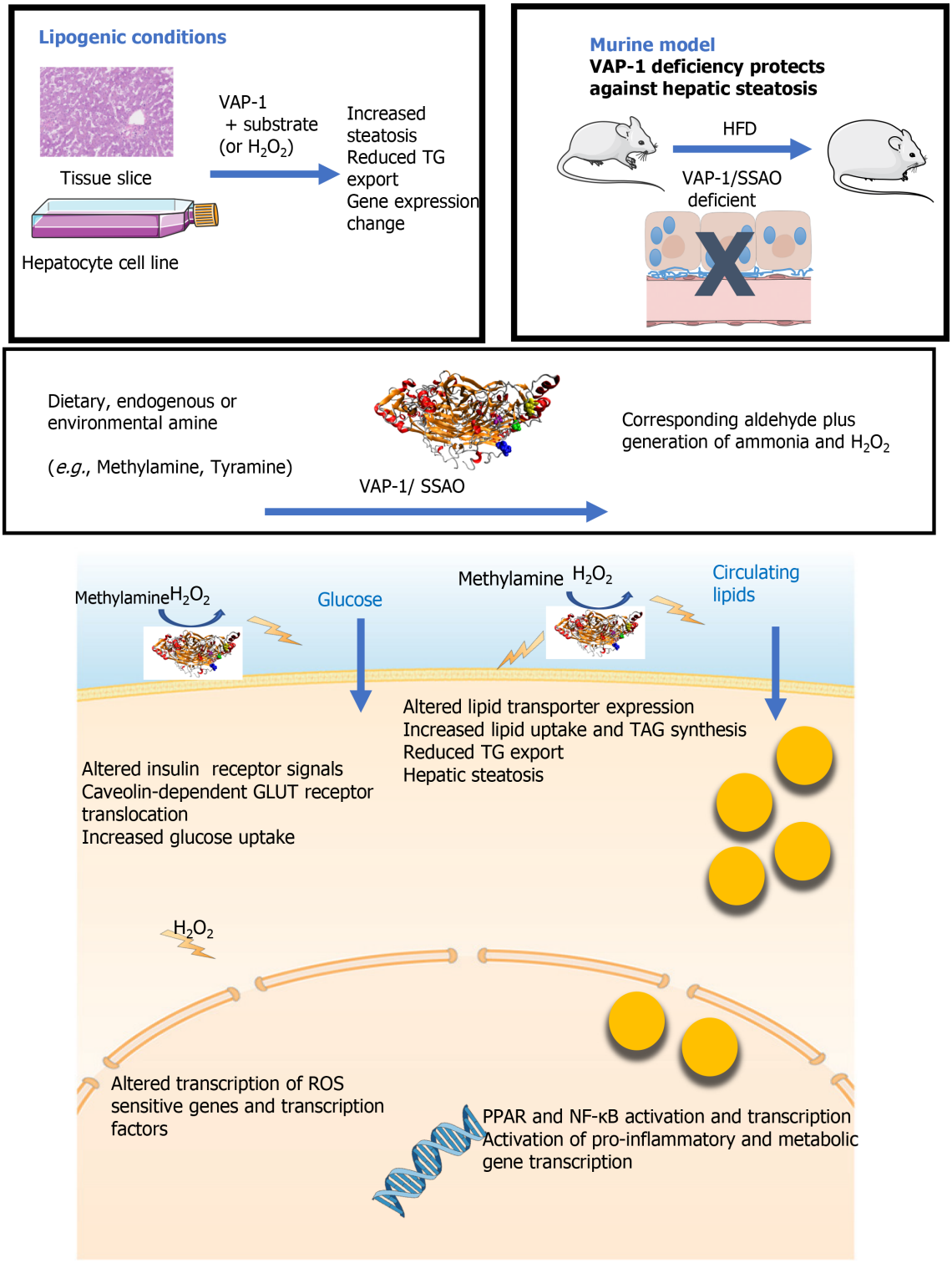



**Figure 5 Graphical summary the impact of vascular adhesion protein-1 on the hepatic pathogenesis of non-alcoholic fatty liver disease.** Exposure of human liver tissue or hepatocytes in culture to vascular adhesion protein-1 (VAP-1) in the presence of endogenous or exogenous (methylamine) substrate led to a reduction in TG export and increased steatosis. In tissue this was accompanied by changes in metabolic gene expression. VAP-1 deficient mice are protected against hepatic steatosis when fed a high fat diet. These findings can be explained by the enzymatic capacity of VAP-1 to reduce amine substrates to the corresponding aldehyde accompanied by generation of potent signaling molecules such as hydrogen peroxide. Addition of hydrogen peroxide to our culture systems recapitulated the effects of VAP-1 activation. Our previous studies suggest that increased inflammation, steatosis and fibrosis in the context of non-alcoholic fatty liver disease in part relate to the ability of VAP-1 to support leukocyte recruitment across endothelial cells, to prime hepatic glucose uptake and to activate hepatic stellate cells. We now show additional effects on transcription of key lipid transporter molecules and transcription factors. The upregulation of FABP4, FABP2, FATP3-5 and LRP1 along with the VLDLR which alter uptake and intracellular targeting of lipid molecules, Transport of fatty acids to the nucleus by receptors such as FABP2 will also activate nuclear receptors such as PPARs and NF-κB, hence influencing gene transcription. VAP-1: Vascular adhesion protein-1; HFD: High fat diet.

## ARTICLE HIGHLIGHTS

### *Research background*

Current standard of care for non-alcoholic fatty liver disease (NAFLD) patients varies according to disease stage, but includes lifestyle interventions common insulin sensitizers, antioxidants and lipid modifiers. However, to date specific therapies for have shown little histological or fibrosis stage improvement in large clinical trials and there is still no licensed therapy for NAFLD. Given the high prevalence, limited treatment options and significant screening costs for the general population, new treatments are urgently required.

### *Research motivation*

Vascular adhesion protein-1 (VAP-1) is an enzyme with proven contributions to systemic and hepatic glucose handling, inflammation and fibrosis. We now show an additional role in hepatic steatosis.

### *Research objectives*

In the current investigation, we aimed to assess the potential for inhibition of the amine oxidase enzyme VAP-1 to modify hepatic lipid accumulation in NAFLD.

### *Research methods*

We have used a combination of human cell cultures, a murine model and human precision cut liver slices to understand the contribution of the semicarbazide sensitive amine oxidase enzyme VAP-1 to lipid handling in NAFLD. This molecule is of increasing therapeutic interest due to its ability to regulate hepatic inflammation and fibrosis.

### *Research results*

VAP-1 increases lipid accumulation and reduces triglyceride export by hepatocytes. This is linked to alterations in expression of key lipid transporters including FABP1, 2 and 4, FATP2-5 and LRP1 and key regulators such as PPARα. In agreement, VAP-1 deficient mice are protected against steatosis on high fat diet.

### *Research conclusions*

We suggest the multifaceted effects of VAP-1 within the liver in NAFLD make it an interesting target for pharmacological intervention.

### *Research perspectives*

We would argue that the increased pharmaceutical interest in amine oxidase inhibitors is well placed.

## ACKNOWLEDGEMENTS

We acknowledge the supervisory support of Katrina McAulay at Unilever, Lee Claridge and Chris Weston in the Centre for Liver Research and staff at the Birmingham Biomedical Services Facility for assisting with murine studies. We are also grateful to Janine Fear for cryo-sectioning liver samples and PCLS.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 949-964

DOI: 10.4254/wjh.v12.i11.949

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Basic Study

# Aceclofenac-induced hepatotoxicity: An ameliorative effect of *Terminalia bellirica* fruit and ellagic acid

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**Author contributions:** Pandey AK conceptualized and designed the study; Gupta A performed the experiments, collected the data, and wrote the manuscript; Gupta A and Pandey AK analyzed and interpreted the data; Pandey AK critically reviewed and finalized the manuscript. All authors have read and approved the final manuscript.

**Institutional animal care and use committee statement:** The study was reviewed and approved by the Institutional Animal Ethics Committee, University of Allahabad, Prayagraj, India (IAEC/AU/2019(1)/16).

**Conflict-of-interest statement:** The authors declare that there are no conflicts of interest.

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

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.



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## Abstract

### *BACKGROUND*

Aceclofenac (ACF), a widely used nonsteroidal anti-inflammatory drug, has been associated with a number of severe cases of clinical hepatotoxicity. *Terminalia bellirica,* an evergreen tree, is known to have several ethnomedicinal uses including antioxidant and hepatoprotective effects. Hence *T. bellirica* fruit extracts and its phytoconstituent ellagic acid (EA) are expected to provide protection against oxidative stress and liver damage produced by long-term use of ACF.

### *AIM*

To evaluate the antioxidant and hepatoprotective activities of *T. bellirica* fruit extracts and EA against ACF-induced toxicity in albino Wistar rats.

### *METHODS*

The *in vitro* antioxidant activities of *T. bellirica* fruit ethyl acetate and aqueous extracts were measured by metal ion chelation and nitric oxide radical scavenging assays. The *in vivo* antioxidant and hepatoprotective effects of *T. bellirica* extracts (200 mg/kg) and EA (40 mg/kg) in ACF-induced hepatotoxic rats were assessed in serum and liver tissue after oral administration for 21 d. Silymarin (40 mg/kg) was used as a standard control. Oxidative stress markers in the blood (ferric reducing ability of plasma and lipid peroxidation inhibition) and liver tissues (superoxide dismutase, catalase and malondialdehyde) were analyzed using standard protocols. Liver function markers such as alkaline phosphatase, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, lactate dehydrogenase, γ-glutamyl transferase, creatinine, total protein, and uric acid were evaluated in rat serum.

### *RESULTS*

The *T. bellirica* fruit ethyl acetate extract exhibited superior metal ion chelating and nitric oxide radical scavenging abilities during *in vitro* antioxidant assays as compared to aqueous extracts. Oral administration of ACF in rats (15 mg/kg) for

**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** India

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 16, 2020
**Peer-review started:** June 16, 2020
**First decision:** August 22, 2020
**Revised:** September 5, 2020
**Accepted:** September 22, 2020
**Article in press:** September 22, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Carrier P
**S-Editor:** Zhang L
**L-Editor:** Webster JR
**P-Editor:** Wang LL

21 d produced oxidative stress and adversely affected liver function suggesting liver injury. Treatment with extracts (ethyl acetate and aqueous), EA and silymarin accounted for a significant reduction in the adverse effects of ACF on oxidative stress and liver function markers in serum and hepatic tissue in rats. Histopathological evaluation of the liver indicated that the extracts and EA significantly decreased the degree of liver damage. The *in vivo* efficacy of EA was higher than *T. bellirica* fruit extracts. Of these extracts, ethyl acetate extract revealed comparatively better antioxidant and hepatoprotective activity.

***CONCLUSION***

Ellagic acid and *T. bellirica* fruit extracts exhibited considerable hepatoprotective and antioxidant activities in long-term ACF-treated rats.

**Key Words:** *Terminalia bellirica*; Ellagic acid; Aceclofenac; Hepatotoxicity; Antioxidant; Histopathology



**Core Tip:** Hepatotoxicity is the most serious adverse effects of aceclofenac (ACF). In this study, ACF-induced hepatic damage in rats was investigated. ACF administration (15 mg/kg/d) for 21 d produced severe hepatotoxicity and oxidative stress as demonstrated by abnormal elevations in serum and tissue markers. Co-administration of *Terminalia bellirica* fruit extracts (200 mg/kg) and ellagic acid (40 mg/kg) significantly attenuated ACF-induced hepatotoxicity. These results showed that supplementation with the test compounds led to restoration of serum liver function markers (SGOT, GPT, GGT, LDH, ALP, total protein, urea, uric acid, creatinine) and hepatic antioxidant status (superoxide dismutase, catalase, TBARS). Hence *T. bellirica* fruit extracts and ellagic acid have the potential to act as a hepatoprotectant and antioxidant in the treatment of drug-induced hepatotoxicity and oxidative stress. To the best of our knowledge, this is the first study to evaluate the therapeutic efficacy of *T. bellirica* fruit extracts and ellagic acid as hepatoprotective agents against ACF-induced hepatotoxicity.

**Citation:** Gupta A, Pandey A. Aceclofenac-induced hepatotoxicity: An ameliorative effect of *Terminalia bellirica* fruit and ellagic acid. *World J Hepatol* 2020; 12(11): 949-964
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/949.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.949

## INTRODUCTION

Oxidative stress is characterized as disparity between the free radical generation and antioxidant defense mechanisms. As a consequence, free radicals attack biomolecules including lipids, proteins and DNA, thus leading to the development of various ailments at cellular and organ levels which ultimately precipitate in a disease etiology viz., hepatotoxicity, inflammation, cancer, diabetes, cardiovascular, and neurodegenerative disorders *etc*.[1,2]. Oxidative stress not only causes DNA damage, lipid peroxidation, and protein oxidation but also produces interference in the physiologic adaptation phenomenon and regulation of intracellular signal transduction mechanisms[3]. Antioxidants (enzymatic and non-enzymatic) existing in the living system are typically effective in neutralizing the adverse effects of free radicals. Numerous synthetic antioxidants are presently used in several food and pharmaceutical sectors although they are reported to produce toxicity. Hence, there is a growing demand from consumers for the utilization of natural antioxidants due to their virtuous efficacy and fewer side effects on health[4,5].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently recommended for the management of pain and inflammatory conditions. They obstruct the activity of cyclooxygenase-1 and -2 enzymes[6]. Aceclofenac (ACF), an established NSAID, is a phenylacetic acid derivative and chemically termed 2-[(2′,6′-dichlorophenyl) amino] phenylacetoxyacetic acid. In humans, ACF is metabolized to 4′-hydroxyaceclofenac *via*

cytochrome P450 2C9b (CYP2C9)[7]. ACF has been shown to arouse selective inhibition of COX-2 as a consequence of limited and continued biotransformation to diclofenac[8]. Prolonged administration of ACF harms gastrointestinal mucosa by irritant action, affecting mucosal permeability and/or prevention of prostaglandin synthesis. Moreover, it exhibits analgesic, antipyretic, and anti-inflammatory activities, and inhibits the arachidonic acid pathway. In addition, prolonged consumption of ACF is associated with upper gastrointestinal complications, mainly perforated and bleeding peptic ulcer[9]. Previous studies on NSAIDs documented that they have adverse effects on the liver; but the incidence of these side effects is inconclusive. However, studies related to ACF-induced liver injuries are very limited.

Silymarin (SLM) is a polyphenolic flavonoid extracted from the fruit and seed of *Silybum marianum* (milk thistle). It is a well-recognized therapeutic agent for hepatic injury and has been used in the treatment of liver cirrhosis and severe hepatitis. SLM is also useful in the mitigation of damage inflicted by toxic compounds[10]. SLM has been shown to provide protection against hepatic, renal, neuronal, and gastric injury[11]. The hepatoprotective potential of SLM is related to its stabilizing action on cytoplasmic membranes[12]. Studies on different animal models have unveiled the notable therapeutic action of SLM on hepatic injury of diverse etiology[13].

*Terminalia bellirica* Roxb. (Combretaceae) is a perennial plant widely found in tropical regions and frequently observed in South-East Asia[14]. Its fruit has been used in various ailments in the indigenous medical system to cure cough, asthma, diarrhea, dyspepsia, anemia, cancer, fever and inflammation, and to promote rejuvenation[15,16]. It is one of the ingredients in "Triphala", an ayurvedic formulation rich in antioxidants which is believed to promote health, immunity and longevity[17], and is used for the treatment of various disorders including fever, constipation, chronic ulcers, anemia, asthma and jaundice[18]. Chemical profiling of *T. bellirica* fruit revealed that gallic acid was one of the major active components of the fruit (2.6 mg/g of total polyphenols). However, other phytochemicals such as ellagic acid, ethyl gallate, chebulagic acid and β-sitosterol have also been reported to be present in noteworthy concentrations[19,20]. *T. bellirica* fruit has been scientifically proven to possess antibacterial, antifungal, antioxidant, antidiabetic and hepatoprotective effects[21,22]. The combination of three lignans and a flavan from *T. bellirica* fruit extract showed significant anti-HIV, anti-malarial and antifungal activity *in vitro*[23]. Ellagic acid (EA) is a polyphenolic compound present in *T. bellirica* fruit in a considerable amount (1.3-2.2 mg/g of total polyphenols), and has been studied extensively due to its medicinal attributes[24,25]. The antioxidant effect of EA is attributed to its free radical scavenging and metal ion chelating abilities, along with enhancement of cellular antioxidant defense. EA also protects cells from free radical-mediated DNA damage[26,27]. However, there are no reports on the therapeutic potential of *T. bellirica* fruit extracts and EA as a hepatoprotectant against ACF-induced toxicity. The present study reports the antioxidant and hepatoprotective activities of *T. bellirica* fruit ethyl acetate (Eth) and aqueous (AQ) extracts as well as EA against ACF-induced oxidative stress and hepatotoxicity. To the best of our knowledge, this is the first study to assess the antioxidant and hepatoprotective effects of *T. bellirica* fruit extracts (Eth and AQ) and EA against ACF-induced liver injury in albino Wistar rats.

## MATERIALS AND METHODS

### Chemicals

Silymarin (Sigma), ellagic acid (Himedia Laboratories Pvt Ltd.), aceclofenac (Ipca Laboratory), ferrozine, ferric chloride, sodium nitroprusside, sulfanilamide, napthyldiamine, phosphoric acid, potassium chloride, and ferrous sulfate (Sisco Research Laboratory (SRL) Pvt. Ltd.) were procured from scientific suppliers. Biochemical kits for estimation of creatinine, uric acid, total protein, and serum enzymes (GPT, ALP, GOT, LDH, GGT) were purchased from Erba Transasia Bio-medicals Ltd. All other laboratory chemicals such as dimethyl sulfoxide, trichloroacetic acid, thiobarbituric acid, bovine serum albumin, butylated hydroxyanisole, sodium hydroxide, and hydrogen peroxide were also procured from SRL, India.

### Collection of plant fruits and their extraction

Fruits of *T. bellirica* were bought from Prayagraj local market and ground into a fine powder. A total of 100 g fruit powder was sequentially extracted with ethyl acetate and water in Soxhlet apparatus[28]. The extract was dried under reduced pressure.

### Experimental animals

Wistar rats (either sex) of a similar age group (weight 150-200 g) were used in the experiments. They were maintained in a temperature (24 ± 2°C) and humidity (40% ± 5%) controlled environment with a 12 h light/dark cycle. Rats were provided with a standard rodent diet and water *ad libitum*. The study was carried out according to the Guidelines of Institutional Animal Ethics Committee, University of Allahabad, India in agreement with the Committee for the Purpose of Control and Supervision of Experiments on Animals.

### Experimental design and treatment schedule

The rats were distributed into six groups with five rats in each group. These groups were as follows: Group-I - normal rats; Group-II - ACF treated (15 mg/kg); Group-III - ACF treated rats administered with the standard drug (Silymarin 40 mg/kg); Group-IV- ACF treated rats administered with ellagic acid (40 mg/kg); Group-V- ACF treated rats co-administered with the aqueous extract (200 mg/kg) and Group-VI- ACF treated rats co-administered with the ethyl acetate extract (200 mg/kg) of *T. bellirica* fruit. During the experimental period, all the rats received a single oral dose of the drugs or extracts or the combinations thereof for 21 d.

### Measurement of body weight and relative weight

The weight of the rats was measured before administration of the drugs and test compounds every day until sacrifice. The relative liver weight was also determined after sacrifice by the formula: Relative liver weight = [liver weight/body weight] × 100.

### Blood collection

4 mL blood was drawn by puncturing the rat's heart. Of which, 2.5 mL blood was used for clot formation and serum was separated at 2500 rpm for 10 min. The remaining blood was placed into anticoagulant ampoules and kept in a cold environment before processing. The plasma was separated after centrifugation at 3000 rpm for 10 min. Both the serum and plasma were stored at -70 °C for further analysis.

### Assessment of in vitro antioxidant activity

**Metal ion chelating activity:** Ferrous ion chelation by the *T. bellirica* fruit AQ and Eth extracts was assessed using the method described by Dinis *et al*[29] with minor modifications[30]. Extracts were dissolved in distilled water instead of methanol. A small amount (200 µL) of extract samples was combined with ferric chloride (50 µL, 2 mmol/L). Ferrozine (200 µL, 5 mmol/L) was added to start the reaction followed by vigorous shaking, and then left for 10 min at room temperature. Butylated hydroxytoluene was used as a positive control. Absorbance was recorded at 562 nm. Chelating activity was estimated by the following formula:

% Metal ion chelating ability = [(A0-A1)/A1] × 100.

Where A0 and A1 are absorbance of the control and test samples, respectively.

**Nitric oxide radical scavenging activity:** Nitric oxide (NO) radical scavenging activity was determined by the method of Green *et al*[31]. To test extracts (0.5 mL), 1.0 mL of sodium nitroprusside (0.01 mol/L in PBS) was added and incubated at 25°C for 3 h followed by the addition of an equal volume of Griess reagent and left for 30 min at room temperature. The concentration of test compounds ranged between 10-100 µg/mL in the final reaction mixture. Ascorbic acid was used as a standard and absorbance was measured at 546 nm. The NO radical scavenging activity was determined using the formula:

% NO scavenging = [(Ac-As)/Ac] × 100.

Ac and As designate absorbance values of the control and test samples, respectively.

**Lipid peroxidation inhibition assay:** Lipid peroxidation inhibition (LPOI) by the *T. bellirica* fruit extracts was measured by the method of Halliwell *et al*[32] in 10% rat liver homogenate. BHA was used as a control. The % LPOI was determined using the following formula:

% Lipid peroxidation inhibition = [(A0-A1)/A0] × 100.

Where A0 and A1 are the absorbance of the control and test samples, respectively at 532 nm.

**Estimation of total antioxidant activity by the ferric reducing antioxidant power assay:** The ferric reducing antioxidant power (FRAP) assay[33] is a method for measuring total antioxidant potential of test compounds. To 0.05 mL plasma, FRAP

reagent (4.5 mL) was added and absorbance was recorded at 593 nm after 5 min. The final concentration of *T. bellirica* fruit extracts in the reaction mixture was 44.44 μg/mL, while the concentration of EA and SLM was 8.89 μg/mL. Ferrous sulfate (100-1000 μmol/mL) was used to create a calibration curve and the result was expressed as the FRAP value (μM $FeSO_4 \cdot 7H_2O$ equivalent/L plasma).

### Biochemical analysis

**Assessment of liver function markers in serum:** The biochemical parameters including SGPT, SGOT, GGT, LDH, ALP, total protein, uric acid, and creatinine were assayed using commercially available kits (Erba Diagnostics Kits).

**Preparation of liver tissue homogenate:** The liver tissue homogenate (10% w/v) was prepared in phosphate buffer (0.1 mol/L, pH-7.4 with 0.15 mol/L KCl). Crude homogenate was centrifuged (1000 × *g* for 30 min, 4°C) and the supernatant was used for estimation of antioxidant enzymes and other biochemical analytes.

### Assessment of antioxidant status in tissue homogenate

**Estimation of malondialdehyde (MDA) in liver homogenate:** Lipid peroxidation was assayed in tissue homogenate using the method of Niehaus and Samuelsson[34]. Thiobarbituric acid reagent (2 mL) was added to tissue homogenate (100 μL) and the content was boiled for 1 h followed by measurement of absorbance at 532 nm. The peroxidation product was represented as nM MDA/mg protein using the extinction coefficient of $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$.

**Determination of total protein in liver tissue homogenate:** The total protein present in the liver homogenate was measured by the method of Lowry *et al*[35].

**Superoxide dismutase activity:** The superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund[36]. One unit of enzyme activity represents 50% inhibition of pyrogallol autooxidation per min.

**Catalase activity:** The catalase (CAT) activity was measured by assessing the reduction in the absorbance of $H_2O_2$ at 240 nm for 3 min at the interval of 30 s[37]. One unit of CAT activity is defined as micromoles of $H_2O_2$ disintegrated per min using the molar absorbance of $H_2O_2$ (43.6 $M^{-1}$ $cm^{-1}$).

**Histological analysis of liver:** The liver biopsies from rats were fixed in 10% formalin, dehydrated in graded alcohol, and then embedded in paraffin wax blocks. The paraffin-block was sliced (5 μm) successively using a rotary microtome. The liver slices were stained with hematoxylin and eosin (H and E) on albumin-coated sterilized glass slides[38]. After mounting in DPX, the sections were studied for histological changes under a light microscope (× 40 magnification).

### Statistical analysis

All the experiments were performed in triplicate. Results are represented as mean ± SD. GraphPad Prism software was used to create the graphs. *P* values (< 0.05) were considered significant.

## RESULTS

### Assessment of in vitro antioxidant activity

**Metal ion chelation activity:** *T. bellirica* fruit extracts (AQ and Eth) exhibited marked concentration-dependent metal ion chelating activity (13%-85%) (Figure 1). The degree of discoloration showed the chelating efficacy of the fruit extracts. Highest chelation potential was observed for the Eth extract (85.38%, $IC_{50}$ 168 μg/mL) followed by the AQ extract (56.42%, $IC_{50}$ 220 μg/mL). Butylated hydroxytoluene showed 90% metal ion chelation activity at a concentration of 100 μg/mL.

**Nitric oxide radical scavenging activity:** NO radical scavenging activity of *T. bellirica* fruit extracts was evaluated at different concentrations (10-100 μg/mL) and the results were expressed in terms of % NO radical scavenging activity (Figure 2). Considerable radical scavenging activity was observed in the test compounds during *in vitro* assay. The AQ extract exhibited comparatively lower NO radical scavenging activity (16%-66%, $IC_{50}$ 70 μg/mL) than the Eth extract (26%-83%, $IC_{50}$ 48 μg/mL) at all test

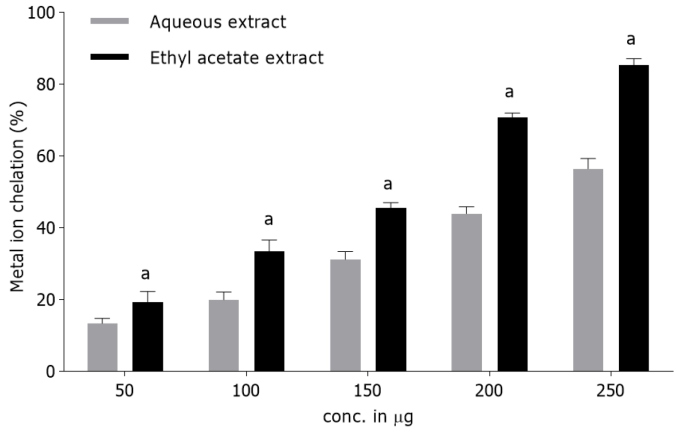


**Figure 1 *In vitro* metal ion chelating activity of *Terminalia bellirica* fruit aqueous and ethyl acetate extracts.** The chelating activity was measured at different concentrations (50-250 µg/ml). Butylated hydroxytoluene was included for comparison and absorbance was measured at 562 nm. Results are presented as mean ± SD of triplicates. $^{a}P < 0.05$ as compared to butylated hydroxytoluene.

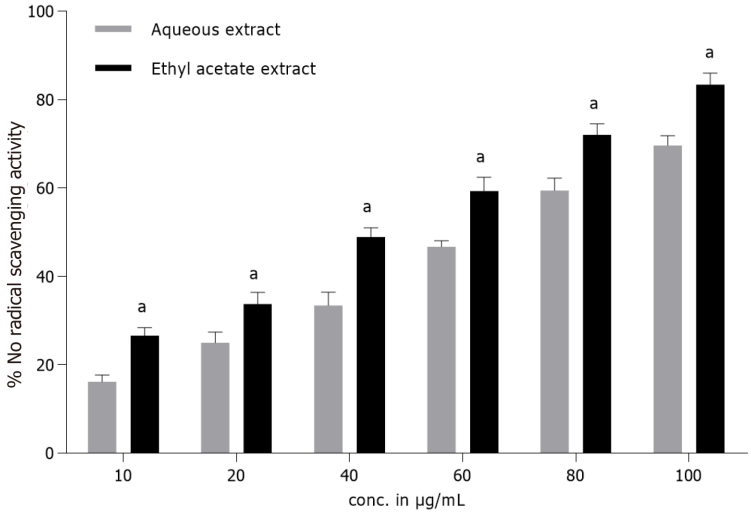


**Figure 2 *In vitro* nitric oxide radical scavenging activity of *Terminalia bellirica* fruit aqueous and ethyl acetate extracts at different concentrations.** Butylated hydroxytoluene was included as a standard and absorbance was measured at 546 nm. Results are presented as mean ± SD of triplicates. $^{a}P < 0.05$ as compared to butylated hydroxytoluene.

concentrations. BHA (0.33-3.3 µg/mL) accounted for 37%-84% activity.

**Lipid peroxidation inhibition activity**: Fruit extracts of *T. bellirica* displayed dose-dependent anti-lipid peroxidative activity during *in vitro* assay. The Eth extract exhibited comparatively higher inhibitory response against $Fe^{2+}$-triggered lipid peroxidation in liver homogenate signifying its lipo-protective efficacy. The LPOI values for the Eth and AQ extracts at a concentration of 5 mg/mL were 75% and 63%, respectively (Figure 3). However at lower concentration (1 mg/mL), the LPOI values for the Eth and AQ extracts were about 23% and 15%, respectively. Standard antioxidant BHA (2 mg/mL) under similar experimental conditions produced about 85% inhibition of lipid peroxidation.

### *Ameliorative effect of fruit extracts and ellagic acid on aceclofenac toxicity in vivo*

**Assessment of change in body weight and relative liver weight:** A noteworthy decline in body weight was observed in ACF-treated rats (group II) in comparison with untreated rats (group I) (Table 1). Group I rats showed approximately 18.83% gain in body weight during the same time period. The percentage loss in body weight in group II rats (20.50%) was markedly higher than that in group I ($P < 0.0001$) and groups III-V ($P < 0.005$). Co-administration of EA and *T. bellirica* fruit extracts (AQ and

**Table 1 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on body weight and liver weight of aceclofenac treated rats**

| Groups | Body weight, change (%) | Absolute liver, weight (g) | Relative liver, weight (%) |
|---|---|---|---|
| Group I | 18.83 ± 1.15 | 4.62 ± 0.16 | 2.85 ± 0.29 |
| Group II | -20.50 ± 6.87[a] | 6.56 ± 0.41[a] | 3.95 ± 1.29[a] |
| Group III | -09.17 ± 3.78[b] | 4.82 ± 0.19[b] | 3.05 ± 0.35[b] |
| Group IV | -11.06 ± 3.77[b] | 5.17 ± 0.87[b] | 3.13 ± 0.46[b] |
| Group V | -18.50 ± 6.26[b] | 5.70 ± 1.09[b] | 3.44 ± 0.58[b] |
| Group VI | -14.83 ± 3.21[b] | 5.03 ± 0.95[b] | 3.21 ± 0.64[b] |

Group I: Control rats; Group II: Aceclofenac (ACF) treated rats; Group III: ACF + Silymarin treated rats; Group IV: ACF + Ellagic acid treated rats; Group V: ACF + Aqueous extract treated rats; Group VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD ($n$ = 5). Silymarin was used as a positive control.

[a]Represents a significant difference compared with the control ([a]$P$ < 0.0001).

[b]Represents a significant difference compared with group II ([b]$P$ < 0.005).

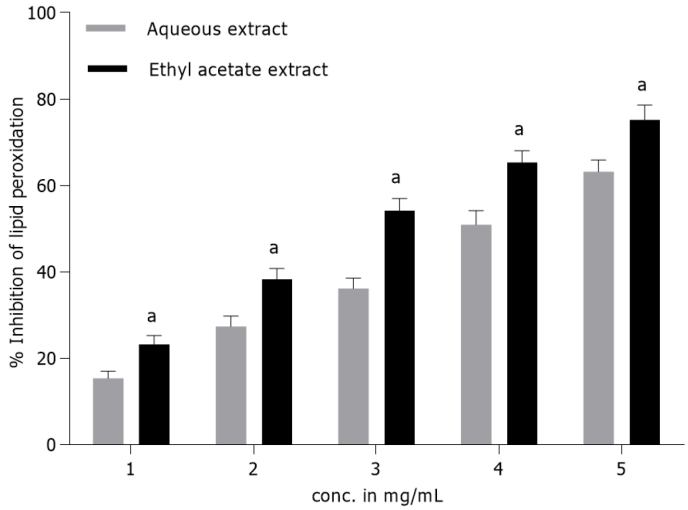


**Figure 3 Percentage inhibition of lipid peroxidation in rat liver homogenate by *Terminalia bellirica* fruit extracts at different concentrations.** Butylated hydroxytoluene (2 mg/mL) was included for comparison and accounted for approximately 85% lipid peroxidation inhibition. The results are shown as mean ± SD of triplicates ([a]$P$ < 0.05).

Eth) with ACF exhibited a restorative effect (81%-89% recovery) on body weight. Standard drug SLM showed maximum recovery potential (90.83%) followed by EA (88.94%), Eth (85.17%) and AQ (81.50%). Furthermore, an inverse correlation was observed between body weight and relative liver weight. Relative liver weight increased from 2.85% in the control to 3.95% in ACF treated rats ($P$ < 0.0001), while treatment with EA and *T. bellirica* fruit extracts (Eth and AQ) showed a restorative effect on the liver weight of rats. In comparison to group II rats, the recovery following administration of EA and *T. bellirica* fruit extracts was statistically significant ($P$ < 0.005) (Table 1).

**Assessment of total antioxidant activity by FRAP Assay:** The therapeutic effect of *T. bellirica* fruit extracts (Eth and AQ) and EA on plasma FRAP are shown in Figure 4. In group II rats, the administration of ACF resulted in a marked decrease ($P$ < 0.05) in plasma FRAP (5.76 μmol/L) as compared to group I. This indicated a reduction in the antioxidant potential of plasma with a simultaneous rise in oxidative stress. Co-administration of EA (group IV), AQ (group V) and Eth (group VI) with ACF caused a significant improvement ($P$ < 0.05) in plasma antioxidant capacity.

**Assessment of change in serum markers:** The measurement of various markers of hepatic function is used in the diagnosis and treatment of a variety of diseases. The effects of ACF and test compound combination (*T. bellirica* fruit extracts and EA) on serum biomarkers including total protein, creatinine, urea, SGOT, SGPT, LDH, GGT,

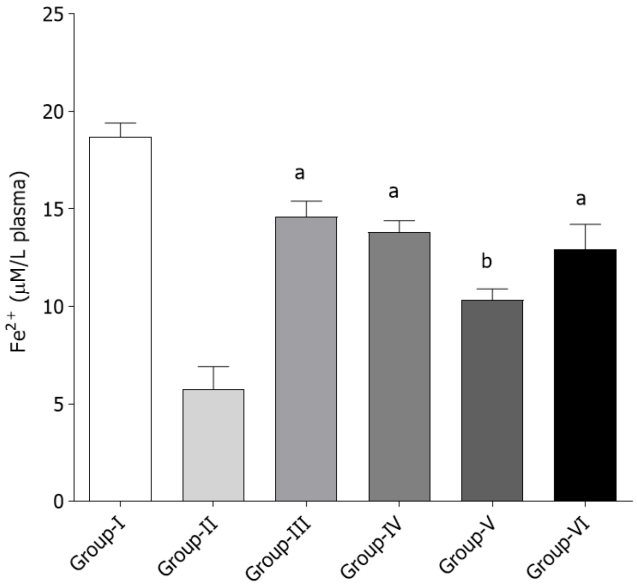


**Figure 4 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on the ferric reducing ability of plasma in aceclofenac-treated rats.** Group I-normal control; Group II-Aceclofenac (ACF) treated rats; Group III-ACF + Silymarin treated rats; Group IV-ACF + Ellagic acid treated rats; Group V- ACF + Aqueous extract treated rats; Group VI: ACF + Ethyl acetate extract treated rats. Silymarin was used as a positive control. The data are represented as mean ± SD ($n$ = 5, $^{a}P < 0.01$; $^{b}P < 0.05$ as compared to group II).

ALP and uric acid are shown in Table 2. Oral administration of ACF led to an elevation in serum creatinine, uric acid, SGOT, SGPT, ALP, LDH, and GGT (group II) ( $P < 0.05$). Co-administration of extracts/EA/SLM with ACF resulted in marked restoration of these biochemical indices.

**Assessment of MDA in liver tissue:** ACF administration for three weeks caused an approximate seven-fold rise in MDA level (18.63 nmol/mg protein) in liver tissues as compared to the control (2.68 nmol/mg protein) (Table 2). SLM treatment reduced the level of MDA by up to 1.7-fold (4.5 nmol/mg protein) in group III rats. *T. bellirica* fruit AQ and Eth extract-treated groups also accounted for a noteworthy reduction in hepatic tissue MDA level (12.28 and 9.46 nmol/mg protein, respectively). Furthermore, co-administration of EA with ACF resulted in a comparatively better recovery in hepatic MDA level (6.74 nmol/mg protein) as compared to *T. bellirica* fruit extracts.

**Assessment of antioxidant enzyme in liver tissue homogenate:** ACF treatment in group II rats caused a noteworthy reduction ($P < 0.05$) in hepatic antioxidant enzyme activity *i.e.*, SOD (12.04 U/mg protein) and catalase (3.21 U/mg protein) as compared to the control group (SOD-31.09 U/mg protein and catalase-8.45 U/mg protein). Appreciable restoration ($P < 0.05$) in hepatic tissue catalase activity was observed in the EA-treated groups (7.19 U/mg protein) followed by the Eth (6.23 U/mg protein) and AQ (5.77 U/mg protein) groups as compared with group II. Moreover, co-administration of EA and Eth and AQ extracts with ACF also caused appreciable enhancement ($P < 0.05$) in SOD enzyme activity (27.24, 21.15, 19.80 U/mg protein, respectively). It was observed that EA showed comparatively similar enzymatic activity to SLM treatment (29.11 U/mg protein) (Table 2).

**Histopathological changes in liver:** Histological sections of the normal rat liver slices showed intact hepatocytes with sinusoidal spaces and evenly distributed cytoplasm (Figure 5A). Oral administration of ACF resulted in severe hepatic damage as confirmed by immense hepatocellular deterioration, necrosis, sinusoidal dilatation, infiltration of inflammatory cells and cytoplasmic vacuolation (Figure 5B). However, treatment with EA and *T. bellirica* fruit extracts in ACF treated rats reduced hepatic damage and associated alterations and thereby improved liver structure and function (Figure 5D-F). Administration of SLM displayed relatively higher hepatoprotective efficacy (Figure 5C). The histopathological improvement observed in liver sections with EA, *T. bellirica* extracts and SLM in ACF treated rats had a direct correlation with liver weight, body weight and serum liver function markers along with tissue

**Table 2 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on serum hepatic function markers in aceclofenac treated rats**

| Groups | Total protein (g/dL) | ALP (IU/L) | SGOT (IU/L) | SGPT (IU/L) | Uric acid (mg/dL) | Creatinine (mg/dL) | LDH (IU/L) | GGT (IU/L) |
|---|---|---|---|---|---|---|---|---|
| Group I | 6.05 ± 0.45 | 87.16 ± 14.19 | 69.37 ± 10.24 | 58.19 ± 09.13 | 1.71 ± 0.37 | 0.80 ± 0.33 | 304.76 ± 10.23 | 4.27 ±1.08 |
| Group II | 3.64 ± 0.14[a] | 157.32 ± 26.16[a1] | 165.40 ± 12.51[a1] | 158.43 ± 8.24[a1] | 6.87 ± 0.22[a] | 3.12 ± 1.31[a] | 689.34 ± 11.21[a1] | 9.29 ± 2.15[a1] |
| Group III | 5.72 ± 0.17[b1] | 93.49 ± 9.08[b1] | 85.21 ± 16.34[b1] | 69.09 ± 4.71[b1] | 2.32 ± 0.13[b1] | 1.37 ± 0.18[b1] | 401.19 ± 3.89[b1] | 5.87 ± 0.24[b] |
| Group IV | 5.52 ± 0.31[b1] | 96.65 ± 11.25[b1] | 89.56 ± 9.13[b1] | 74.98 ± 11.38[b1] | 2.34 ± 0.24[b1] | 1.49 ± 0.17[b1] | 449.54 ± 15.09[b1] | 6.18 ± 0.51[b1] |
| Group V | 4.75 ± 0.38[b2] | 121.49 ± 16.13[b2] | 140.17 ± 4.75[b1] | 132.11 ± 14.17[b1] | 3.38 ± 0.52[b2] | 2.72 ± 0.18[b2] | 587.37 ± 7.94[b2] | 7.69 ± 0.27[b2] |
| Group VI | 5.60 ± 0.11[b2] | 107.17 ± 12.37[b1] | 113.6 ± 4.89[b1] | 109.25 ± 04.85[b1] | 2.53 ± 0.19[b2] | 2.16 ± 0.06[b1] | 505.18 ± 11.45[b1] | 6.68 ± 0.39[b2] |

Group-I: Control rats; Group-II: Aceclofenac (ACF) treated rats; Group-III: ACF + Silymarin treated rats; Group-IV: ACF + Ellagic acid treated rats; Group-V: ACF + Aqueous extract treated rats; Group-VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD ($n$ = 5). Silymarin was used as a positive control. ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; GGT: Glutamyl transferase.

[a]Represents a significant difference compared with the control.

[a1]$P < 0.0001$.

[b]Represents a significant difference compared with group II.

[b1]$P < 0.0005$.

[b2]$P < 0.005$.

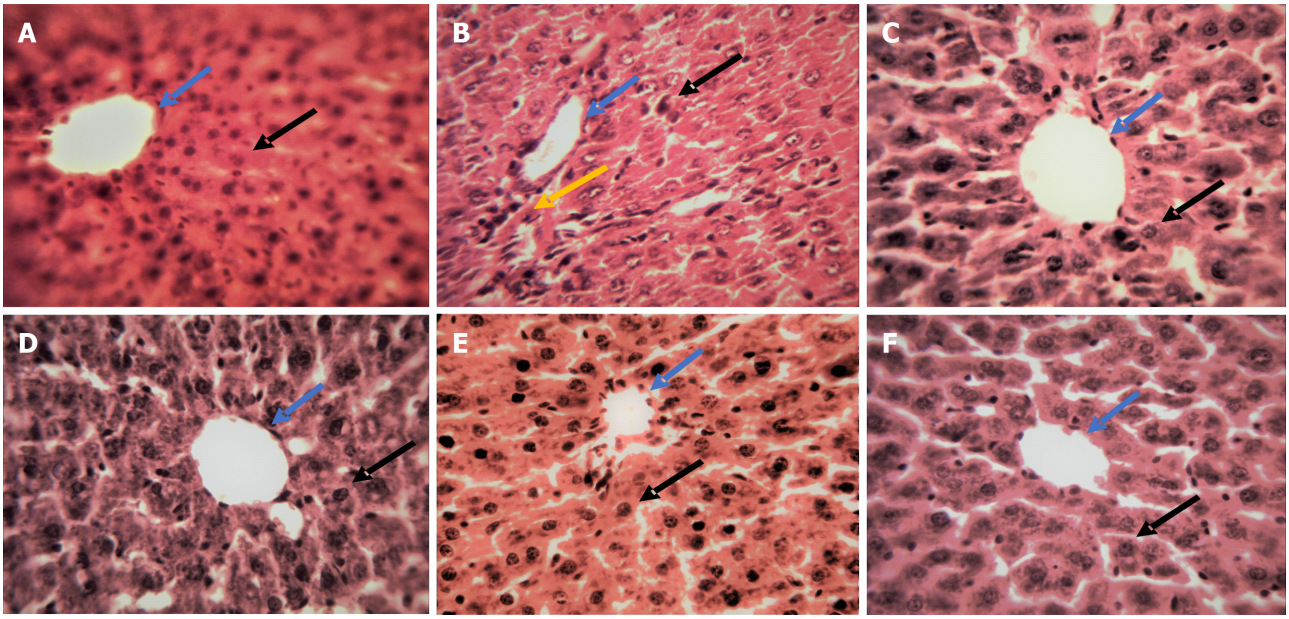


**Figure 5 Histopathological changes in liver slices after oral administration of *Terminalia bellirica* fruit extracts, ellagic acid and Silymarin in Aceclofenac-treated rats.** A: Control; B: Aceclofenac (ACF) treated rats; C: ACF + Silymarin treated rats; D: ACF + Ellagic acid treated rats; E: ACF + Aqueous extract treated rats; and F: ACF + Ethyl acetate extract treated rats. The blue color arrow represents central vain, black arrow represents a nucleus and the yellow arrow represents inflammatory cells.

antioxidants.

## DISCUSSION

The antagonistic properties of drugs and synthetic antioxidants have drawn the attention of scientists to explore new sources of natural antioxidants and hepatoprotectants which are more potent in mitigating oxidative stress and averting

the initiation of disease[4,39]. Antioxidants hinder the oxidation of critical biomolecules by preventing the cascade of oxidizing chain reactions[5]. Interestingly, few studies on the antioxidant properties of *T. bellirica* fruit have been carried out[16,22,40]. However, this is the first study to assess the antioxidant and hepatoprotective attributes of *T. bellirica* fruit extracts and its constituent EA against ACF-induced liver injury in albino Wistar rats.

Antioxidant evaluation of *T. bellirica* fruit Eth and AQ extracts showed significant radical scavenging activities during *in vitro* metal ion chelation and NO radical scavenging assays. It was measured by a pink color complex formed due to ferrozine and $Fe^{2+}$ interaction. Metal chelating ability showed the potential of the test compounds to protect lipids from oxidative damage[2]. In the present study, *T. bellirica* fruit Eth extract ($IC_{50}$ 168 μg/mL) showed higher ion chelating ability as compared to the AQ extract ($IC_{50}$ 220 μg/mL) (Figure 1). The chelation process promotes the lipophilicity of the metal ion and thereby favoring its penetration through the lipoid membrane. This action diminishes the production of $OH^{\bullet}$ radical and thus averts the beginning of lipid peroxidation[41]. Previous studies have also confirmed the positive association between metal chelation and lipoprotective activities[42]. Moreover, it has been recognized that chelating agents act as secondary antioxidants by forming bonds with metals thus lowering their redox potential and stabilizing the oxidized state of the metal ion[43].

Nitric oxide is required during inflammatory processes but higher concentrations are toxic to tissues including vascular damage and other ailments. Sodium nitroprusside in the presence of oxygen generates nitrite ions at physiological pH, which is analyzed by a specific method[44]. The nitrite radical undergoes diazotization reaction with sulfanilamide and subsequent coupling with naphthyl ethylene diamine generates pink chromophore. The radical scavenging activity of fruit extracts may be attributed to its competition with oxygen to react with nitric oxide[45]. In the experiment, the Eth extract (83%, $IC_{50}$ 48 μg/mL) showed comparatively better NO scavenging activity than the AQ extract (74%, $IC_{50}$ 57 μg/mL) (Figure 2). The occurrence of p-hydroxyl groups in the aromatic ring structure and conjugated double bonds that make the electrons more delocalized are structural prerequisites for potent radical scavenging action by the extracts and BHA. The p-hydroxy system possesses electron-donating properties and is a radical target. The number, positions of OH-groups and the type of group replacements are mainly accountable for phenylpropanoids functioning as effective antioxidant[46], anti-inflammatory, enzyme modulator or antiproliferative agents[47].

Lipid peroxidation is a free radical-triggered redox process associated with inflammation and biochemical changes in the lipids[42,48]. It rapidly starts with the action of hydroxyl radicals generated during the Fenton reaction in the presence of iron (II)[49]. In this study, the Eth extract (5 mg/mL) exhibited a 75% decrease in peroxidation product suggesting its lipoprotective ability, while comparatively less activity was observed with the AQ extract (63%) (Figure 3). The protection accorded by the test extracts could be ascribed to the metal ion ($Fe^{3+}$) chelation which is crucial for the production of hydroxyl radicals[41]. The antioxidants break the oxidation chain reaction initiated by free radicals through transfer of reducing equivalents ($H^{+}$) from the phenolic hydroxyl groups, thus producing a stable end product that does not promote further lipid oxidation. *T. bellirica* fruit has been shown to possess potent chelating ability and therefore it may exhibit appreciable inhibitory action on lipid peroxidation[40]. These results are corroborated by a recently published study from our laboratory, which advocated that *T. bellirica* fruit Eth extract had comparatively higher antioxidant activity than the AQ extract during *in vitro* analysis[16].

FRAP provides a direct assessment of the antioxidant or reducing capacity of the samples. Reduction of $Fe^{3+}$-TPTZ complex to $Fe^{2+}$-TPTZ complex by the test compounds is the basis for measurement of this ability producing a blue color which is measured at 593 nm[33]. The absorbance of the reaction mixture is directly correlated with the reducing ability of the sample. The FRAP value is an indicator of the hydrogen or electron-donating ability of test samples[50]. The FRAP value shown by the EA treated rat group was significantly higher ($P < 0.05$) than that in the *T. bellirica* fruit extract treated groups (Figure 4).

Phytoconstituents isolated from *T. bellirica* fruit were previously reported to be antioxidant, anti-inflammatory and hepatoprotective agents. Triterpenoidal compounds (*e.g.*, oleanolic and ursolic acids) are extensively found in food and therapeutic herbs[51]. The hepatoprotective efficacy of oleanolic acid against carbon tetrachloride ($CCl_4$) and ursolic acid against ethanol-induced liver injury has been confirmed[52,53]. Both compounds individually exhibited significant *in vitro* antioxidant and anti-inflammatory activities in PC12 cell lines exposed to 1-methyl-4-

phenylpyridinium ion or $H_2O_2$[54]. Moreover, oleanoic acid triggered expression of phase II response genes and stimulated the antioxidant enzymes and transcription factor (Nrf2)[55]. It also blocked the NF-κB pathway which was further substantiated by a high binding affinity towards NF-κB subunits (p50 and p52), TNF-α and COX-2 during *in silico* experiments[55,56].

The pharmacological activity of *T. bellirica* fruit extracts evaluated in this study can be accredited mainly to the higher amount of phenolic compounds and flavonoids. Tannins are phenolic compounds responsible for the bitter taste of foods and beverages[57]. A significant correlation between tannin content and total antioxidant activity has been established previously[58]. Additionally, tannins extracted from the acetone extract of natural products showed potent antioxidant properties compared with the low molecular-weight phenolic compounds derived from the same plant samples[57]. Recently, researchers evaluated the hepatoprotective and antioxidant attributes of gallic acid and EA against $CCl_4$-induced hepatic injury in mice[59,60]. Gallic acid produced superior DPPH scavenging activity than EA[59] and methyl gallate[61]. This activity could be ascribed to the availability of a free carboxyl group.

Administration of ACF for 21 d led to a fall in body weight demonstrating the adverse effect of long-term treatment in Wistar rats. It has been reported that AFC caused gastric ulcers in an animal model leading to difficulty in food intake that culminated in malnourishment[62]. The unusual rise in liver weight in ACF treated rats seems to be due to its toxic behavior. Previous studies suggested that a decline in body weight during hepatic injury is associated with the interplay of adiponutrin and abdominal fat[63]. However, treatment with *T. bellirica* fruit extracts (Eth and AQ) and EA suppressed the toxic effect of ACF in rats and improved body weight. This result was more pronounced in SLM treated rats.

The activity of reactive oxygen species (ROS), mitochondrial stress, immune response, and idiosyncratic reactions are the prime causes of hepatic injury resulting from prolonged use of ACF. However, the precise mechanism of its toxic behavior is still unclear[7]. Altered serum and tissue biomarkers along with histological changes are clinical indicators of liver damage. Abnormally high levels of SGOT, SGPT, ALP, uric acid, creatinine, LDH and GGT in the circulation are directly correlated with severe liver injury[6,11]. In the current study, prolonged ACF intake caused hepatic damage in rats, as evidenced by a marked rise in serum and tissue markers as compared to the control rats. These study results are in line with previous studies[7,64]. The findings suggested that *T. bellirica* fruit Eth and AQ extract (200 mg/kg) and EA (40 mg/kg) treatment reduced ACF-induced elevations in the levels of these parameters towards the normal range revealing improvement in hepatic function (Table 2). Similar results have been observed in SLM treated rats.

The intracellular antioxidant enzymes such as SOD and CAT act as the first line of defense against oxidative damage in hepatic tissue. SOD catalyzes the dismutation of superoxide ($O_2^-$) radical into $H_2O_2$ and oxygen, which is one of the chief cellular defense mechanisms[3]. Reduced SOD and CAT activities in ACF administered rats indicated the diminished potential of ROS scavenging action. In ACF fed rats, treatment with *T. bellirica* fruit extracts and EA enhanced the level of SOD in hepatic tissue (Table 3). A similar trend was also observed in the SLM treated group. Additionally, CAT also oozes into the extracellular fluid as a result of tissue damage. Inside the cell it has the potential to act as a strong antioxidative agent, and thus increases cell survival[1]. Decreased CAT activity in ACF-fed rats revealed lowered tissue protection ability. Co-administration of *T. bellirica* fruit extracts and EA in ACF-treated rats significantly enhanced the activity of CAT (Table 3). Previous studies on EA suggested that the hepatoprotective activity may be associated with its antioxidant and radical scavenging properties. EA might be responsible for increasing enzymatic protein synthesis or reducing the ROS-mediated loss of CAT activity[65]. It seems possible that CAT might act as an essential autocrine antioxidant and survival element. Furthermore, ROS produced as an offshoot of ACF treatment, also destroy liver tissues by stimulating lipid peroxidation. Increased level of MDA confirmed the higher rate of lipid peroxidation causing tissue damage and breakdown of cellular antioxidant defense systems[66-68]. Co-administration of *T. bellirica* fruit extract (200 mg/kg) and EA (40 mg/kg) with ACF appreciably reduced lipid peroxidation in rats.

Histological analysis of ACF-treated rat liver slices also corroborated abnormal alterations observed in serum markers and tissue antioxidants. ACF produced notable impairment in the anatomical features of liver tissue encompassing pathological irregularities such as inflammatory infiltration, the formation of vacuoles and focal necrosis. *T. bellirica* fruit extracts (Eth and AQ) and EA treatment significantly lowered the number of damaged hepatocytes. Furthermore, the hepatoprotective potential of extracts and EA was also confirmed by the dearth of necrotic and inflammatory lesions

**Table 3 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on antioxidant markers in liver tissue homogenate of aceclofenac treated rats**

| Groups | MDA (nmol/mg protein) | SOD (U/mg protein) | Catalase (U/mg protein) |
|---|---|---|---|
| Group I | 2.68 ± 0.08 | 31.09 ± 0.71 | 8.45 ± 0.17 |
| Group II | 18.63 ± 1.43[a] | 12.04 ± 1.49[a] | 3.21 ± 0.84[a] |
| Group III | 4.50 ± 0.18[b1] | 29.11 ± 0.35[b1] | 7.59 ± 0.41[b1] |
| Group IV | 6.74 ± 0.14[b1] | 27.24 ± 0.57[b1] | 7.19 ± 0.37[b1] |
| Group V | 12.28 ± 0.12[b2] | 19.80 ± 0.62[b2] | 5.77 ± 1.19[b2] |
| Group VI | 9.46 ± 0.19[b1] | 21.15 ± 0.49[b2] | 6.23 ± 0.92[b2] |

Group-I: Control rats; Group-II: Aceclofenac (ACF) treated rats; Group-III: ACF + Silymarin treated rats; Group-IV: ACF + Ellagic acid treated rats; Group-V: ACF + Aqueous extract treated rats; Group-VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD ($n$ = 5). Silymarin was used as a positive control. SOD: Superoxide dismutase; MDA: Malondialdehyde.

[a]Represents a significant difference compared with the control ([1]$P$ < 0.0001).

[b]Represents a significant difference compared with group II.

[b1]$P$ < 0.0001.

[b2]$P$ < 0.005.

in rat tissue sections. Comparatively better hepatoprotective efficacy was observed in SLM and EA treated rats.

## CONCLUSION

The results of the current study suggest that the administration of *T. bellirica* fruit extracts and EA have considerable hepatoprotective efficacy against ACF-induced oxidative stress and hepatic damage in Wistar rats. ACF adversely altered the levels of serum liver function markers and tissue antioxidants. Abnormal levels of biomarkers might be the result of peroxidation reactions and biotransformation of ACF in liver. These reactions are accountable for oxidative damage to cellular components. *T. bellirica* fruit extracts and EA treatment significantly ameliorated the hepatic injury inflicted by ACF. However, further evaluation is warranted to reveal the complete mechanism of action of different phytoconstituents present in *T. bellirica* fruit which might be helpful in the development of a new therapeutic agent of natural origin.

## ARTICLE HIGHLIGHTS

### *Research background*

Hepatotoxicity is one of the common side effects of nonsteroidal anti-inflammatory drugs (NSAIDs). Aceclofenac , a prodrug in the aryl-acetic acid class, is an oral NSAID effective in the treatment of painful inflammatory diseases. Chronic use of aceclofenac damages gastrointestinal mucosa by irritant action, causing an alteration in mucosal permeability and/or suppression of prostaglandin synthesis.

### *Research motivation*

Previous studies on *Terminalia bellirica* fruit revealed that it possesses a wide range of bioactive compounds that are accountable for its antioxidant and radical scavenging potential against various types of experimental models. Moreover, its fruit has a positive impact on NSAIDs-induced liver injury. Therefore, in this study we explored the therapeutic attributes of *T. bellirica* fruit and ellagic acid against aceclofenac-induced hepatotoxicity and oxidative stress.

### *Research objectives*

The major objectives were to evaluate the antioxidant and hepatoprotective activities of *T. bellirica* fruit ethyl acetate (Eth) and aqueous (AQ) extracts and its constituent ellagic acid against aceclofenac-induced hepatotoxicity in albino Wistar rats.

### Research methods

The antioxidant activities of *T. bellirica* fruit extracts were measured i*n vitro* by metal ion chelation and nitric oxide radical scavenging assays. The *in vivo* antioxidant and hepatoprotective effects of *T. bellirica* extracts (200 mg/kg) and ellagic acid (40 mg/kg) in aceclofenac-induced hepatotoxic rats were evaluated in serum and liver tissue. The ferric reducing ability of plasma and lipid peroxidation inhibition were measured in blood. Liver function markers such as ALP, GPT, GOT, LDH, γ-glutamyl transferase, creatinine, total protein, and uric acid were evaluated in rat serum and superoxide dismutase, catalase and malondialdehyde were analyzed in liver tissues using standard protocols.

### Research results

*T. bellirica* fruit Eth extract ($IC_{50}$ 168 μg/mL) showed higher ion chelating ability than the AQ extract ($IC_{50}$ 220 μg/mL). Similarly, the Eth extract ($IC_{50}$ 48 μg/mL) showed comparatively better NO scavenging activity than the AQ extract ($IC_{50}$ 57 μg/mL). The Eth extract also decreased lipid peroxidation by 75% indicating its lipoprotective ability. The ferric reducing ability of plasma value in the EA treated rat group was significantly higher ($P < 0.05$) than that in the *T. bellirica* fruit extract treated groups *in vivo*. *T. bellirica* fruit extracts and ellagic acid treatment suppressed the toxic effect of aceclofenac in rats and improved the body weight coupled with restoration of serum liver function markers and tissue specific antioxidants.

### Research conclusions

The results of the current study suggest that the administration of *T. bellirica* fruit extracts and ellagic acid exhibited considerable hepatoprotective efficacy against aceclofenac-induced oxidative stress and hepatic damage in Wistar rats. Abnormal levels of biomarkers may have occurred due to peroxidation reactions and biotransformation of aceclofenac in liver. These reactions were responsible for oxidative damage to cellular components. *T. bellirica* fruit extracts and ellagic acid treatment significantly ameliorated the hepatic injury induced by aceclofenac.

### Research perspectives

*T. bellirica* fruit extracts and its phytoconstituent ellagic acid exhibited appreciable radical scavenging, antioxidant and hepatoprotective activity in aceclofenac-induced liver injury. However, further evaluation is warranted to reveal the complete mechanism of action of different phytoconstituents present in *T. bellirica* fruit which might be helpful in the development of a new therapeutic agent of natural origin.

## ACKNOWLEDGEMENTS

Both authors gratefully acknowledge UGC-SAP and DST-FIST facilities of the Department of Biochemistry, University of Allahabad, Prayagraj.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 965-975

DOI: 10.4254/wjh.v12.i11.965

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Basic Study

# Obeticholic acid attenuates human immunodeficiency virus/alcohol metabolism-induced pro-fibrotic activation in liver cells

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**Author contributions:** New-Aaron M and Dagur RS performed the experiments, acquired, analyzed and interpreted the data; New-Aaron M and Osna NA drafted the manuscript; Ganesan M, Kharbanda KK and Poluektova LY contributed to critical revision, editing of the manuscript; Osna NA contributed to conception, design and funding of the study; all authors approved the final version of the article.

**Supported by** The National Institutes of Health, No. R01AA027189-01A1.

**Institutional review board statement:** The study was reviewed and approved by the Subcommittee for Research and Development Review Board at Veterans Affairs Medical Center, Omaha, United States.

**Conflict-of-interest statement:** Moses New-Aaron, Murali Ganesan, Raghubendra Singh

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## Abstract

### *BACKGROUND*

The morbidity and mortality of human immunodeficiency virus (HIV)-infection is often associated with liver disease, which progresses slowly into severe liver dysfunction. There are multiple insults which exacerbate HIV-related liver injury, including HIV-associated dysregulation of lipid metabolism and fat turnover, co-infections with hepatotropic viruses and alcohol abuse. As we reported before, exposure of hepatocytes to HIV and alcohol metabolites causes high oxidative stress, impairs proteasomal and lysosomal functions leading to accumulation of HIV in these cells, which end-ups with apoptotic cell death and finally promotes development of liver fibrosis.

### *AIM*

To study whether obeticholic acid (OCA) prevents HIV/ethanol metabolism-induced hepatotoxicity and subsequent activation of hepatic stellate cells (HSC) by HIV$^+$ apoptotic hepatocyte engulfment.

### *METHODS*

Huh7.5-CYP (RLW) cells were exposed to HIV and acetaldehyde-generating system (AGS) in the presence or absence of OCA. In the cells, we measured the

Dagur, Kusum K. Kharbanda, Larisa Y Poluektova , Natalia A. Osna. All authors have nothing to disclose.

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

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** July 16, 2020
**Peer-review started:** July 16, 2020
**First decision:** September 14, 2020
**Revised:** September 16, 2020
**Accepted:** October 5, 2020
**Article in press:** October 5, 2020
**Published online:** November 27, 2020

**P-Reviewer:** MD DEA
**S-Editor:** Zhang H
**L-Editor:** A

expression of HIV-related markers: HIVgagRNA-by real-time polymerase chain reaction (PCR), p24- by western blot, HIV DNA-by semi-nested PCR, integrated HIV DNA-by ddPCR. Lysosomal and proteasomal activities were measured using fluorometrically-labeled substrates. For hepatocyte apoptosis, cleaved caspase 3 and cleaved PARP were visualized by western blot and cytokeratin 18- by M30 ELISA-in supernatants. Apoptotic bodies were generated from untreated and HIV-treated RLW cells exposed to UV light. Pro-fibrotic activation of HSC was characterized by Col1A1 and transforming growth factor-β mRNAs, while inflammasome activation- by NLRP3, caspase 1, interleukin (IL)-6, IL-1β mRNA levels.

*RESULTS*

In RLW cells, OCA treatment attenuated HIV-AGS-induced accumulation of HIVgagRNA, HIV DNA and p24. OCA suppressed reactive oxygen species production and restored chymotrypsin-like proteasome activity as well as cathepsin B lysosome activity. OCA also decreased HIV-AGS-triggered apoptosis in RLW cells. Exposure of HIV-containing apoptotic hepatocytes to HSC prevented activation of inflammasome and induced pro-fibrotic activation in these cells.

*CONCLUSION*

We conclude that by suppressing oxidative stress and restoring proteasomal and lysosomal functions impaired by HIV and ethanol metabolism, OCA decreases accumulation of HIV in hepatocytes, leading to down-regulation of apoptosis in these cells. In addition, OCA reverses pro-fibrotic and inflammasome-related activation of HSC triggered by engulfment of HIV-containing apoptotic hepatocytes, potentially contributing to suppression of liver fibrosis development.

**Key Words:** Human immunodeficiency virus; Liver; Obeticholic acid; Alcohol; Hepatocytes; Fibrosis



**Core Tip:** We investigated the ability of obeticholic acid (OCA) to reverse pro-fibrotic effects of human immunodeficiency virus (HIV) and ethanol metabolism in liver cells. Based on our previous studies, hepatocyte apoptosis occurs under combined exposure of cells to HIV and ethanol metabolites. The subsequent engulfment of HIV-containing apoptotic hepatocytes by hepatic stellate cells induced pro-fibrotic activation in these cells, thereby promoting fibrosis development. Here, we demonstrated that OCA attenuates hepatocyte apoptosis by preventing accumulation of HIV components in liver cells exposed to virus and acetaldehyde-generating system (AGS) mimicking natural ethanol metabolism in primary hepatocytes. These beneficial effects of OCA are attributed to suppression of oxidative stress leading to restoration of HIV-AGS-impaired proteasomal and lysosomal functions in liver cells. OCA also reduces activation of inflammasome in hepatic stellate cells and their pro-fibrotic activation. Thus, anti-fibrotic properties of OCA can be used for combined treatment of HIV-infected alcohol abusers with a high risk of liver fibrosis development.

**Citation:** New-Aaron M, Ganesan M, Dagur RS, Kharbanda KK, Poluektova LY, Osna NA. Obeticholic acid attenuates human immunodeficiency virus/alcohol metabolism-induced pro-fibrotic activation in liver cells. *World J Hepatol* 2020; 12(11): 965-975
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/965.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.965

## INTRODUCTION

Liver disease is a second-leading cause of mortality in human immunodeficiency virus (HIV)-infected patients[1]. While effective anti-retroviral treatment (ART) has

**P-Editor:** Ma YJ

dramatically decreased the onset of acquired immunodeficiency syndrome, the morbidity and mortality are often associated with liver disease, which progresses slowly into severe liver dysfunction. This progression is related not only to the ART-induced hepatotoxicity, but to HIV properties likely associated with progression of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NASH)[2,3]. There are multiple insults, which promote HIV-related liver injury, including dysregulation of lipid metabolism and fat turnover, co-infections with hepatotropic viruses and alcohol abuse[4,5].

Recently, we have shown that alcohol metabolite, acetaldehyde promotes accumulation of HIV proteins in hepatocytes, inducing oxidative stress and cell apoptosis[6]. Furthermore, the engulfment of these HIV-containing hepatocytes by non-parenchymal cells, macrophages and hepatic stellate cells (HSC) causes inflammasome activation in macrophages and pro-fibrotic activation of HSC[6]. This indicates that massive apoptosis of infected hepatocytes may induce continuous activation of HSC leading to liver fibrosis development. Since acetaldehyde-triggered HIV replication in hepatocytes is abortive[6], HIV accumulation cannot be fully controlled by ART and requires the treatment with additional anti-fibrotic drugs.

One of the promising drugs with anti-fibrotic activity is obeticholic acid (OCA). In 2016, OCA became the United States Food and Drug Administration-approved to treat primary biliary cholangitis and currently is at phase 3 trial (Regenerate) to treat fibrosis caused by NASH[7]. OCA is a farnesoid-X receptor (FXR) agonist, which binds to the FXR in the nucleus of liver and intestinal cells. Multiple genes are activated by this pathway, including the control of metabolism of bile acids, lipids, glucose, and amino acids. FXR is highly expressed on hepatocytes and immune cells, and is involved in pathogenesis of viral hepatitis, alcohol-and non-alcohol-induced liver disease[8]. In NASH-fibrosis, OCA regulates liver injury progression *via* targeting of gut microbiota[9]. Currently, it is not quite clear whether OCA directly modulates hepatocyte apoptosis: While some studies reported the lack of anti-apoptotic effects of OCA on these cells[10], other studies demonstrated the reduction of hepatocyte apoptosis by OCA-mediated suppression of metabolic stress and prevention of subsequent *p53* activation, with further anti-fibrotic and anti-inflammatory downstream effects[11]. These studies were mainly performed on experimental *in vivo* models, which makes difficult to exclude the effects of OCA on gut microbiota, narrowing down the mechanisms to only OCA-regulated hepatocyte apoptosis. None of published *in vivo* or *in vitro* studies have been performed in the context of the effects of OCA on HIV- and alcohol-induced liver injury. However, activation of HSC by engulfment of HIV-expressing apoptotic hepatocytes generated as a downstream event in HIV and ethanol metabolism-induced oxidative stress is one of the reasons for liver fibrosis progression, which serves as an important target to prevent end-stage liver disease development. Thus, based on already characterized mechanisms of liver injury progression triggered by the combination of HIV with acetaldehyde[6], we aimed to investigate whether OCA protects from apoptotic hepatocyte death and from activation of HSC by engulfment of apoptotic HIV-infected hepatocytes.

## MATERIALS AND METHODS

This is the original (basic) study performed at University of Nebraska Medical Center, Omaha, NE, United States. Here, for the first time, *in vitro* approach is used to characterize the ability of OCA to reverse the pathology induced by HIV and ethanol metabolism in liver parenchymal and non-parenchymal cells. In our study, OCA has been tested as an anti-fibrotic drug, which affects the pathogenesis of HIV-alcohol-induced liver fibrosis development.

### *Reagents and media*

High glucose Dulbecco's modified eagle medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, United States), Trizol was from Life Technologies, primer probes and real-time polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems by Thermo Fisher Scientific, CA , United States. Other reagents, all analytical grade quality, were from Sigma (St. Louis, MO, United States).

### *Cells and treatments*

As experimental prototype of human primary hepatocytes, we used Huh7.5-CYP (RLW) cells. These cells have reduced innate immunity and can be infected with HIV.

To metabolize ethanol, they were stably transfected by CYP2E1, but do not express alcohol dehydrogenase (ADH). To overcome this limitation and mimic natural ethanol metabolism in primary hepatocytes, we treated RLW cells with acetaldehyde-generating system (AGS), which contains yeast ADH as a source of enzyme, NAD as a co-factor, and 50 mmol/L ethanol as a substrate for ADH and continuously produce physiologically relevant amount of acetaldehyde (Ach) without toxic effects. We have characterized and successfully used these cells and AGS for HCV-based ethanol liver studies[12-14]. Cells were pre-treated for 24 h with AGS and then exposed to $HIV_{ADA}$ (MOI 0.1) for 48 h. To investigate the effects of OCA, cells were pre-treated with OCA (50 μmol/L) for 4 h before experiment.

### *HIV RNA, HIV DNA, integrated HIV DNA, western blot and reactive oxygen species*

HIV RNA was detected by RT-PCR; HIV DNA was detected by semi-nested PCR; integrated HIV DNA was measured by digital droplet PCR; reactive oxygen species (ROS) were quantified by DCF (2′,7′–dichlorofluorescein fluorescence method); western blot was performed as described. The details of all these methods were already published[6].

### *Apoptosis measurements:*

Apoptosis in RLW cells was measured by caspase 3 and PARP cleavage in cell lysates (western blot) and M30 Apoptosense ELISA (Duapharma group, Inc. West Chester, OH, United States) in cell supernatants.

### *Activities of proteasome and cathepsins*

Proteasome activities and cathepsin B and L activities were assayed fluorometrically as described previously[15,16].

### *Apoptotic bodies*

Apoptotic bodies (AB) were generated from uninfected and HIV-infected RLW cells by exposure to UV light and characterized as shown previously[13].

### *Hepatic stellate cells and treatments with AB*

As the source of human HSC, we used commercially available human cell line, LX2 (EMD Millipore, cat SCC064) grown based on instructions from the manufacturer. AB from RLW cells (AB Hep), both uninfected (control) and HIV-infected ($AB_{HIV}$), were incubated with LX2 cells for 2-8 h at 1: 3 ratio and then pro-fibrotic markers [Col1A1, transforming growth factor (TGF)-β] and inflammasome-related parameters [NLRP3, caspase 1, interleukin (IL)-6, IL-1β] were quantified by RT-PCR.

### *Statistical analyses*

Data were analyzed using GraphPad Prism v7.03 software (GraphPad, La Jolla, CA, United States). Data from at least three duplicate independent experiments were expressed as mean ± SEM. Comparisons among multiple groups were performed by one-way ANOVA, using a Tukey post-hoc test. For comparisons between two groups, we used Student's *t*-test. A *P* value of 0.05 or less was considered significant.

## RESULTS

Our previous studies on primary human hepatocytes exposed to ethanol and RLW cells treated with AGS demonstrated, first, that incubation of ADH-non-expressing RLW cells with AGS recapitulated the effects of ethanol on ethanol-metabolizing hepatocytes and second, that the highest levels of apoptotic hepatocyte death were observed when cells were exposed to both insults (ethanol/AGS and HIV)[6]. That is why in this paper, we presented the data on ability of OCA to protect cells from the harmful effects of AGS on HIV-infected hepatocyte-like RLW cells to mimic ethanol metabolism observed in primary human hepatocytes. For OCA screening, we did not use primary human hepatocytes since their supply was limited and they quickly (in 24 h) de-differentiate to loose ethanol-metabolizing capacity.

### *OCA attenuates AGS-HIV-induced apoptotic cell death and oxidative stress*

We found that while AGS + HIV induced caspase 3 cleavage almost three-fold, pre-treatment with OCA suppresses these effects (Figure 1A and B). The same trend (but with lower magnitude of response to AGS) was observed on cleaved PARP, and OCA

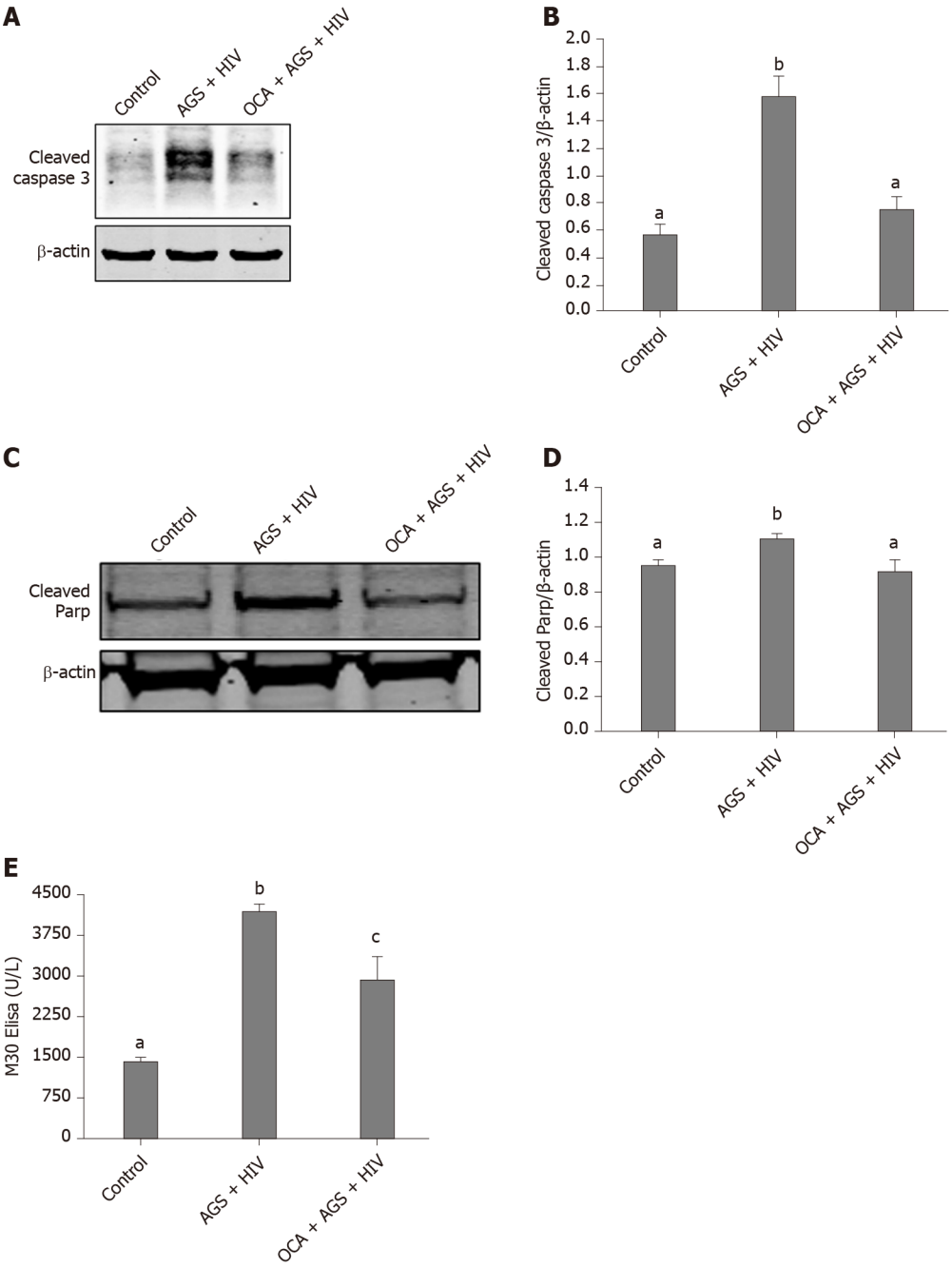


**Figure 1 Obeticholic acid attenuates apoptosis in acetaldehyde-generating system-human immunodeficiency virus-exposed Huh7.5-CYP cells**. A: Cleaved caspase 3 (western blot, representative blot); B: Cleaved caspase 3 cleavage, quantification; C: Cleaved PARP (western blot, representative blot); D: Cleaved PARP, quantification; E: M30 ELISA. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

also suppressed it (Figure 1C and D). Measuring cleaved cytokeratin 18 by M30 ELISA in cell supernatants, we found that OCA attenuated AGS-HIV-induced apoptosis in hepatocytes (Figure 1E). Since apoptosis may be induced by elevated *p53*, we measured the induction of *p53* mRNA by AGS+HIV, in the presence or absence of pan-caspase inhibitor or OCA. As shown on Figure 2A, while AGS and HIV induced *p53* mRNA level two-fold, both OCA and pan-caspase inhibitor reduced *p53* expression only by 14%. In addition, OCA attenuated AGS-HIV-induced ROS production (Figure 2B), thereby protecting liver cells from oxidative stress.

### *OCA suppresses accumulation of HIV in hepatocytes*

We measured the effects of OCA on accumulation of HIVgag RNA (RT-PCR) and p24 (western blot) induced by AGS + HIV exposure to RLW cells. In these experiments, OCA suppressed the treatment-induced HIVgag RNA levels by 50% (Figure 3A) and

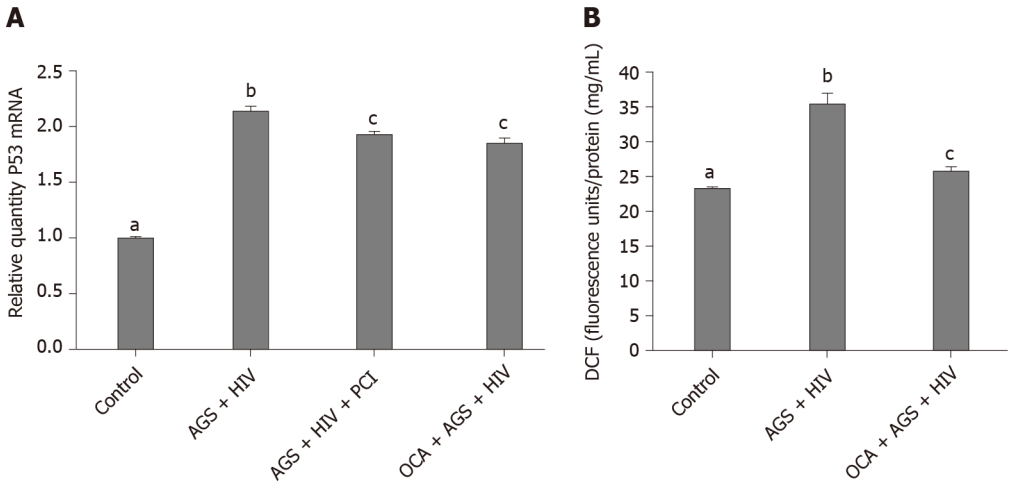


**Figure 2 Effects of obeticholic acid on *p53* mRNA and reactive oxygen species production in human immunodeficiency virus-infected Huh7.5-CYP cells exposed to acetaldehyde-generating system**. A: *p53* mRNA; B: Reactive oxygen species production. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

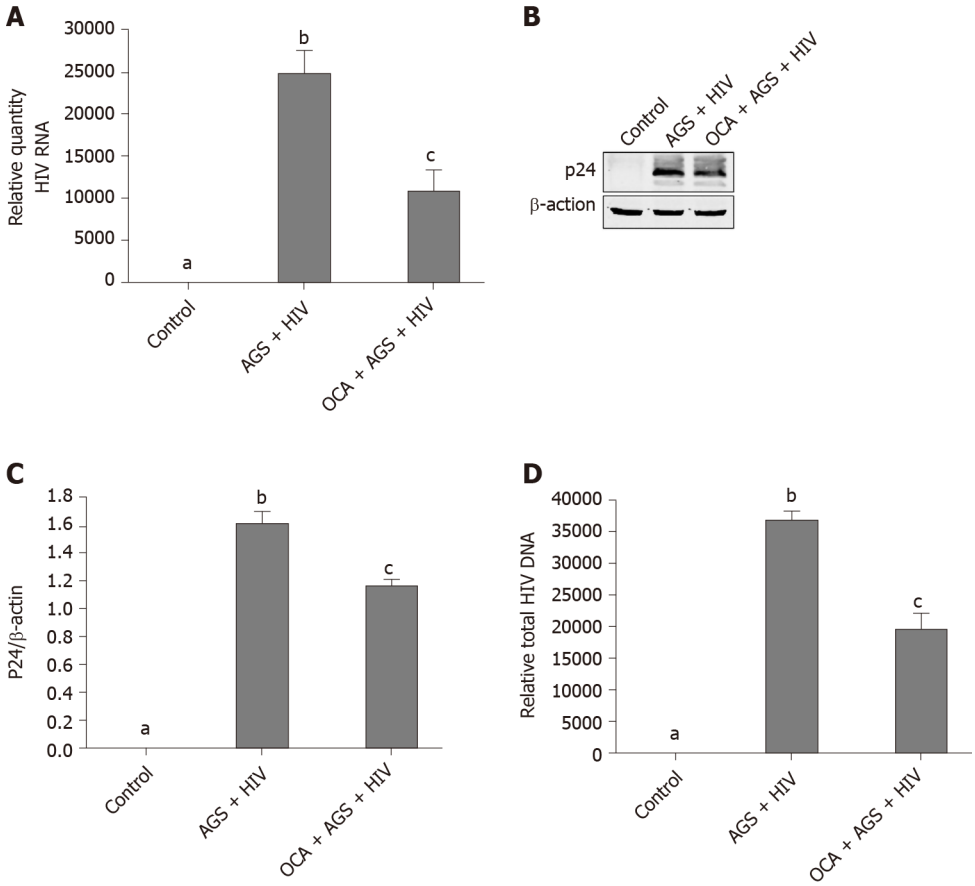


**Figure 3 Obeticholic acid suppresses human immunodeficiency virus expression in Huh7.5-CYP cells exposed to acetaldehyde-generating system + human immunodeficiency virus**. A: Human immunodeficiency virus (HIV) gagRNA; B: p24; C: HIV DNA. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

p24 –by 26% (Figure 3B and C). OCA also decreased the level of HIV DNA in RLW cells by about 60% (Figure 3D), with no integration of HIV DNA in cell genome. As has been shown by us before, the accumulation of HIV components in RLW cells is controlled by HIV-AGS-decreased lysosomal and proteasomal activities[6]. In fact, we observed partial restoration of chymotrypsin-like proteasome and cathepsin B and L lysosomal activities by OCA; however, OCA did not restore AGS-HIV-affected trypsin-like proteasome activity (Figure 4A-D).

### *OCA reduces pro-fibrotic activation of HSC by engulfment of HIV-containing apoptotic hepatocytes*

AB generated from uninfected (control) or HIV-infected RLW cells were exposed to LX2 cells, followed by measurement of Col1A1 and TGF-β mRNAs by RT-PCR. We found that HIV-containing apoptotic cells induced more prominent pro-fibrotic activation of HSC than uninfected apoptotic cells; OCA suppressed this pro-fibrotic HSC activation by uninfected AB and to most extend, by $AB_{HIV}$ engulfment (Figure 5A and B).

To characterize the effects of OCA on inflammasome activation in HSC, we measured NRLP3, caspase 1 and pro-inflammatory cytokines, IL-6 and IL-1β mRNA expression induced by engulfment of uninfected AB and HIV-containing AB, Up-regulation of NRLP3, caspase 1, IL-6 and IL-1 β levels by HIV-containing AB was successfully prevented by OCA treatment (Figure 6).

## DISCUSSION

As previously shown, HIV accumulation induced by pre-exposure of cells to ethanol metabolites, mainly acetaldehyde produced by AGS, induces oxidative stress and apoptosis in hepatocytes. This is beneficial due to infected hepatocytes clearance before the integration of HIV DNA into human genome occurs. However, intensive hepatocyte apoptosis may have detrimental outcomes since HIV-containing apoptotic hepatocytes induce pro-fibrotic activation of HSC, thereby promoting fibrosis development[6]. Here, we investigated whether OCA protects from AGS-HIV-induced hepatocyte apoptosis, which causes HSC activation by $AB_{HIV}$ engulfment to drive liver fibrosis development.

In our model, OCA pre-treatment attenuated apoptosis (caspase 3 and PARP cleavage as well as cleaved cytokeratin 18 expression) in AGS-HIV-exposed liver cells. Unlike suppression of *p53* expression on liver cells by OCA reported in HIV-non-infected cells by others[11], here, OCA mildly suppressed AGS-HIV-induced *p53* mRNA. Apoptotic hepatocyte death was triggered by oxidative stress induced by AGS and HIV in CYP2E1-expressing RLW cells. In our hands, OCA indeed suppressed ROS production, thereby attenuating oxidative stress, which corroborated the data obtained on different models[17,18]. Here, the suppression of oxidative stress by OCA restores proteasome and lysosome functions, which increases the degradation of HIV proteins[19,20] and thus, diminishes the expression of HIV gag RNA and p24 gag protein in infected RLW cells. However, as we established before, the prevention of HIV and AGS- induced apoptotic hepatocyte death by exposure to pan-caspase inhibitor causes accumulation of cells with integrated HIV DNA[6], which is an unwanted event. Importantly, while OCA pre-treatment suppresses apoptosis in HIV-infected hepatocytes, there was no increase in hepatocytes expressing integrated HIV DNA.

In addition to beneficial effects of OCA on attenuation of HIV-AGS-induced hepatocyte death and reduction of HIV markers expression, OCA also reverses pro-fibrotic activation (based on Col1A1 and TGF-β mRNA levels) of HSC by engulfment of HIV-containing apoptotic hepatocytes. Similar event was observed by[10] on CCl4-injured mice, indicating that OCA controls HSC activation triggered *via* multiple mechanisms. One of this mechanisms contributing to liver fibrosis progression is an activation of inflammasome pathway in HSC[21]. In fact, we observed the reversing effect of OCA on this pathway in HSC, which supports anti-fibrotic activity of OCA. Thus, the suppression of pro-fibrotic activation by OCA in HSC is crucial for liver fibrosis development.

In fact, *in vivo* protection from early alcohol-induced liver damage by OCA has been already demonstrated on alcohol-fed uninfected mice without disclosing the mechanisms, by which this happened[22]. Furthermore, in monocytes from peripheral blood of HIV-infected patients, the expression of nuclear receptors, including FXR, was reported to be low[23], and the treatments with FXR agonists like OCA might play a beneficial role. Importantly, in HIV-infected alcohol-abused patients, the efficacy of

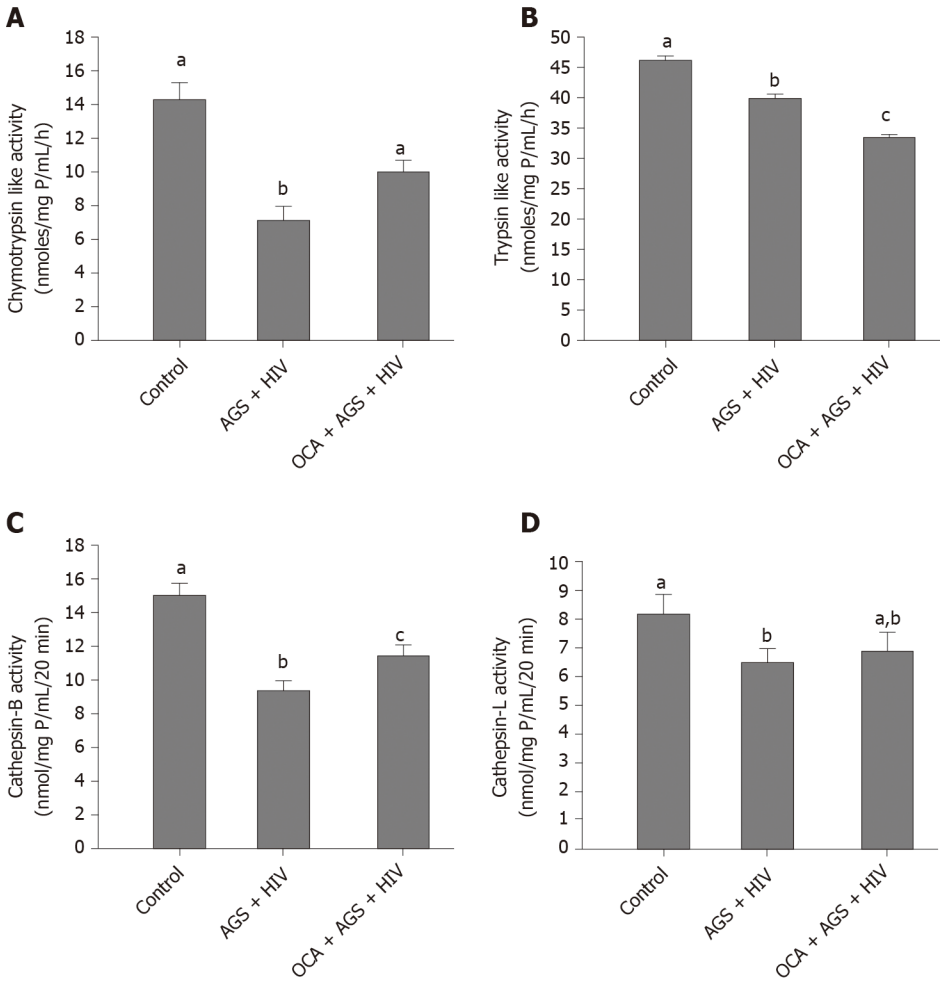


**Figure 4 Restorative effects of obeticholic acid on proteasomal and lysosomal activities in acetaldehyde-generating system-human immunodeficiency virus-exposed Huh7.5-CYP cells**. A: Chymotrypsin-like activity, proteasome; B: Trypsin-like activity, proteasome; C: Cathepsin B activity, lysosome; D: Cathepsin L activity, lysosome. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

OCA treatment has never been tested. Furthermore, these studies have not been done in the context of HIV-affected liver function. Our experiments provides *in vitro* evidence for protective effects of OCA from liver fibrosis progression induced by HIV and ethanol metabolism. The major limitation of this innovative study is that the results are currently based only on in vitro, but not *in vivo* experiments. Nevertheless, these *in vitro* experiments are necessary to characterize the exact mechanisms, by which OCA prevents HIV/ethanol metabolism-induced liver injury. These mechanisms are difficult to identify by *in vivo* studies, due to multiple triggers of liver fibrosis progression. We plan to confirm the *in vitro* effects of OCA in future by *in vivo* studies on humanized mice model since only these mice can be infected with human live HIV and fed the liquid ethanol-containing diet. Thus, our current *in vitro* study pioneers in justifying OCA inclusion to the treatment scheme of HIV-infected alcohol abusers with high risk of liver fibrosis development.

## CONCLUSION

In conclusion, in HIV-infected hepatocytes exposed to continuously released acetaldehyde, OCA attenuates apoptotic death of infected cells and pro-fibrotic activation of HSC by engulfment of apoptotic HIV$^+$ hepatocytes, thereby protecting from liver fibrosis development.

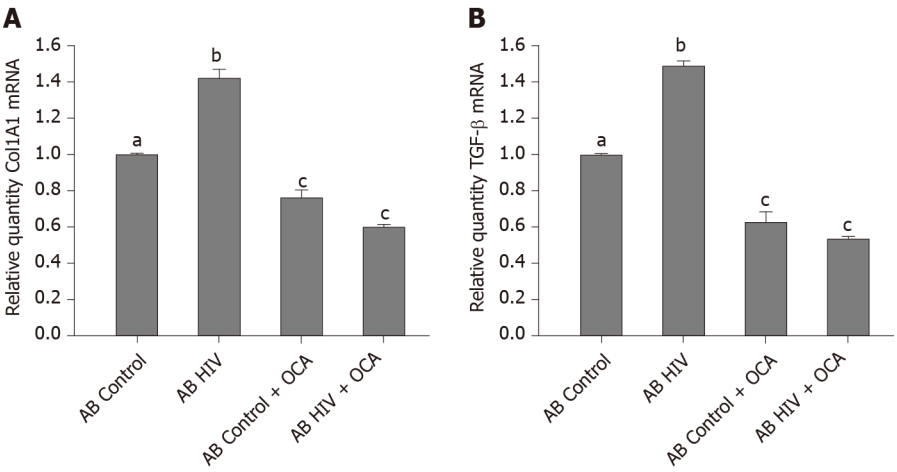


**Figure 5 Anti-fibrotic effects of obeticholic acid on hepatic stellate cells activated by engulfment of apoptotic Huh7.5-CYP cells**. A: Col1A1 mRNA; B: Transforming growth factor-β mRNA. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. TGF: Transforming growth factor; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

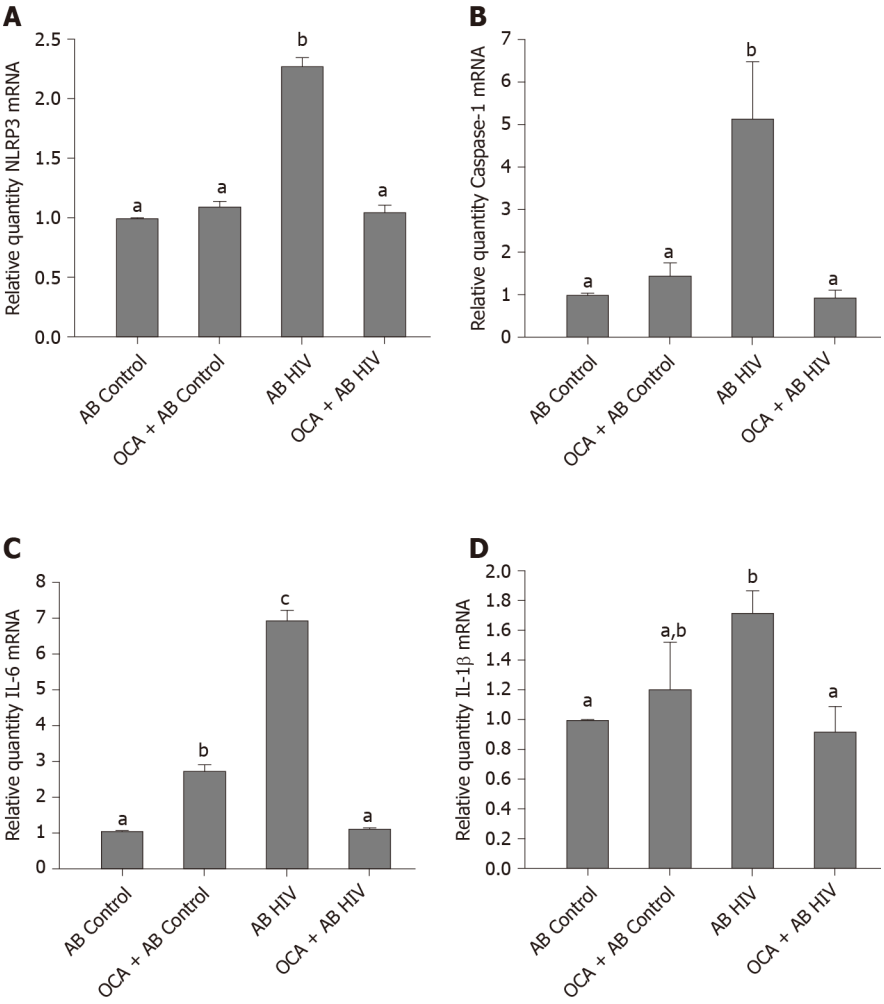


**Figure 6 Obeticholic acid suppresses acetaldehyde-generating system-human immunodeficiency virus-induced activation of inflammasome, mRNAs**. A: NLRP3; B: Caspase 1; C: Interleukin (IL)-6; D: IL-1β. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at $P \leq 0.05$. IL-6 and IL-1β: -interleukins B and IL-1β; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

## ARTICLE HIGHLIGHTS

### Research background

Due to frequent association of morbidity and mortality of human immunodeficiency virus (HIV)-infection with liver injury, the inclusion of drugs with anti-fibrotic activity to the treatment of people living with HIV (PLWH) is pathogenically important. Alcohol consumption is known to speed up liver fibrosis development. Previously, we have shown that the exposure of hepatocytes to HIV and ethanol metabolites causes high oxidative stress, impairs proteasomal and lysosomal functions leading to accumulation of HIV in these cells, which end-ups with apoptotic cell death and finally, promotes progression to liver fibrosis.

### Research motivation

The combined exposure of hepatocytes to HIV and alcohol induces hepatotoxicity and pro-fibrotic activation of hepatic stellate cells (HSC). Since HIV replication in hepatocytes is abortive, it cannot be fully controlled by antiretroviral therapy (ART) and thus, to prevent liver fibrosis progression in alcohol-abused PLWH, ART should be combined with the drugs suppressing apoptosis without enhancing HIV DNA integration in hepatocytes and decreasing pro-fibrotic activation of liver non-parenchymal cells.

### Research objectives

The objective of this study was to investigate whether obeticholic acid (OCA) prevents HIV/ethanol metabolism-induced activation of HSC by $HIV^+$ apoptotic hepatocyte engulfment, thereby diminishing liver fibrosis.

### Research methods

The study was performed on hepatocyte-like Huh7.5-CYP (RLW) cells infected with HIV ADA and exposed to acetaldehyde-generating system (AGS) in the presence or absence of OCA. As an end-point, we have measured expression of HIV-related markers (HIVgagRNA-by real-time polymerase chain reaction (PCR), p24- by western blot, HIV DNA-by semi-nested PCR, integrated HIV DNA-by ddPCR) and non-HIV-related parameters (lysosomal and proteasomal activities, hepatocytes apoptosis). We also characterized pro-fibrotic activation and inflammasome induction in HSC (LX2 cells) by HIV-containing apoptotic hepatocytes internalization.

### Research results

We found that OCA attenuated HIV-AGS-induced accumulation of HIVgagRNA, HIV DNA and p24. It suppressed ROS production, restored chymotrypsin-like proteasome activity as well as cathepsin B lysosome activity and decreased apoptosis in RLW cells. Exposure of HIV-containing apoptotic hepatocytes to OCA prevented activation of inflammasome and pro-fibrotic activation of HSC.

### Research conclusions

By suppressing oxidative stress and restoring proteasomal and lysosomal functions impaired by HIV and ethanol metabolism, OCA decreases accumulation of HIV in hepatocytes, leading to down-regulation of apoptosis in these cells. OCA also reverses pro-fibrotic and inflammasome-related activation of HSC triggered by engulfment of HIV-containing apoptotic hepatocytes, potentially contributing to suppression of liver fibrosis development.

### Research perspectives

Our *in vitro* studies are in the frame of pre-clinical characterization of anti-fibrotic effects of OCA in alcohol-exposed PLWH with a high risk of liver fibrosis development. At the next step, these *in vitro* effects will be confirmed by *in vivo* experiments on humanized mice infected with HIV and fed ethanol diet.

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World Journal of Hepatology

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*World J Hepatol* 2020 November 27; 12(11): 976-992

DOI: 10.4254/wjh.v12.i11.976

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Basic Study

# Screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry

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**Author contributions:** Khan M was involved in this project from the design to execution and writing of this manuscript, optimized the expression and purification of hepatitis C virus non-structural protein 3 protease, extracted antioxidant compounds and tested their inhibition using the *in vitro* fluorescence resonance energy transfer assay, contributed to the LCMS/MS analysis to identify phenolics/flavonoids in plant extracts and evaluated their interaction using a modeling approach; Rauf W contributed to the design and execution of the experiments and LCMS/MS data analysis; Habib F performed the LCMS/MS analysis; Rahman M provided help with the purification of hepatitis C virus non-structural protein 3 and manuscript writing; Iqbal M conceived the idea, planned all of the experiments, and contributed to the data analysis, and manuscript writing.

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## Abstract

### *BACKGROUND*

Hepatitis C virus genotype 3a (HCV G3a) is highly prevalent in Pakistan. Due to the elevated cost of available Food and Drug Administration-approved drugs against HCV, medicinal natural products of potent antiviral activity should be screened for the cost-effective treatment of the disease. Furthermore, from natural products, active compounds against vital HCV proteins like non-structural protein 3 (NS3) protease could be identified to prevent viral proliferation in the host.

### *AIM*

To develop cost-effective HCV genotype 3a NS3 protease inhibitors from citrus fruit extracts.

### *METHODS*

Full-length NS3 without co-factor non-structural protein 4A (NS4A) and codon optimized NS3 protease in fusion with NS4A were expressed in *Escherichia coli*. The expressed protein was purified by metal ion affinity chromatography and gel filtration. Citrus fruit extracts were screened using fluorescence resonance energy transfer (FRET) assay against the protease and polyphenols were identified as

**Institutional review board statement:** It is certified that the protocol of the study, screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry was approved by the institutional review board.

**Conflict-of-interest statement:** There is no financial, commercial or other conflict of interest with any author.

**Data sharing statement:** All data generated or analyzed during this study are included in this manuscript and its supplementary material. Any additional information, if required, may be asked from the corresponding author (hamzamgondal@gmail.com).



**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Pakistan

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** July 27, 2020

potential inhibitors using electrospray ionization-mass spectrometry (MS)/MS technique. Among different polyphenols, highly potent compounds were screened using molecular modeling approaches and consequently the most active compound was further evaluated against HCV NS4A-NS3 protease domain using FRET assay.

*RESULTS*

NS4A fused with NS3 protease domain gene was overexpressed and the purified protein yield was high in comparison to the lower yield of the full-length NS3 protein. Furthermore, in enzyme kinetic studies, NS4A fused with NS3 protease proved to be functionally active compared to full-length NS3. So it was concluded that co-factor NS4A fusion is essential for the purification of functionally active protease. FRET assay was developed and validated by the half maximal inhibitory concentration ($IC_{50}$) values of commercially available inhibitors. Screening of citrus fruit extracts against the native purified fused NS4A-NS3 protease domain showed that the grapefruit mesocarp extract exhibits the highest percentage inhibition 91% of protease activity. Among the compounds identified by LCMS analysis, hesperidin showed strong binding affinity with the protease catalytic triad having S-score value of -10.98.

*CONCLUSION*

Fused NS4A-NS3 protease is functionally more active, which is effectively inhibited by hesperidin from the grapefruit mesocarp extract with an $IC_{50}$ value of 23.32 μmol/L.

**Key Words:** Hepatitis C virus genotype 3a; Non-structural protein 3 protease; Fluorescence resonance energy transfer assay; Citrus extract; Mass spectrometry; Hesperidin



**Core Tip:** The manuscript describes the screening of active metabolites in citrus fruit extracts against hepatitis C virus genotype3a non-structural protein 3 (HCV-G3a NS3) protease. In this study, conditions have been optimized to get highly purified and functionally active protein HCV NS3. Further, fluorescence resonance energy transfer assay was used to screen the citrus extracts against NS3 protease. By using liquid chromatography coupled with tandem mass spectrometry/mass spectrometry analysis and bioinformatics modeling approaches, the observed activity of citrus extracts against HCV genotype3a NS3 protease was ascribed to hesperidin. Fluorescence resonance energy transfer assay confirmed the inhibitory potential of hesperidin against NS4A-NS3 protease domain with an half maximal inhibitory concentration value of 23.32 μmol/L.

**Citation:** Khan M, Rauf W, Habib F, Rahman M, Iqbal M. Screening and identification of bioactive compounds from citrus against non-structural protein 3 protease of hepatitis C virus genotype 3a by fluorescence resonance energy transfer assay and mass spectrometry. *World J Hepatol* 2020; 12(11): 976-992
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/976.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.976

## INTRODUCTION

Hepatitis C virus (HCV) is responsible for chronic hepatitis C disease in humans[1]. HCV infection remains asymptomatic and the virus persists in approximately 80% of untreated cases, which may ultimately lead to liver cirrhosis and finally hepatocellular carcinoma[2]. HCV is a major global cause of morbidity and mortality affecting more than 170 million people[3]. Annually, 400000 patients die worldwide due to HCV infection[4]. In Pakistan, the prevalence of HCV infection is estimated to range from 4%-6%[5-7]. Seven pathogenic HCV genotypes with subtypes have been identified[8] and genotype 3a is predominant in Pakistan[7].

**Peer-review started:** July 27, 2020
**First decision:** August 22, 2020
**Revised:** September 3, 2020
**Accepted:** September 16, 2020
**Article in press:** September 16, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Rybicka M
**S-Editor:** Zhang L
**L-Editor:** Filipodia
**P-Editor:** Li JH

In recent years, direct acting antiviral drugs have revolutionized HCV treatment. Sofosbuvir (a non-structural protein [NS] 5B inhibitor) and daclatasvir (a non-structural protein 5A [NS5A] inhibitor) have demonstrated sustained virologic response of more than 90% and have far fewer adverse events compared to previous treatment options. Sofosbuvir was the first nucleotide analogue that was found to be effective alone against HCV without interferon combination, which opened new gateways for development of additional direct acting antiviral drugs with excellent therapeutic outcomes[9]. The earlier Food and Drug Administration (FDA)-approved NS3 protease inhibitors, boceprevir and telaprevir were only effective against HCV genotype 1 (mainly prevalent in western countries) and less effective against genotype 3a, most prevalent genotype in Pakistan. However, the recently FDA-approved NS3 protease inhibitor glecaprevir has shown broad or pan-genotypic activity against HCV with much reduced side effects, especially in combination with pibrentasvir (ABT-530, NS5A inhibitor) as a single tablet. However, the high cost of protease inhibitors limits their use in resource-limited countries rendering the global eradication of HCV infection a difficult goal.

According to recent World Health Organization report, globally, the landscape for traditional and complimentary medicines has been steadily expanding[10] and a large number of world populations rely on traditional medicines that use natural products for treatment of viral and other infections[11].

Efforts have been made to identify extracts and natural products isolated from citrus family to inhibit HCV genotype 3a NS3 protease, because citrus fruits are considered as a treasure trove of several active natural metabolites, including coumarins, alkaloids, flavonoids, limonoids, essential oils, phenolic acids and carotenoids. The anti-oxidative[12-18], anti-inflammatory[19-21], and anti-cancer properties[22-24], as well as cardiovascular[25], neuroprotective[26,27] and hepatoprotective effects[28,29] of citrus and their extracts have been extensively reported[30-33]. Moreover, citrus metabolites have been used in many Asian countries as traditional medicinal herbs for treatment of digestive disorders, common cold and influenza, constipation and diarrhea, fluid retention, irritable bowel syndrome, persistent headaches, skin disorders, anxiety, depression, allergies, osteoarthritis, rheumatoid, prostate disorders as well as stomach and breast cancer[34,35].

In the current study, we have successfully produced a highly purified, stable and functionally active HCV NS3 protease of genotype 3a in fusion with its co-factor NS4A. After validating the fluorescence resonance energy transfer (FRET) assay using commercially available protease inhibitors, the extracts from citrus × paradisi (grapefruit), citrus sinesis (orange), citrus aurantinum (bitter orange), citrus reticulata (mandarin) and citrus limon (lemon) were collected, enriched and evaluated. The most active extract was analyzed and characterized using high-performance liquid chromatography coupled with tandem mass spectrometry (LCMS/MS). Candidate compounds from most active extracts were docked against the HCV NS3 protease structure to identify the most promising natural product, which was acquired and further evaluated to inhibit HCV NS3 protease using a FRET assay. As a result, this study has pinpointed the most active natural product from citrus family against NS3 protease of HCV genotype 3a.

## MATERIALS AND METHODS

### *Plasmid constructs*

The pET11a-$His_6$-NS3 construct was provided by Dr. Ikram Anwar (former PhD student, Drug Discovery and Structural Biology Lab, NIBGE, Pakistan). In pET11a-$His_6$-NS3, the nucleotide sequence of full-length NS3 (encoding both protease and helicase domains comprising amino acid 1 to amino acid 631) of hepatitis C virus genotype 3a was cloned into Bam HI/Hind III restriction sites of pET11a vector (Novagen, Madison, WI, United States) using respective restriction enzymes[36]. The NS3 sequence in the pET11a-$His_6$-NS3 construct was placed in the reading frame with the N-terminal $His_6$-tag (Supplementary Figure 1A).

Another construct inserted with the NS3 protease domain in fusion with the core of NS4A activator peptide at the C-terminal was named pET11a-$His_7$-NS4A-NS3 ( Supplementary Figure 1B) and synthesized by GenScript (Piscataway, NJ, United States); further description is given in the Supplementary information.

### *Expression of full-length NS3 and NS3 protease in Escherichia coli*

For expression of full-length $His_6$-NS3 (contain a serine protease and an RNA helicase)

competent cells of BL21-CodonPlus (DE3)-RIL and *Escherichia coli* BL21 (DE3) were transformed with pET11a-$His_6$-NS3. For expression of the NS3 protease, pET11a-$His_7$-NS4A-NS3 was transformed into *E. coli* BL21 (DE3) cells considering the codon-optimized nucleotide sequence of NS3 protease. Further details of the expression experiments are given in the Supplementary information.

### Purification of full-length NS3 and NS4A-NS3 protease domain

For purification, *E. coli* cell paste of pET11a-$His_6$-NS3/BL21-CodonPlus (DE3)-RIL or pET11a-$His_7$-NS4A-NS3/BL21 (DE3) was resuspended in ice-cold 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.6 added with 20% glycerol, 0.5 mol/L NaCl, 2.5 mmol/L β-mercaptoethanol and 0.4% Triton X-100[36] or 25 mmol/L HEPES (pH 7.5), 1 mol/L NaCl, 25 mmol/L Imidazole and 10% glycerol (buffer A). Both proteins were purified by Nickel affinity chromatography and gel filtration; the detailed procedure is presented in the Supplementary information.

### Activity measurement of full-length NS3 and NS4A-NS3 protease domain

The FRET assay was performed to determine protease activity, using the depsipeptide substrate Ac-Asp-Glu-Asp-(EDANS)-Glu-Glu-Abu-ψ-[COO]-Ala-Ser-Lys(DABCYL)-NH2 (AnaSpec, San Jose, CA, United States). The NS3 Protease domain cleaves the depsipeptide substrate, which results in the generation of fluorescence that can be read continuously on a fluorescence plate reader (THE SPARK®; TECAN, Morrisville, NC, United States) at their desired excitation (355 nm) and emission (510 nm) wavelengths. The synthetic peptide KKGCVVIVGHIELGK obtained from LifeTein LLC (Somerset, NJ, United States)[37] corresponding to the central part of HCV NS4A was the co-factor necessary for NS3 protease activity. To increase its solubility, two N-terminal lysine residues were also added[37,38]. To analyze the activity of $His_6$-NS3, the enzyme was further diluted to 1 nM in an assay buffer (50 mmol/L HEPES pH 7.5; 10 mmol/L DTT; 0.4% triton X-100, 40% glycerol, and 3% dimethylsulfoxide conc. in each well) and pre-incubated with NS4A-peptide (25 μmol/L) at 30°C for 10 min. Whereas, NS4A-NS3 protease domain was diluted to a final concentration of 0.5 nmol/L in the assay buffer and then was incubated for 10 min at 30°C (no separate cofactor was added). To initiate the reaction, substrate (10 μL) was added in a 2-fold serial dilution with maximum concentration of 4 μmol/L. Lastly, two places in 96-well plates were used as a control containing reaction mixture without substrate. Inner filter effect corrections of substrate were done as previously described[39]. To determine the kinetics of enzyme (*K*m, *k*cat and *k*cat/*K*m), the Michaelis-Menton equation was fitted to the data by non-linear regression using GraphPad Prism® software (GraphPad Software Inc., San Diego, CA, United States).

### Validation of NS4A-NS3 protease domain and FRET inhibition assay

Inhibition experiments using NS4A-NS3 protease domain of genotype-3a were performed according to a previous protocol[40]. After correcting inner filter corrections, the assay was validated using commercial-based inhibitors: Ciluprevir, telaprevir, asuanaprevir, and danoprevir (AdooQ® Bioscience, Irvine, CA, United States). To this end, the NS4A-NS3 protease domain was pre-incubated with inhibitor before adding depsipeptide substrate (1 μmol/L). Half maximal inhibitory concentration ($IC_{50}$) values were obtained using GraphPad Prism® software (GraphPad Software Inc.)[41]. The reaction was monitored at wavelengths 355 nm (excitation) and 510 nm (emission), for 20, 30, or 60 min. All activity measurements were done in triplicate. The final percentage of enzyme inhibition was calculated as average from three independent experiments[42]. Errors were calculated as the standard deviation (SD), and $IC_{50}$ values were calculated as previously described.

### Preparation of extracts

Five varieties of citrus fruits (citrus × paradisi [grapefruit], citrus sinesis [orange], citrus aurantinum [bitter orange], citrus reticulata [mandarin], and citrus limon [lemon]) were used in this study. The different parts of dried and crushed citrus plants materials were provided by Jiaherb Inc. (Pine Brook, NJ, United States) and Sanjiang Bio (Walnut, CA, United States). These powdered extracts were packed in opaque storage bags and stored at -20°C for future use[43]. The extraction procedure is further described in the Supplementary information.

### Inhibition of HCV NS4A-NS3 protease activity by plant extracts

An opaque 96-well plate was used to perform enzyme inhibition assays as described in the Supplementary information. All extracts were tested at a final 3-fold dilution

ranging from 1.566 μg/mL to 3.33 mg/mL. The inhibition was calculated as the average from three independent experiments[42]. Errors were calculated as the SD, and $IC_{50}$ values were calculated as mentioned above.

### LCMS analysis of extracts

For electrospray ionization (ESI)-MS/MS analysis, the dried extracts, which indicated the best inhibition activity by FRET assay, were dissolved in LCMS-grade methanol and subjected to ESI-MS/MS analysis using the LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, United States), equipped with an ESI probe as explained in the Supplementary information.

### Modeling predictions of compounds using bioinformatics software

Compounds identified by ESI-MS/MS analysis were tested in docking studies using molecular operating environment (MOE) software by means of the following communications, *i.e.* Intel [R] xenon [R] CPU E5620@2.40 GHz system, which has 3.8GB RAM having 11.4 [X 86_64] operating system[44,45]. Structures of identified compounds were constructed using chembiodraw ultra 14.0 software. HCV NS4A-NS3 protease domain (receptor protein) was modeled by SWISS-MODEL. Three dimensional (3D) protonation and energy minimization were performed using standard MOE parameters. Then docking analysis with default MOE parameters was used to check the interaction of selected ligands to the receptor protein and find the correct ligand conformation. After docking, best conformations were analyzed depending on least S-score values for hydrogen bonding/π-π interactions[46].

### Evaluation of pure natural product (hesperidin)

After the identification of specific natural product (hesperidin) by ESI-MS/MS analysis and its further interaction with NS3 protease by molecular docking, hesperidin (90% pure; Jiaherb) was subjected to inhibitory activity analysis. Inhibition experiments using the NS4A-NS3 protease domain were performed as previously described[40] and $IC_{50}$ values were calculated.

## RESULTS

### Expression and purification of full-length NS3 and NS4A-NS3 protease domain

To test the efficacy of selected inhibitors against the HCV-NS3 protease domain, full-length NS3 (containing the serine protease and an RNA helicase domains) and a NS4A-NS3 protease domain were produced by recombinant means. To this end, expression of full-length $His_6$-NS3 from pET11a-$His_6$-NS3 expression vector ( Supplementary Figure 1) was performed in *E. coli* BL21 (DE3) and BL21-CodonPlus (DE3)-RIL as described in the Methods section. High expression levels of $His_6$-NS3 were obtained in BL21-CodonPlus (DE3)-RIL compared to BL21 (DE3) cells, as detected by the appearance of a 68.3 kDa band on a Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Figure 1A and B). Therefore, further expression of $His_6$-NS3 was performed in BL21-CodonPlus (DE3)-RIL. For the NS4A-NS3 protease domain (22.5 kDa), high expression levels were achieved in *E. coli* BL21 (DE3) using the pET11A-$His_7$-NS4A-NS3 expression vector (Figure 1C, Supplementary Figures 1 and 2).

Full-length $His_6$-NS3 and $His_7$-NS4A-NS3 protease domain were purified to homogeneity by Ni-NTA affinity chromatography. A high level of purity (> 95%) was achieved for the $His_7$-NS4A-NS3 protease domain compared to $His_6$-NS3, as determined by Coomassie blue-stained SDS-PAGE gels (Supplementary Figure 3). To remove impurities from the affinity-purified $His_6$-NS3 samples, gel filtration was performed that yielded a protein of high purity (Figure 1D, Peaks 1 and 2). For the $His_7$-NS4A-NS3, native NS4A-NS3 protease domain of high purity was obtained by cleaving the $His_7$-tag from the protein with TEV protease and purification by nickel-affinity chromatography and gel filtration (Supplementary Figures 1D and 4). Purification yield of full-length $His_6$-NS3 and NS4A-NS3 protease domain 0.55 and 6 mg per liter culture volume was obtained, respectively.

### Activity analyses of full-length $His_6$-NS3 and NS4A-NS3 protease domain

The activity of the purified full-length $His_6$-NS3 and NS4A-NS3 protease domain were measured using a FRET-based assay as described in the Methods section. An increase in activity of the NS4A-NS3 protease domain was observed upon an increase in

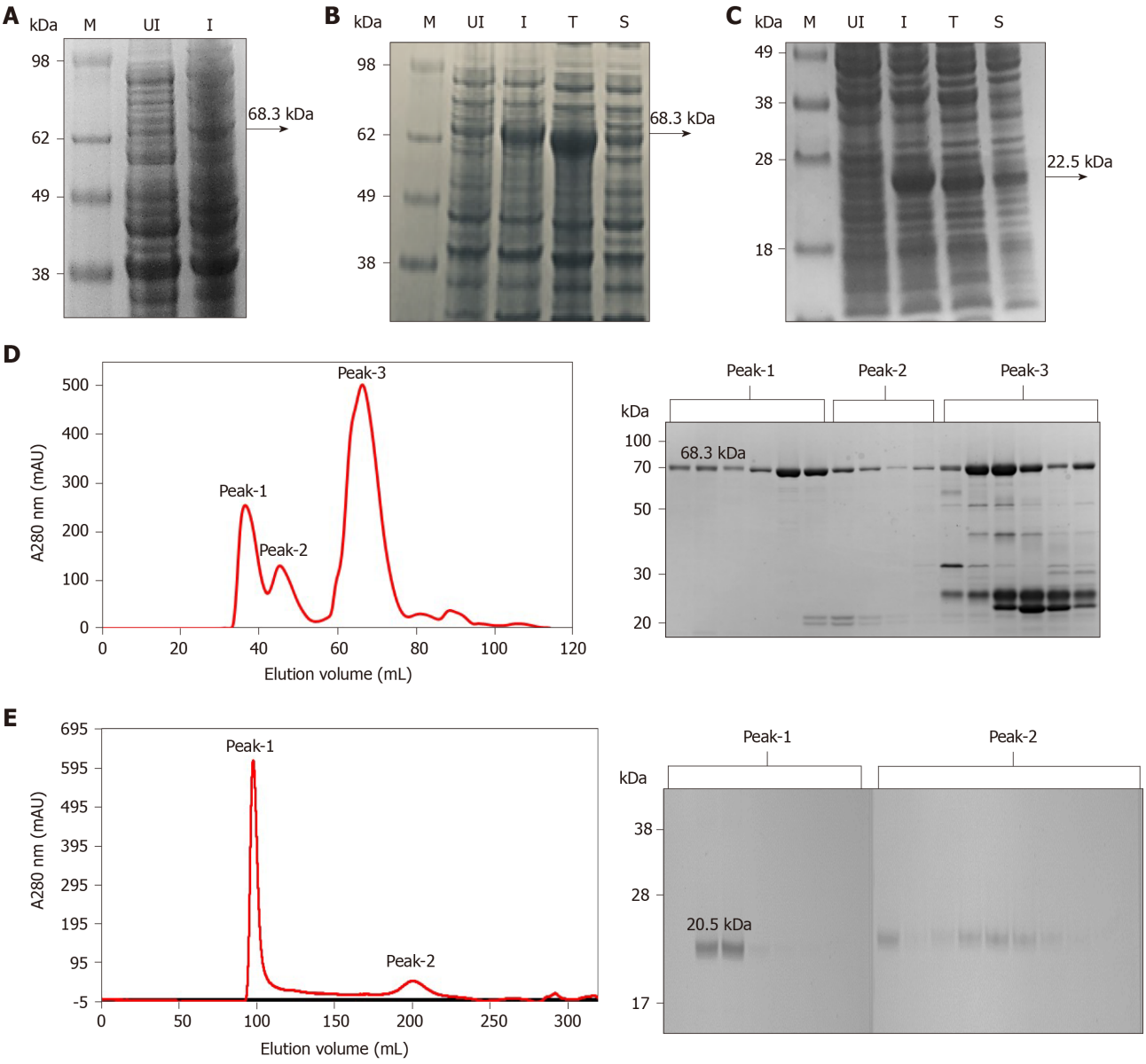


**Figure 1 Expression and purification of full-length His6-NS3 and non-structural protein 4a-non-structural protein 3 protease domain.** A: Analysis of the full-length $His_6$–NS3 protein (68.3 kDa) expressed in *Escherichia coli* BL21 (DE3) using pET11a-$His_6$-NS3. Lane M: SeeBlue™ Pre-Stained Protein Marker (LC5625); Lane UI: Uninduced BL21 (DE3) cells and Lane I: Induced BL21 (DE3) cells; B: Analysis of soluble and insoluble fractions of the $His_6$–NS3 protein expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells using pET11a-$His_6$-NS3. Lane M: Invitrogen Cat 1891868 See Blue® Plus2 Pre-Stained ladder; Lane UI: Uninduced cells; Lane I: Induced cells; Lane T: Total cell lysate of induced cells; Lane S: Soluble fraction of lysed cells; C: Analysis of soluble and insoluble fractions of the $His_7$–non-structural protein 4a-non-structural protein 3 (NS4A-NS3) protease domain expressed in *E. coli* BL21 (DE3) cells using pET11a-$His_7$-NS4A-NS3. Lane M: SeeBlue™ Pre-Stained Protein Marker (LC5625); Lane UI: Uninduced cells; Lane I: Induced cells; Lane T: Total cell lysate of induced cells; Lane S: Soluble fraction of lysed cells; D: Analysis of the Ni-NTA $His_6$–NS3 protein by gel filtration, presence of proteins in peak 1, 2 and 3 is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; and E: Analysis of the native NS4–NS3 protease domain by gel filtration, presence of proteins in peak 1 and 2 is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

depsipeptide substrate concentration (0.0625 to 6 μM) whereas with full-length $His_6$-NS3, no obvious activity was detected although the experiment was repeated several times (Supplementary Figure 5A and B). Moreover, the kinetic data revealed that the NS4A-NS3 protease domain exhibited a *K*m of 6.39 μM and catalytic efficiency ($k_{cat}/K_m$) of 0.015 $\mu M^{-1}.s^{-1}$ (15 $nM^{-1}.s^{-1}$) (Figure S5C) and qualified for conducting assays for an intensive search for inhibitors.

Furthermore, before embarking upon inhibition of the NS4A-NS3 protease domain by natural extracts/compounds, validation of the inhibition assay was done using commercial inhibitors. BILN 2061, VX-950, asuanaprevir and danoprevir inhibited the activity of the NS4A-NS3 protease domain with $IC_{50s}$ of 52.28 ± 13.08, 69.59 ± 13.42, 69.42 ± 13.48 and 24.42 ± 8.04 nM, respectively (Figure 2). The obtained $IC_{50}$ values validated the assay and suggested that the activity of the purified NS4A-NS3 protease

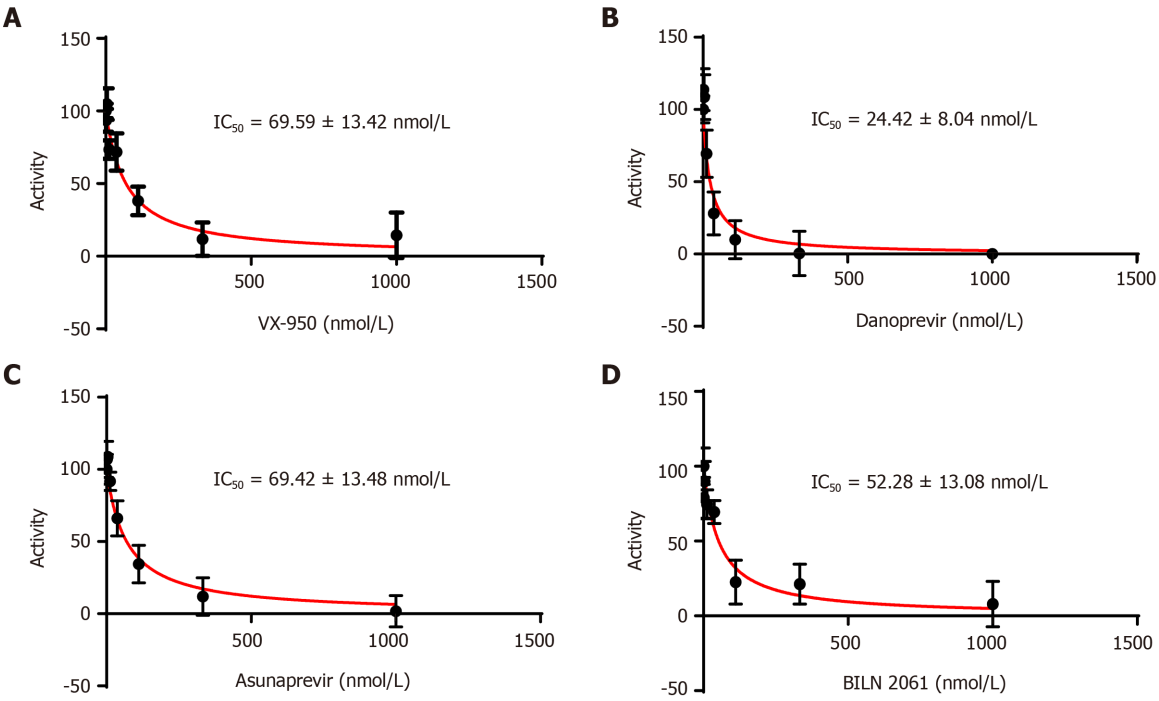


**Figure 2 Inhibition of non-structural protein 4a-non-structural protein 3 protease domain by commercial inhibitors.** A: VX-950; B: Danoprevir; C: Asunaprevir; and D: BILN 2061 and determination of inhibition constant ($IC_{50}$).

domain was inhibited by commercial inhibitors and the conditions for FRET assay were properly optimized.

### *Screening of citrus extracts against NS4A-NS3 protease domain of genotype 3a using the FRET assay*

After validating the FRET assay, 14 extracts of citrus fruit varieties were collected. Extracts from different parts of citrus (described in methods) were evaluated against the NS4A-NS3 protease domain of genotype 3a. Among them, top five extracts exhibited highest percentage inhibition of protease activity were selected. Selected extracts strongly inhibited the activity of NS4A-NS3 protease domain, *i.e.* grapefruit mesocarp extract, showed highest 91% inhibition of protease with an $IC_{50}$ value of 7.51 ± 0.87 μg/mL; orange extract (exhibited 86% inhibition of protease with an $IC_{50}$ value of 33.39 ± 1.52 μg/mL; bitter orange extract showed 85% inhibition of protease with an $IC_{50}$ value of 40.24 ± 1.60 μg/mL; mandarin extract showed 82% inhibition with an $IC_{50}$ value of 65.40 ± 1.81; and lemon extract inhibited 80% of protease activity with an $IC_{50}$ value of 74.60 ± 1.86 μg/mL. Among these extracts, grapefruit mesocarp extract showed highest percentage inhibition (91%) with lowest $IC_{50}$ value of 7.51 ± 0.87 μg/mL against NS4A-NS3 protease domain of genotype 3a using the FRET assay (Figure 3).

### *ESI-MS/MS analysis of grapefruit mesocarp extract*

The grapefruit mesocarp extract exhibiting the best activity against NS4A-NS3 protease domain was subjected to ESI-MS/MS analysis to reveal the identification of active natural product(s). Among the tested extraction solvents, n-hexane proved to be the best in minimizing the noise and showing maximum polyphenol natural products at negative ion mode (Figure 4). The full scan mass spectrum followed by the fragmentation through collision induced dissociation (CID) of the ion peaks of extract in negative ion mode $[M-H]^{1-}$ revealed the presence of aliphatic and aromatic organic acids (with and without glycans), lactones (bergapten at m/z 215 and (R)-marmin at m/z 331), flavonoids, *i.e.* alpinetin at m/z 269, hesperitin at m/z 301, polymethoxyflavone at m/z 435, naringenin arabinofuranose at m/z 535, naringin at m/z 579, hesperidin at m/z 609, neohesperidin at m/z 610 and anthocyanin (cyanidin-3-O-sophoroside chloride at m/z 645). At positive ionization mode $[M+H]^{1+}$ the synephrine, limonene and tangeretin were identified. These compounds were recognized by tandem mass spectrometry and hesperidin analysis data (as a representative) as described below.

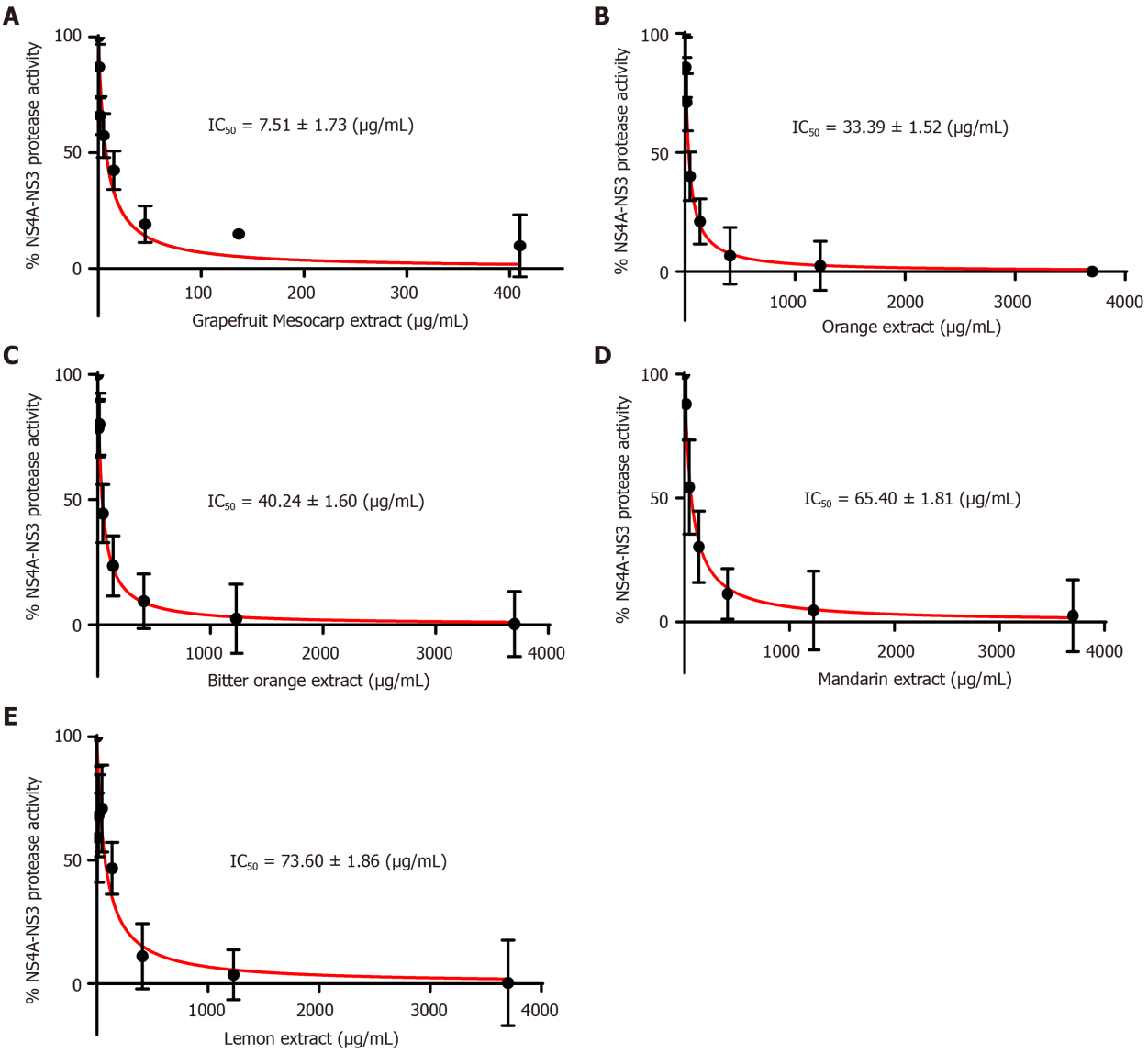


**Figure 3 Percentage inhibition pattern and inhibition constant calculation of non-structural protein 4a-non-structural protein 3 protease domain by different citrus fruit extracts inhibitors.** A: Grapefruit mesocarp extract; B: Orange extract; C: Bitter orange extract; D: Mandarin extract; and E: Lemon extract. $IC_{50}$: Inhibition constant.

Among all of the identified compounds, hesperidin flavonoid was the most abundant natural product in grapefruit mesocarp extract, giving a base peak at m/z 609.3 in negative ion mode. To confirm the hesperidin structure, the molecular ion at m/z 609.3 was fragmented (@CID 15.0) that yielded the daughter ions at m/z 463 (as a minor peak) and at m/z 301 as a base peak by losing one and two glycans, respectively, as described in Figure 5A. The peak at m/z 301 (hesperitin) was further subjected to $MS^3$ fragmentation (@CID20.0), which produced 19 daughter ions, *i.e.* m/z 286 (-$CH_3^{\bullet}$), further generating the fragments at m/z 268 (-$H_2O$), m/z 258 (-CO) and m/z 257 after double bond rearrangement (Figure 5B). The ion peak at m/z 258 generated two signals at m/z 151 and m/z 125 by the loss of B and C rings, respectively. The hesperitin (m/z 301) generated a peak at m/z 283 by loss of $H_2O$ producing the alkyne adduct and/or a fragment with extended conjugation on C ring, which further produced an entity at m/z 241 after losing $CO_2$. The alkyne adduct at m/z 283 can yield m/z 268 by losing a methyl radical. The ion peaks at m/z 257, m/z 242 and m/z 227 were produced by the loss of $CO_2$, followed by that of $CH_3^{\bullet}$ and $O^{\bullet}$, respectively, from hesperitin (m/z 301). The ion peak at m/z 151 can be produced directly from m/z 301 and/or any of the other adducts having C ring intact through retro Diels-Alder reactions. Notably, m/z 151 accompanied by m/z 125 are the signature ion peaks generated from most of the flavonoid aglycones during their tandem mass spectrometry[47]. The fragment at m/z 301 also generated fused ring adducts at m/z 259, m/z 215, m/z 199 and m/z 185. All of the ion peaks confirmed

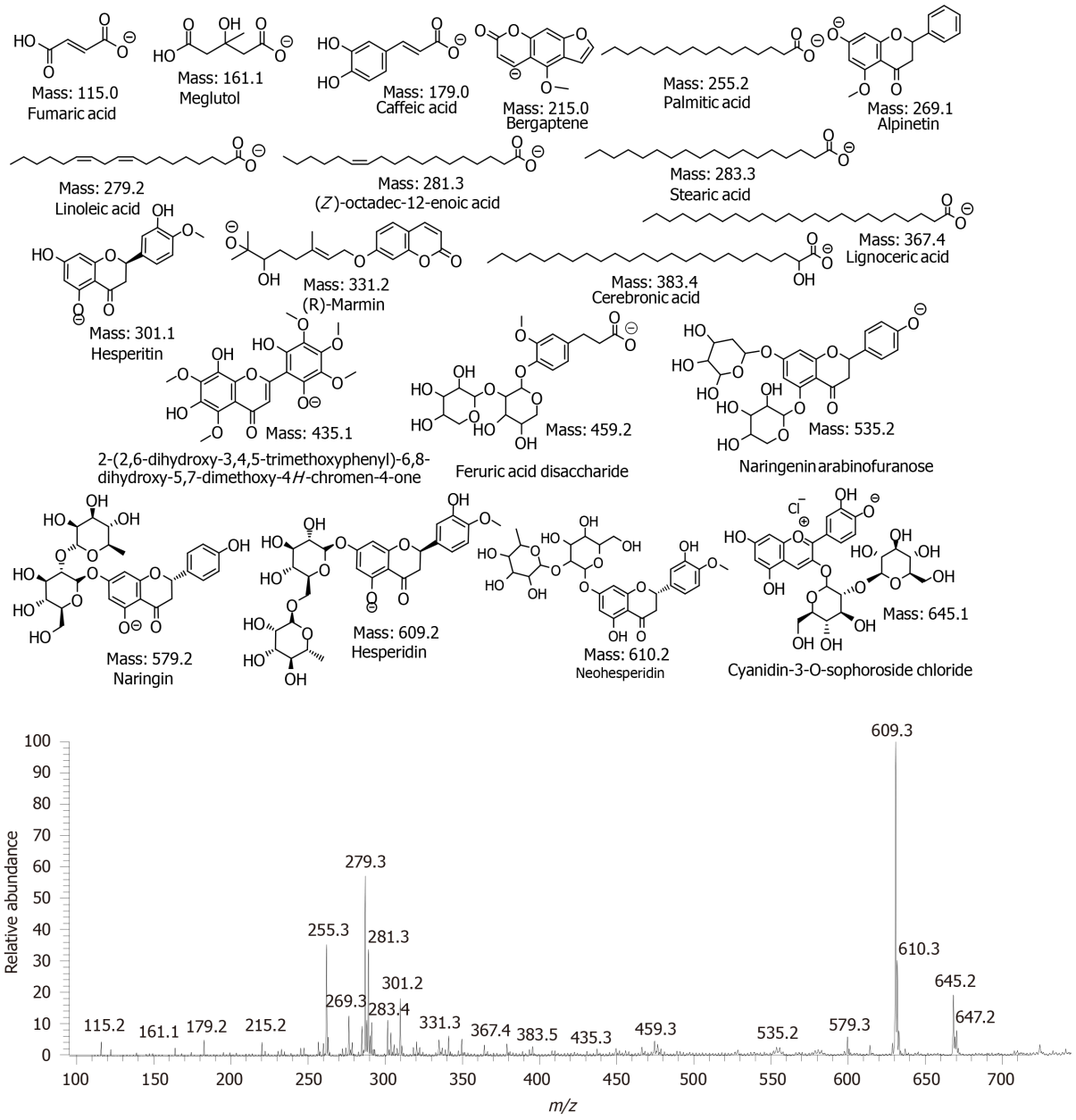


**Figure 4 Electrospray ionization mass spectrometry analysis of grapefruit mesocarp extract on negative ion mode.**

the hesperidin structure[47,48]. Additionally, the data were fully correlated with fragmentation of authentic hesperidin sample.

### *Modeling predictions of compounds using bioinformatics software*

The homology model of HCV NS4A-NS3 protease domain was developed using the HCV NS3/NS4A protease crystal structure (PDB ID: 3P8N) as a template that has 76.80% sequence identity with the template confirming a high-quality model. The homology model (Figure 6A) was in a closed flap conformation with GMQE and QMean scores of 0.81 and -1.77, respectively. The model was further validated by the values of the ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/) (98% in the most favored region). Analysis with verify 3D software showed that 84.62% of the residues averaged a 3D-1D score of ≥ 0.2, and the overall quality factor measured ERRAT software was 93.4%.

Among the identified citrus phytochemicals from the grapefruit mesocarp extract, hesperidin exhibited a good docking score [S-score = -10.98] and most importantly, it bound tightly with the catalytic residues of HCV NS4A-NS3 protease domain (HIS 57, ASP 81, and SER 139) (Figure 6B). Hence, it may be worth further exploring against NS4A-NS3 protease domain of HCV genotype 3a.

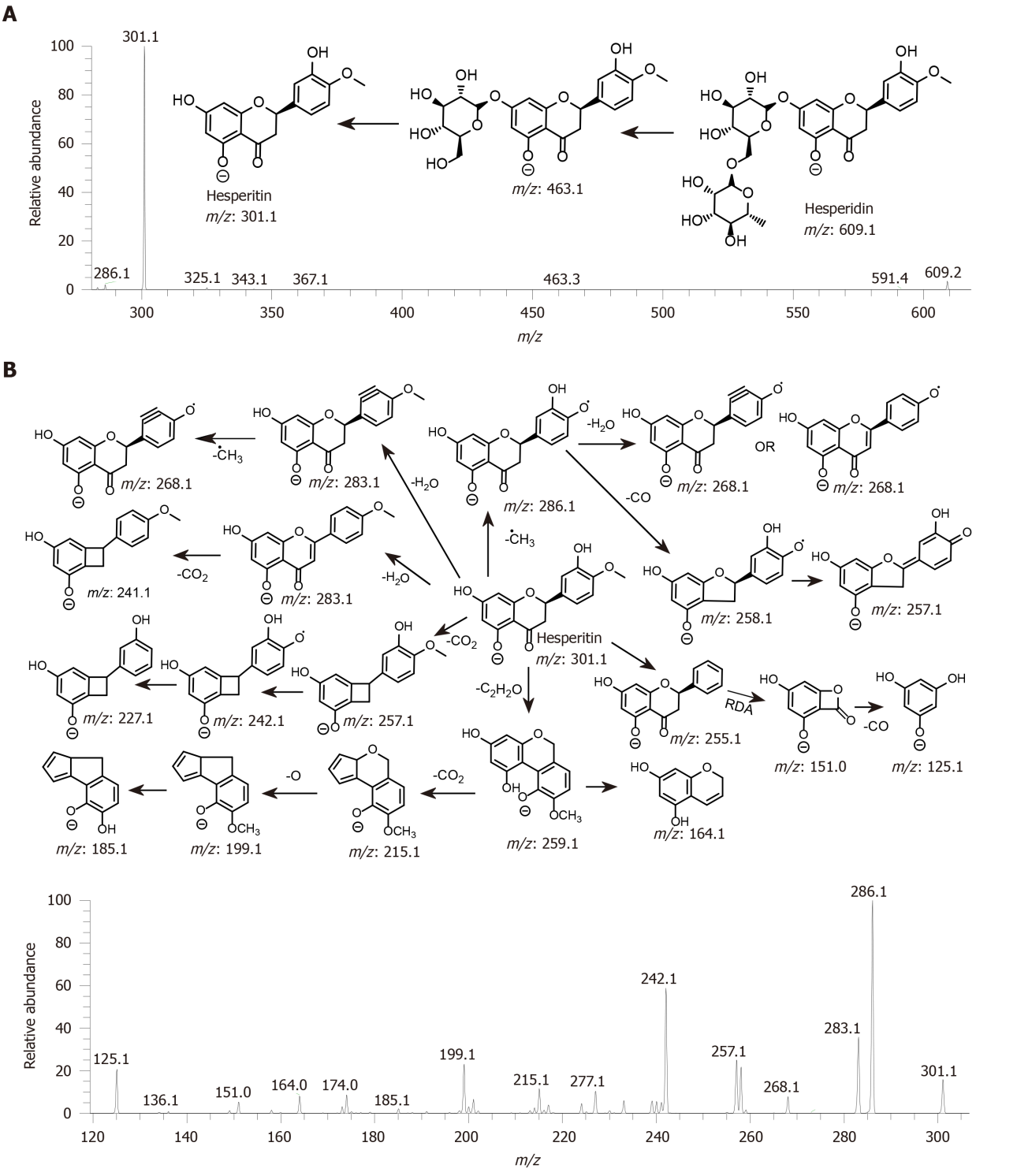


**Figure 5 Proposed fragmentation of hesperidin based on quasi-electrospray ionization -MS$^n$ (up to MS$^3$) spectra in negative ion mode.**

### *Evaluation of pure flavonoids*

The ESI-MS/MS analysis of the most active extract (grapefruit mesocarp) followed by simulation data, reveals that hesperidin is the most abundant and highly active inhibitor of the NS4A-NS3 protease domain of genotype-3a. The investigations inspired us to acquire hesperidin from commercial sources (Jiaherb) and evaluate its inhibition potential. Inhibition experiments with NS4A-NS3 protease domain of genotype-3a were performed according to the protocol described in methods. Hesperidin was tested at a concentration range of 1.23 μg/mL-1.67 mg/mL in the FRET assay, which gave $IC_{50}$ value of 23.32 μmol/L (Figure 6C).

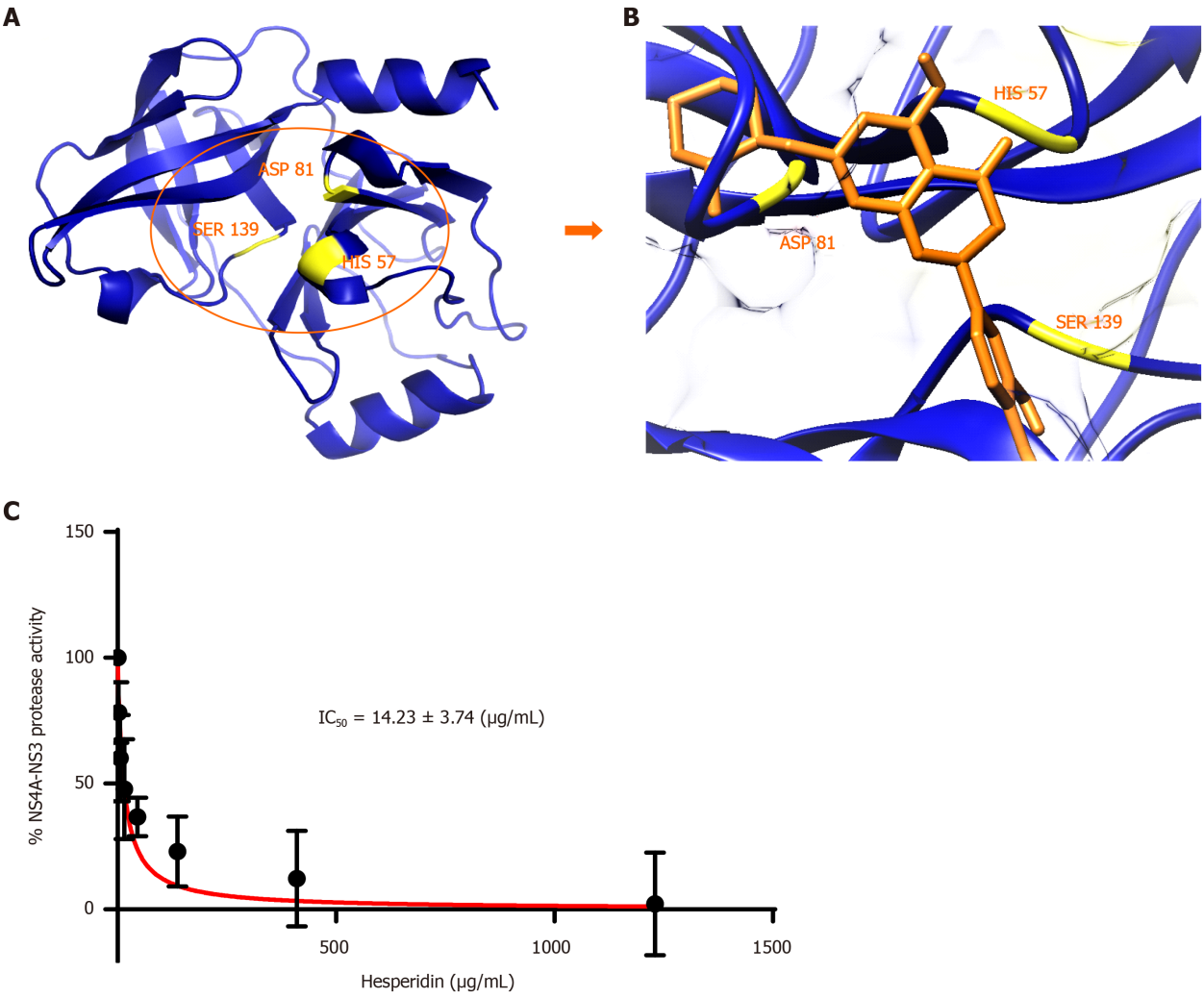


**Figure 6 Inhibition studies of hepatitis C virus non-structural protein 4a-non-structural protein 3 protease domain by hesperidin.** A: Hepatitis C virus (HCV) non-structural protein 4a-non-structural protein 3 (NS4A-NS3) protease domain model built by Swiss Model in the blue color cartoon. The active site residues are represented in yellow color for clarification; B: Docking of Hesperidin inside the receptor-binding site of HCV NS4A-NS3 protease domain (HIS 57, ASP 81 and SER 139). Hesperidin is represented in orange color. Blue ribbon represents protease domain of HCV NS3; and C: Inhibition constant ($IC_{50}$) calculation of HCV NS4A-NS3 protease domain by hesperidin.

## DISCUSSION

A multifunctional NS3 of HCV comprising of serine protease domain at N-terminal and a C-terminal RNA helicase domain. The protease domain requires the NS4A activator peptide to cleave 3 of its 4 polyprotein processing sites[49,50] and therefore provides an attractive target for inhibition of viral replication[51]; the protease is much more efficient at cleaving the fourth in the presence of NS4A. In the present study, full-length NS3 without cofactor NS4A and NS3 protease domain in fusion with co-factor NS4A from a codon-optimized NS4A-NS3 protease domain open reading frame were expressed in *E. coli* and purified (Figure 1). The NS4A-NS3 protease domain showed a higher purification yield than full-length NS3. Full-length NS3 did not display any detectable protease activity. This might be because the NS4A was not fused with the sequence of full-length NS3 and a separate synthetic NS4A peptide was used in activity assay buffer which may not bind to the proper site in NS3 to increase protease cleavage efficiency[52]. Another possible reason is that the fusion provides a higher effective local concentration of the activator. On the other hand, the native NS4A-NS3 protease domain, containing fused cofactor, showed significant activity with $K_m$ and $k_{cat}/K_m$ of 6.39 µmol/L and 0.015 $\mu M^{-1}.s^{-1}$, respectively. Moreover, inhibition of the NS4A-NS3 protease domain using known commercial inhibitors (BILN 2061, VX-950, asuanaprevir and danoprevir) validated the activity assay (Figure 2), which suggests that native purified protein can be employed to search for natural inhibitors/compounds against HCV.

Citrus fruits are important for human health because of their highly nutritious and

pharmacological activities[51]. They are rich in bioactive compounds including flavonoids[53]. Flavonoids have inhibitory action against a number of viruses as well. For example, quercetin, rutin, morin, dihydroquercetin, dihydrofisetin, pelargonidin chloride, leucocyanidin and catechin have activity against up to seven viruses, including herpes simplex virus leucocyanidin[54], respiratory syncytial virus[55,56], poliovirus[57,58], sindbis virus[59] and dengue virus[60]. Because of their abundance and possible medicinal uses, citrus extracts were included in the present study. Different plant parts (mesocarp, seeds, pericarp and pulp) of grapefruit, orange, bitter orange, mandarin and lemon were used to extract the bioactive compounds from them. FRET assay inhibition study of the tested extracts revealed that grapefruit mesocarp extract proves to be the more active against NS4A-NS3 protease domain having lowest $IC_{50}$ value (7.51 ± 0.87 μg/mL) (Figure 3).

ESI-MS/MS analysis of the grapefruit mesocarp extract demonstrated significantly higher abundance of hesperidin as compared to other metabolites (Figure 4). The structure of hesperidin was confirmed using the tandem mass spectrometric technique (Figure 5). Molecular docking studies of the identified flavonoids against the NS4A-NS3 protease domain (HCV 3a) revealed that hesperidin demonstrated a potential inhibitory activity against the NS4A-NS3 protease domain, exhibiting S-score value of -10.98 and strong binding affinity with the catalytic site residues (his 57, asp 81 and ser 139) (Figure 6A and B). These assumptions were proved when hesperidin (90% purity) was evaluated against NS4A-NS3 protease domain using the FRET assay, which gave sub-nanomolar $IC_{50}$ (23.32 μmol/L) (Figure 6C). This indicated that the predominant inhibition of grapefruit mesocarp extract was due to the presence of hesperidin in it. Notably, hesperidin was tested against different viruses, *i.e.* sindbis virus, vaccinia virus, parainfluenza virus, herpes simplex virus types 1 and 2, poliovirus, and vesicular stomatitis virus[59,61,62]. Hesperetin has exhibited an inhibitory effect with an $IC_{50}$ value of 20.5 μg/mL (about 68 μM) against sindbis virus infection, by plaque assay[59]. Moreover, previously docking studies on hesperidin and narirutin showed their inhibition potential against avian influenza virus H1N1[63], which were consistent with our results, and their inhibitory activity was experimentally confirmed in another study[64]. In this study, we found that hesperidin actively inhibited HCV NS3 protein with an $IC_{50}$ value of 23.32 μmol/L. Additionally, hesperidin was reported to be active involving host-factor, *i.e.* suppression of mitogen-activated protein kinase signaling pathways in H1N1 infection and this pathway is also activated by HCV[65,66].

Various natural products exhibit the potential to be anti-HCV protease inhibitors. Among them, epigallocatechin-3 gallate targets both viral cell entry and RNA replication steps[67] into both primary human hepatocytes and hepatoma cell lines[68]. It also exhibits an antiviral activity against all HCV genotypes, tested in the HCV pseudotyped particles (HCVpp system)[69]. Other natural products including quercetin, honokiol, 3-hydroxy caruilignan C and excoecariphenol D corilagin exhibit anti-HCV behaviors, which have been tested *in vivo* or in cellular models. Quercetin exhibited inhibition of HCV NS3 protease[70]. It has an ability to minimize the production of virus by inhibiting both NS3 as well as heat shock proteins which are required for the replication of HCV[71]. Honokiol prevents HCV infection by interfering with their cell entry and replication process[72], 3-hydroxy caruilignan C also exhibited anti-HCV activity at both RNA and protein levels[73]. Excoecariphenol D corilagin inhibited HCV NS3-4A protease with an $IC_{50}$ values within a range of 3.45-9.03 μmol/L, whereas excoecariphenol D and corilagin significantly showed potential inhibition of HCV RNA in huh 7.5 cells[74]. Our findings suggested that hesperidin showed lowest $IC_{50}$ value = 23.32 μmol/L (Figure 6C) as compared to previously reported natural products including epigallocatechin-3 gallate ($IC_{50}$ value = 5-21 μmol/L), honokiol ($IC_{50}$ value = 4.5 μmol/L), 3-hydroxy caruilignan C ($IC_{50}$ value = 37.5 μmol/L) and excoecariphenol D corilagin ($IC_{50}$ value = 12.6 and 13.5 μmol/L)[75].

Although FDA-approved, direct-acting antiviral drugs such as mavyret are available in market, they are very expensive and are not affordable for many HCV patients in resource limited and developing countries. So, there is a need to study natural products and to identify potent phytochemicals, which inhibits HCV replication and can be developed into inexpensive anti-HCV drugs. In conclusion, the present study identified hesperidin as a potential new inhibitor of HCV protease. Because hesperidin is an important bioflavonoid widely found in grapefruit mesocarp[76], its cheap availability may make it an interesting candidate as anti-HCV drug prospects which might replace other more expensive drugs.

## CONCLUSION

In this study, HCV NS3 protease fused with co-factor NS4A was found to be functionally more active compared to full-length NS3. Citrus fruit extracts were screened using FRET assay against NS4A fused protease. Among these extracts, grapefruit mesocarp showed the highest percentage inhibition (91% of protease activity). LCMS data revealed the high abundance of hesperidin in the most active extract, which was subsequently subjected to docking studies showing strong binding interaction of hesperidin with the catalytic site residues of NS4A-NS3 protease domain (S-score = -10.98). Hesperidin inhibited NS4A-NS3 protease domain with an $IC_{50}$ value of 23.32 μM in FRET assay.

## ARTICLE HIGHLIGHTS

### *Research background*

Hepatitis C virus genotype 3a (HCV G3a) is highly prevalent in many countries including Pakistan. FDA-approved drugs have significantly contributed in effective control of the disease but are expensive and not affordable to a large proportion of the infected population.

### *Research motivation*

Medicinal natural products having antiviral potential could be screened for the cost-effective treatment of the disease. Using such products, inhibition assays against vital viral proteins like non-structural protein (NS) 3 protease could be developed to prevent viral proliferation in the host.

### *Research objectives*

This study developed cost-effective HCV G3a NS3 protease inhibitors from citrus fruit extracts.

### *Research methods*

Codon optimized NS3 protease domain fused with NS4A as well as full-length NS3 constructs were cloned in pET11a expression vector. Both constructs were expressed in *Escherichia coli* BL21 (DE3) cells and purification was performed using Ni-affinity chromatography followed by gel filtration. The fluorescence resonance energy transfer assay was developed and validated using commercial inhibitors. Furthermore, extracts from different citrus species, were screened on the basis of percentage inhibition. The components of the most active extract were identified using electro spray ionization-mass spectrometry/mass spectrometry technique. Docking was performed with Molecular operating environment software to screen out the potent natural product, which was acquired in purified form and evaluated against NS3/4A protease using fluorescence resonance energy transfer assay.

### *Research results*

We successfully overexpressed and purified genotype 3a NS3 protease domain fused with NS4A and the yield was also higher than full-length NS3 protein. Inhibition of NS3 protease fused with NS4A protein was tested against different citrus extracts and grapefruit mesocarp extract showed highest percentage inhibition of protease activity (91%). Hesperidin was identified as the inhibiting compound in the extract having docking S-score value of -10.98.

### *Research conclusions*

NS3 protease fused with co-factor NS4A was found functionally more active. Hesperidin from the grapefruit mesocarp extract showed the inhibition against NS4A-NS3 protease domain with an $IC_{50}$ value of 23.32 μmol/L.

### *Research perspectives*

Hesperidin flavonoid may be further explored as potential antiviral agent against HCV as an affordable option for infected population.

## ACKNOWLEDGEMENTS

We would like to thank Dr. David Waugh, (Head, Protein Engineering Core, Macromolecular Crystallography Laboratory, NCI, NIH, United States) for providing the help to MK in cloning and optimizing the expression and purification of HCV NS3 protease as well as proof reading of the MS. Thanks to Taekjip Ha (Distinguished Professor, Department of Biophysics, Johns Hopkins University, Baltimore, MD 21218) and Chun Ying Lee (Graduate Student in Myong's lab, Department of Biophysics, Johns Hopkins University, Baltimore, MD 21218) for providing training and facility to MK to develop FRET assay. Thanks to Dr. Cheryl Winkler (Head, Molecular Genetic Epidemiology Studies Section, Basic Research Laboratory, NCI, NIH, United States), for hosting MK at NCI, NIH, to complete the research work and proof reading of the MS.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 993-1003

DOI: 10.4254/wjh.v12.i11.993

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

# Cannabis use history is associated with increased prevalence of ascites among patients with nonalcoholic fatty liver disease: A nationwide analysis

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**Author contributions:** Choi CJ designed the study, acquired data, performed statistical analysis, drafted, and edited this manuscript; Weiss SH supervised the design and reviewed statistical analysis of the study and edited the manuscript; Nasir UM acquired data, and edited the draft; Pyrsopoulos NT designed and supervised the study, and edited the manuscript.

**Institutional review board statement:** The activities described in our study do not meet the regulatory definition of human subjects research, and therefore our study was deemed not requiring approval by Rutgers Institutional Review Board (IRB).

**Conflict-of-interest statement:** Dr. Pyrsopoulos is a recipient of research grants from Mallinckrodt, Valeant, Gilead, Bayer, Beigene, Genfit, Prometheus, Grifols, Intercept, Zydus, and Eisai. Dr. Pyrsopoulos also has served as a

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## Abstract

### *BACKGROUND*

Recent studies have revealed the endocannabinoid system as a potential therapeutic target in the management of nonalcoholic fatty liver disease (NAFLD). Cannabis use is associated with reduced risk for NAFLD, we hypothesized that cannabis use would be associated with less liver-related clinical complications in patients with NAFLD.

### *AIM*

To assess the effects of cannabis use on liver-related clinical outcomes in hospitalized patients with NAFLD.

### *METHODS*

We performed a retrospective matched cohort study based on querying the 2014 National Inpatient Sample (NIS) for hospitalizations of adults with a diagnosis of NAFLD. The NIS database is publicly available and the largest all-payer inpatient database in the United States. The patients with cannabis use were selected as cases and those without cannabis were selected as controls. Case-control matching at a ratio of one case to two controls was performed based on sex, age, race, and comorbidities. The liver-related outcomes such as portal hypertension, ascites, varices and variceal bleeding, and cirrhosis were compared between the groups.

consultant for Bayer, and Eisai. The other authors have no potential conflicts of interest to report.

**STROBE statement:** The authors have read the STROBE Statement—a checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—a checklist of items.



**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D, D, D
Grade E (Poor): 0

**Received:** June 4, 2020
**Peer-review started:** June 4, 2020
**First decision:** July 30, 2020
**Revised:** August 27, 2020
**Accepted:** October 23, 2020
**Article in press:** October 23, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Chao HW, Moretti R
**S-Editor:** Fan JR
**L-Editor:** A
**P-Editor:** Wang LL

*RESULTS*

A total of 49911 weighed hospitalizations with a diagnosis of NAFLD were identified. Of these, 3820 cases were selected as the cannabis group, and 7625 non-cannabis cases were matched as controls. Patients with cannabis use had a higher prevalence of ascites (4.5% *vs* 3.6%), with and without cannabis use, $P = 0.03$. The prevalence of portal hypertension (2.1% *vs* 2.2%), varices and variceal bleeding (1.3% *vs* 1.7%), and cirrhosis (3.7% *vs* 3.6%) was not different between the groups, with and without cannabis use, all $P > 0.05$. Hyperlipidemia, race/ethnicity other than White, Black, Asian, Pacific Islander or Native American, and higher comorbidity score were independent risk factors for ascites in the cannabis group. Among non-cannabis users, obesity and hyperlipidemia were independent protective factors against ascites while older age, Native American and higher comorbidity index were independent risk factors for ascites.

*CONCLUSION*

Cannabis was associated with higher rates of ascites, but there was no statistical difference in the prevalence of portal hypertension, varices and variceal bleeding, and cirrhosis.

**Key Words:** Nonalcoholic fatty liver disease; Fatty liver; Cannabis; Marijuana use; Liver diseases; Hospitalization



**Core Tip:** Recent studies showed the lower prevalence of nonalcoholic fatty liver disease (NAFLD) among cannabis users compared to non-cannabis users, therefore suggestive of cannabis's modulatory role in the development of NAFLD. However, our case-control matching analysis, based on sex, age, race, and comorbidity, showed cannabis use as independently associated with higher rates of ascites in patients with NAFLD. A conceivable explanation for the finding is the dominant effect of cannabinoid receptor type 1 through its hepatic profibrotic effects in patients with NAFLD.

**Citation:** Choi CJ, Weiss SH, Nasir UM, Pyrsopoulos NT. Cannabis use history is associated with increased prevalence of ascites among patients with nonalcoholic fatty liver disease: A nationwide analysis. *World J Hepatol* 2020; 12(11): 993-1003
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/993.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.993

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now one of the most common liver diseases worldwide, and its global burden is growing[1]. Weight loss remains the primary treatment for NAFLD, and other treatment modalities have been actively studied, including the use of cannabinoids. Emerging evidence suggests that cannabis may play an important role in the management of various chronic liver diseases[2]. A retrospective population study by Adejumo *et al*[3] has shown a dose-dependent reduction in the prevalence of NAFLD with cannabis use, and cannabis has been suggested to suppress or even reverse the development of NAFLD.

The prevalence of NAFLD parallels that of the obesity pandemic, and NAFLD is considered a metabolic disorder where its pathogenesis involves the complex interplay among hormonal, nutritional, and genetic factors[4]. Aside from a significant weight loss, another cornerstone of the management is to reduce cardiovascular, oncologic, and hepatic risk factors for mortality[4,5]. Perhaps, cannabis use can modulate effects on the development and progression of NAFLD through its metabolic risks and its impact on hepatic steatosis.

Cannabis use is associated with increased appetite and calorie consumption, but paradoxically it is associated with reduced risk of obesity and diabetes[6]. In a cross-sectional data by Kim *et al*[6], active cannabis use provided a protective effect against

NAFLD, independent of metabolic risks. Yet, there has been conflicting evidence whether or not cannabis induces the progression of chronic liver diseases, but a systemic review by Farooqui *et al*[7] showed that when confirmed by liver biopsy, there was no association between cannabis use and prevalence of hepatic fibrosis. This lack of association is contrary to initial cross-sectional studies by Hézode *et al*[8] and Ishida *et al*[9] who suggested increased fibrosis in patients who use marijuana.

Here, we aimed to measure clinical outcomes of cannabis use at the national level among hospitalized patients with NAFLD. Given the previously demonstrated correlation between cannabis use and reduced prevalence of NAFLD, we hypothesized that cannabis would be associated with fewer liver-related complications in patients than in individuals who did not use cannabis. This is the first database study to investigate cannabinoids' effects on liver-related outcomes in hospitalized patients with NAFLD. With our study, we hope to alert clinicians of the possible relevance of ascertainment of cannabis use, which in turn might alter future routine assessments to further probe about cannabis use, especially in light of trends showing recent increases in use in the United States.

## MATERIALS AND METHODS

### Data source and study population

This study is a retrospective analysis of the 2014 Healthcare Cost and Utilization Project-National Inpatient Sample (HCUP-NIS). The HCUP-NIS is the largest all-payer inpatient database in the United States, comprising more than 40 states with more than 7 million annual hospital discharges[3]. Results were extracted from the NIS database by identifying hospitalized patients older than 18 years with a diagnosis of NAFLD, by using the corresponding International Classification of Diseases codes: 571.8 (other chronic nonalcoholic liver disease). We excluded common secondary causes of intrahepatic fat accumulation by excluding alcoholic liver disease, hemochromatosis, viral hepatitis B and C, primary biliary cirrhosis, autoimmune hepatitis, and toxic liver disease. The HCUP-NIS is publicly accessible, de-identified database, and it is considered a limited database. Under Health Insurance Portability and Accountability Act, a limited database does not require a review by the institutional review board (IRB). Therefore, Rutgers IRB approval was deemed not required in our study.

### Model design

Patients with NAFLD with cannabis use were selected as cases, and those without cannabis use were used to produce the control group through case-control matching at a ratio of one case to two controls, on the basis of sex, age, race, and comorbidities. Sex was binary (women or men), race was categorical (White, Black, Hispanic, Asian or Pacific Islander, Native American, and other), and obesity was binary (no obesity or obesity). The age was converted into eight categorical variables for different age groups: 18-27, 28-37, 38-47, 48-57, 58-67, 68-77, 78-87, and 88 or older. The elixhauser comorbidity index (ECI) was used to assess comorbidities in patients. ECI is a method of categorizing comorbidities on the basis of the International Classification of Diseases, which calculates a weighted sum of each of the presence of 29 binary comorbidities[10]. ECI uses 29 of the Agency for Healthcare Research and Quality comorbidity indicators to predict hospital use and in-hospital mortality[11,12]. In our study, the ECI score ranged from -21 to 44, with a mean score of 3. ECI was converted into four groups: ECI ≤ 0, 1-5, 6-10, and ≥ 11.

### Outcomes and predictive variables

The primary outcomes of the study were inpatient mortality, advanced liver disease-related complications including portal hypertension, ascites, varices and variceal bleeding, cirrhosis, and spontaneous bacterial peritonitis (SBP), as presented in Table 1. SBP and inpatient mortality were not included in our final analysis as it was not possible to conduct meaningful analysis due to their small sample sizes. For each hospitalization, baseline demographics and hospital characteristics were obtained. To address potential confounding factors, we added diabetes, hyperlipidemia, and obesity in Table 1 to compare their baseline prevalence between the case control groups.

### Statistical analyses

A biomedical statistical expert (SHW) reviewed statistical analysis. All data analyses

**Table 1 Patient demographics, hospital characteristics, and outcomes among patients with nonalcoholic fatty liver disease, by history of cannabis use**

| | Cannabis users, *n* = 3820 | Non-cannabis users, *n* = 7625 | *P* value |
|---|---|---|---|
| Sex | | | NS[1] |
| Female | 36.0% | 36.1% | |
| Male | 64.0% | 63.9% | |
| Patient age, mean (SD) | 41.4 (12.9) | 42.0 (12.9) | 0.03[2] |
| Patient age, in 10 years age groups[3] | | | NS[4] |
| 18-27 | 16.8% | 16.6% | |
| 28-37 | 25.3% | 25.3% | |
| 38-47 | 20.4% | 20.5% | |
| 48-57 | 25.8% | 25.8% | |
| 58-67 | 11.0% | 11.0% | |
| 68-77 | 0.8% | 0.8% | |
| Race/ethnicity | | | NS[4] |
| White | 60.6% | 60.7% | |
| Black | 21.6% | 21.6% | |
| Hispanic | 13.9% | 13.9% | |
| Asian or pacific islander | 0.4% | 0.4% | |
| Native American | 0.9% | 0.9% | |
| Others | 2.6% | 2.4% | |
| ECI, mean (SD) | 2.1 (9.4) | 3.6 (8.1) | < 0.01[2] |
| ECI, by category | | | NS[4] |
| ≤ 0 | 47.6% | 47.7% | |
| 1-5 | 15.8% | 15.9% | |
| 6-10 | 19.6% | 19.5% | |
| 11 or higher | 16.9% | 16.9% | |
| Insurance | | | < 0.05[4] |
| Medicare | 16.6% | 17.2% | |
| Medicaid | 42.8% | 30.5% | |
| Private | 21.9% | 42.6% | |
| Self-Pay | 13.4% | 6.0% | |
| Others | 5.4%[5] | 3.7% | |
| Cannabis abuse | | | |
| Non-dependent use | 94.1% | 0 (by definition) | |
| Dependent use | 5.9% | 0 (by definition) | |
| Length of stay (days) | 5.1 | 4.9 | 0.18[2] |
| Total hospitalization charges | $42503 | $43183 | NS[2] |
| Comorbidities | | | |
| Diabetes | 29.2% | 34.8% | < 0.05[5] |
| Obesity | 29.5% | 49.4% | < 0.05[5] |
| Hyperlipidemia | 24.2% | 32.4% | < 0.05[5] |
| Clinical outcomes | | | |

| | | | |
|---|---|---|---|
| Portal hypertension | 80 (2.1%) | 165 (2.2%) | NS[5] |
| Ascites | 170 (4.5%) | 275 (3.6%) | 0.03[5] |
| Varices and variceal bleeding | 50 (1.3%) | 130 (1.7%) | 0.11[5] |
| Cirrhosis | 140 (3.7%) | 275 (3.6%) | NS[5] |

[1]Chi-square, 2-tailed.
[2]Student *t*-test, 2-tailed.
[3]Patient ages ranged from 18 to 73, there was no one over 78.
[4]Chi-square, 2-tailed, for 2 by *n* table: Statistical significance demonstrates that the two groups differ.
[5]Condition absent *vs* present in the 2 groups, chi-square, 2-tailed. NS: Not statistically significant; ECI: Elixhauser comorbidity index.

were conducted using SPSS, version 26 (IBM Corp, Armonk, NY, United States). NIS is based on a complex sampling design that includes stratification, clustering, and weighting. Weighting of patient-level observations was implemented to obtain estimates for the entire population in the United States of hospitalized patients with NAFLD. All statistical analyses were two-tailed, with $P < 0.05$ considered statistically significant. Chi-squared tests were performed to compare categorical data, and Student *t*-tests were performed for continuous data. Univariate linear (continuous outcomes) or logistic (dichotomous outcomes) regression analysis were used to calculate unadjusted odds ratios for the primary outcomes. Subsequently, multivariate regression analysis was used to adjust the results for potential confounders. The final multivariate logistic regression model was built by including those factors associated with the outcome in univariate analysis with $P < 0.20$.

## RESULTS

### Patient characteristics and demographics

A total of 3820 patients with NAFLD who had a history of cannabis use were identified in our study. 7625 patients with NAFLD and without cannabis use were matched and placed in the control group. Table 1 summarizes the patients' baseline characteristics. There was no statistical difference of age, sex, race, ethnicity, and ECI between the groups ($P > 0.05$); therefore, these variables were successfully matched. Yet, patients who used cannabis were slightly younger (mean age: 41.4 *vs* 42.0, $P = 0.03$), and had fewer comorbidities (ECI mean: 2.1 *vs* 3.6, $P < 0.01$), with and without cannabis use. However, the differences in the mean age and mean ECI were not clinically different between the groups.

There were statistical differences in types of insurance in the case group compared with the control group. The proportion of Medicare holders was lower in the cannabis group (16.6% *vs* 17.2%), and a proportion of patients with private insurance was also less in cannabis group (21.9% *vs* 42.6%), with and without cannabis use; all $P < 0.05$. Detailed patient demographics and hospital characteristics are presented in Table 1.

There were fewer patients with metabolic derangement such as diabetes (29.2% *vs* 34.8%), obesity (29.5% *vs* 49.4%), and hyperlipidemia (24.2% *vs* 32.4%) in the cannabis group compared to the non-cannabis group. Of note, 94.1% of the cannabis users were coded under non-dependent use, and due to such a high proportion of non-dependent cannabis use, dose-dependent effects of cannabis on different liver-related complications were not able to be explored.

### Liver disease-related complications

The cannabis group had a higher prevalence of ascites [4.5% *vs* 3.6%, OR 1.25; 95% confidence interval (CI): 1.02-1.51]. There was no statistical difference in the prevalence of portal hypertension [2.1% *vs* 2.2%, not statistically significant (NS)], varices and variceal bleeding (1.3% *vs* 1.7%, $P = 0.11$), and cirrhosis (3.7% *vs* 3.6%, NS), with and without cannabis use. There was a small sample size, less than 10 in one of the groups for spontaneous bacterial peritonitis and inpatient mortality, and therefore not included in the analysis. Detailed chronic liver disease-related complications are presented in Table 1.

### Healthcare resource utilization associated with index admission

The mean length of stay was longer for those who used cannabis compared to those

who did not use cannabis (5.1 d *vs* 4.9 d), but this difference was not statistically different, $P = 0.18$. Total hospitalization charges were lower for patients who used cannabis ($42503) compared with those who did not use cannabis ($43183), but this difference was also not statistically different either.

### *Independent predictors of ascites*

The variables found to be independent predictors of ascites among cannabis users were hyperlipidemia (aOR 1.53; 95%CI: 1.08-2.17), racial group other than White, Black, Hispanic, Asian, Pacific Islanders or Native American (aOR 2.57; 95%CI: 1.24-5.31), and higher comorbidity index (aOR 1.08; 95%CI: 1.07-1.10), all $P < 0.05$. Among patients who did not use cannabis, older age (aOR 1.02; 95%CI: 1.01-1.03), Native Americans (aOR 2.60; 95%CI: 1.03-6.61), and higher comorbidity index (aOR 1.05; 95%CI: 1.04-1.06) were independent predictor of ascites. However, in the same group, obesity (aOR 0.61; 95%CI: 0.46-0.81) and hyperlipidemia (aOR 0.59; 95%CI: 0.44-0.80) were independent protective factors against ascites, all $P < 0.05$. The univariate and multivariate logistic regression analysis for ascites is further delineated in Table 2.

## DISCUSSION

Cannabis is produced from *Cannabis sativa*, known as the marijuana plant, which contains more than 60 active chemical compounds called cannabinoids[3,13]. The primary effects of cannabinoids come from activation of cannabinoid receptors: The two types of G-protein coupled cannabinoid receptors, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2)[7,14]. CB1 is primarily expressed in the brain while CB2 in immune tissue, and under normal physiologic conditions both CB1 and CB2 are very weakly expressed by the liver[15]. However, an upregulation of these receptors are markedly induced in the human liver with cirrhosis[16,17]. The levels of CB1 are six times greater in patients with chronic hepatitis C compared to control while twice greater in patients with cirrhosis compared to those at a lower fibrosis state[18-20]. CB1 and CB2 exert opposite effects on the liver; CB1 has profibrogenic properties while activation of CB2 in mice has been associated with antifibrotic and anti-inflammatory properties[7].

The most active and best-studied cannabinoids are tetrahydrocannabinol (THC) and cannabidiol (CBD)[7,13]. Both THC and CBD act on CB-1 and CB2, but they have different affinities for the receptors. CB1 activation has been shown to have pro-inflammatory effects and to be involved in hepatic fibrosis and steatosis[3,21]. For instance, in CB1 null mice, CB1 blockers increase fatty acid oxidation and decrease hepatic inflammation and lipogenesis[22]. In contrast, CB2 exerts anti-inflammatory effects. CB2 activation has also been shown to suppress obesity and steatohepatitis and to protect the liver from ischemia-reperfusion injury[3]. THC preferentially activates the CB1 pathway, whereas CBD triggers the dominant activation of CB2[3]. The clinical impact of receptor expression and the ratio of activated CB1 and CB2 are unclear among patients with NAFLD. Given the previous evidence of opposite hepatic effects of CB1 and CB2, it is conceivable to suspect the overall cannabinoid effects from both receptors. Ascites is the only liver-related complication that was significantly different between cannabis and non-cannabis groups out of the complications tested in our study, (OR 1.25; 95%CI: 1.02-1.51). Therefore, a possible explanation for higher prevalence of ascites is the dominant hepatic effects of activated CB1 compared to CB2.

Because marijuana use remains illegal in many states in the United States, most marijuana consumed is often not produced under a controlled setting. Therefore, marijuana varies in its contents and has a risk of containing other chemical contaminants, and it also varies in the amounts of active cannabinoids[23]. Different modes of administration along with dynamic pharmacokinetic processes of cannabinoids lead to different bioavailabilities of active cannabinoids ingested. The most common administration route is smoking through cigarettes, pipes, or water pipes[24]. Smoking is the principal route of cannabis administration as it provides a rapid and efficient delivery to the brain, and the ability to titrate dose to the desired effect[25]. The different inhalation methods lead to inconsistent delivery of cannabinoids in the body, thus making control of the dose of active cannabinoids difficult[23]. Oral administration of cannabis involves a slower absorption, and therefore it has a slower, more-delayed peak of THC, one of the primary psychoactive components of cannabis[25]. Wall *et al*[26] reported oral THC bioavailability as 10%-20%. Therefore, many cannabis users prefer smoking due to a quick onset of effects, but smoking is generally not recommended for therapeutic use[25]. Unfortunately, the mode of

**Table 2 Independent predictors of ascites among patients with nonalcoholic fatty liver disease, stratified by cannabis use history**

| Cannabis users (*n* = 3820) | | | | | Non-cannabis users (*n* = 7625) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Ascites** | **OR (95%CI)** | ***P* value** | **aOR (95%CI)** | ***P* value** | **Ascites** | **OR (95%CI)** | ***P* value** | **aOR (95%CI)** | ***P* value** |
| | Univariate logistic regression | | Multivariate logistic regression[1] | | | Univariate logistic regression | | Multivariate logistic regression[1] | |
| Diabetes | 1.17 (0.84-1.62) | NS | | | Diabetes | 1.16 (0.91-1.49) | 0.23 | | |
| Obesity | 1.00 (0.71-1.40) | NS | | | Obesity | 0.41 (0.31-0.53) | < 0.01 | 0.61 (0.46-0.81) | < 0.01 |
| Hyperlipidemia | 1.32 (0.94-1.85) | 0.11 | 1.53 (1.08-2.17) | 0.02 | Hyperlipidemia | 0.57 (0.43-0.77) | < 0.01 | 0.59 (0.44-0.80) | < 0.01 |
| Age (continuous) | 0.99 (0.98-1.01) | 0.22 | | | Age (continuous) | 1.02 (0.01-1.03) | < 0.01 | 1.02 (1.01-1.03) | < 0.01 |
| Race/ethnicity (categoric) | | | | | Race/ethnicity (categoric) | | | | |
| White | Reference | | | | White | Reference | | | |
| Black | 1.13 (0.78-1.64) | NS | | | Black | 0.80 (0.58-1.10) | 0.16 | | |
| Hispanic | 0.87 (0.53-1.42) | NS | | | Hispanic | 1.00 (0.70-1.42) | NS | | |
| Asian or pacific islander | [1] | | | | Asian or pacific islander | [1] | | | |
| Native American | [2] | | | | Native American | 1.96 (0.78-4.92) | 0.15 | 2.60 (1.03-6.61) | 0.04 |
| Others | 2.46 (1.24-4.87) | 0.01 | 2.57 (1.24-5.31) | 0.01 | Others | 0.71 (0.29-1.74) | NS | | |
| Comorbidity (continuous) | 1.08 (1.07-1.10) | < 0.01 | 1.08 (1.07-1.10) | < 0.01 | Comorbidity (continuous) | 1.06 (1.05-1.07) | < 0.01 | 1.05 (1.04-1.06) | < 0.01 |
| Sex (female) | 0.80 (0.58-1.09) | 0.15 | | | Sex (female) | 0.99 (0.77-1.27) | NS | | |

[1]Univariate analysis with screening $P < 0.02$ was used to determine variables to include in the final multivariate analysis. Hyperlipidemia, Race/ethnicity, comorbidity index, and sex included in the regression.
[2]Univariate analysis with screening $P < 0.02$ was used again, and obesity, hyperlipidemia, age, race/ethnicity, and comorbidity index were included in the multivariate regression. NS: Not statistically significant.

cannabis use is not systematically recorded in the NIS, and our retrospective study would be unlikely to reliably capture mode data to compare different modes in the cannabis group. A large prospective study in which mode and dose of cannabis would be ascertained and monitored would be of interest to study the effects of dose and mode on the clinical outcomes.

The patients who used cannabis had higher prevalence of ascites compared to non-cannabis group (OR 1.25; 95%CI: 1.02-1.51). Possible explanations for this finding can be related to 1) hypoalbuminemia due to the toxicity of cannabis use or 2) lower body mass, which is a potential indicator for inadequate nutritional status. Hypoalbuminemia was described as a spectrum of toxic reactions from intravenous cannabis use along with fulminant gastroenteritis, toxic hepatitis, acute renal failure, electrolyte disturbances, leukocytosis, anemia, and relative thrombocytopenia from a study of 4 cases of intravenous cannabis use by Payne *et al*[27] in 1975. Serum albumin is the most abundant plasma protein and therefore binding to albumin is a key determinant of the drug efficacy, distribution and possible toxicity of cannabinoids[28]. The increase in plasma albumin may reduce the unbound fraction of cannabinoids, which further reduces the efficacy of the drug[28]. Along the same reasoning, it is conceivable to hypothesize more cannabinoid toxicity in patients with hypoalbuminemia due to more unbound cannabinoids. A study by Blüml *et al*[29] showed an inverse relationship between body mass index (BMI) and illicit drug use including cannabis use among young males, and therefore along with the toxicity of high-dose cannabis use, hypoalbuminemia from poor nutritional status may explain higher rate of ascites in cannabis users due to the low oncotic pressure from hypoalbuminemia. The forementioned explanation for hypoalbuminemia can partly explain the isolated finding of higher rates of ascites in cannabis users without higher rates of portal hypertension, varices and variceal bleeding and cirrhosis. Another explanation of such finding can be related to a lack of long-term follow-up. As the NIS is limited to hospitalized data, a spectrum of clinical presentations of decompensated

cirrhosis such as varices and variceal bleeding and actual clinical diagnosis of cirrhosis outside of hospital may not be captured in our study design. Further studies with a long-term follow-up investigating cannabis use in patients with NAFLD are warranted to further evaluate the rates of clinical manifestations of decompensated cirrhosis.

The patients with cannabis group had a higher rate of ascites compared to the non-cannabis group despite higher baseline rates of metabolic risks in non-cannabis group such as diabetes, hyperlipidemia, and obesity. This suggests that cannabis may not be a magic bullet for the management of NAFLD. In cannabis group, higher comorbidity index was an independent risk factor for ascites, and this is expected as older patients with more comorbidity are associated with worse prognosis in patients with chronic liver diseases. In non-cannabis group, age and higher comorbidity were again independent risk factors for ascites. However, strikingly obesity (aOR 0.61; 95%CI: 0.46-0.81) and hyperlipidemia (aOR 0.59; 95%CI: 0.44-0.88) were independent protective factors against ascites in patients with NAFLD who did not use cannabis. A possible explanation for this finding can be related to nutritional status in patients with ascites. Metabolic derangements such as obesity and insulin resistance predispose to NAFLD[30], and therefore they are risk factors for development of NAFLD. However, at terminal stage of liver disease with evidence of decompensation such as ascites, the increased BMI and appropriate albumin levels can be protective against developing ascites. Previous studies showed that muscle wasting was worse in obese patients[31] with cirrhosis, and these patients are at high risk for fat-and water-soluble vitamin depletion[32]. Therefore, our finding of the association between obesity and reduced prevalence of ascites is surprising. However, a study by Li *et al*[33] showed that patients with higher BMI had lower rates of liver-related mortality compared to lower BMI among patients with cirrhosis and hepatocellular carcinoma. We were not able to measure nutritional status in our study; therefore although obesity often co-exists with malnutrition, we can speculate that our study population with an obesity diagnosis may have better nutrition than those without obesity.

Patients with cirrhosis are at higher risk for poor tolerance of fasting, and therefore aggressive energy restriction is avoided in these patients[34]. Due to the risk of sarcopenia with weight loss, patients who are advised to lose weight should be monitored for changes in body muscle mass and muscle strength[34]. Therefore, increased BMI in the setting of end-stage liver disease may suggest better nutritional status compared to non-obese patients. In the meantime, sarcopenic obesity is associated with a higher rate of mortality in patients with cirrhosis, and therefore further distinction between sarco obesity and obesity is warranted. The impairment of liver lipogenesis is prominent in decompensated NAFLD, and subsequently low levels of cholesterol in advanced NAFLD is not surprising. Therefore, in patients with decompensated NAFLD as seen with ascites, hyperlipidemia and obesity can be associated with less prevalence of ascites due to a better nutritional status in this vulnerable population with advanced liver disease.

Among cannabis users, hyperlipidemia was an independent risk factor for ascites (aOR 1.53; 95%CI: 1.08-2.17). Previous few studies showed significantly diminished level of serum high-density lipoprotein, low-density lipoprotein, and total cholesterol in liver cirrhosis[35,36]. Similarly, some studies showed a decrease in triglyceride in cirrhosis[36]. Liver biosynthesis is reduced with the progression of cirrhosis[37]. Although there were mixed findings of the relationship between cholesterol values and cirrhosis, our findings are compatible with the abovementioned relationship.

This is the national retrospective study to evaluate the clinical effects of cannabis among hospitalized patients with NAFLD at the national level. Strengths of our manuscript are a large sample size as well as the use of case-control matching analysis, where the groups are matched on age, sex, race, comorbidity. To address potential confounding factors, we also examined diabetes, hyperlipidemia, obesity as co-variates in multi-variate analysis.

Despite these strengths, our study has some limitations that are mainly associated with the nature of large population database studies, in which patients are typically not routinely tested for cannabis use upon admission, and the diagnosis of cannabis use is often made from patient reports. Therefore, unless patients are forthcoming with their caregivers regarding cannabis use, cannabis use may be missed or under-coded. The NIS also relies on accurate billing by clinicians to accurately record diseases and complications, which may lead to an underestimate of diagnosis. In addition, monitoring long-term clinical outcomes, such as liver-related complications not recorded in NIS, remains challenging.

Another limitation of this study is the inability to characterize different concentrations or modes of cannabis administration. 94.1% of the cannabis users were coded under non-dependent use, and due to the limited sample size, we could not

provide dose-dependent data. A possible explanation for the low baseline percentage of dependent cannabis use is a lack of available data, limited current routine clinician assessment, or truly low number of patients who abuse cannabis. We were unable to characterize the concentrations and effects of each cannabinoid, as well as the dose-dependent effects of these cannabinoids. Long-term randomized controlled studies with different levels of cannabinoid types and amounts are warranted to better understand each cannabinoid's effects on the cannabinoid system in the body.

## CONCLUSION

In conclusion, this was the first database study investigating progressive liver disease-related clinical outcomes in hospitalized patients with NAFLD. Cannabis was associated with higher rates of ascites, but there was no statistical difference in the prevalence of portal hypertension, varices and variceal bleeding, and cirrhosis. In the cannabis group, hyperlipidemia was an independent risk factor for ascites but in non-cannabis group hyperlipidemia and obesity were independent protective factors against ascites. A large prospective study in which mode and dose of cannabis would be ascertained and monitored would be of interest.

## ARTICLE HIGHLIGHTS

### *Research background*

The impact of cannabis on the progression of chronic liver diseases has been unclear in prior studies. Systemic reviews showed no association between the increased prevalence of hepatic fibrosis and cannabis use, but cannabis use was still associated with a reduced prevalence of nonalcoholic fatty liver disease.

### *Research motivation*

Because of the modulatory effects of cannabis on risk factors for the development of nonalcoholic fatty liver disease (NAFLD), we wanted to measure the correlation between cannabis use and clinical outcomes related to chronic liver diseases. Without clear evidence between the cannabis use and progression of established NAFLD, it is critical for clinicians to educate the patients on the use of cannabis due to limited evidence on cannabinoid effects. Therefore, our study is motivated to alert clinicians of the possible relevance of ascertainment of cannabis use, which in turn might alter future routine assessments to further probe about cannabis use, especially in light of trends showing recent increases in use in the United States.

### *Research objectives*

Our study aimed to assess the association between cannabis use and clinical liver-related outcomes among hospitalized patients with NAFLD.

### *Research methods*

In our study, we performed a retrospective matched cohort study for hospitalized adult patients with NAFLD. Case-control matching at a ratio of one case to two controls was performed based on sex, age, race, and comorbidities to adjust for confounders. The liver-related complications including portal hypertension, ascites, varices and variceal bleeding, and cirrhosis were measured and compared between two groups.

### *Research results*

The cannabis group had a higher prevalence of ascites compared to patients with NAFLD who did not use cannabis. Obesity and hyperlipidemia were independent protective effects against ascites in the non-cannabis group.

### *Research conclusions*

Cannabis use was associated with higher rates of ascites despite higher rates of metabolic risks in the non-cannabis group such as diabetes, hyperlipidemia, and obesity. This suggests that cannabis may not be a magic bullet for the management of NAFLD, and therefore judicious use of cannabis in advanced NAFLD is warranted.

### *Research perspectives*

A large prospective study in which mode and dose of cannabis use would be warranted to further explore the effects of administration mode and dose of cannabis on the liver-related clinical complications.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1004-1019

DOI: 10.4254/wjh.v12.i11.1004

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

**Retrospective Cohort Study**

# Phase angle and non-alcoholic fatty liver disease before and after bariatric surgery

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**Author contributions:** Teixeira J and Fernandes SA performed the data collection; all the authors wrote the paper and approved the final version.

**Institutional review board statement:** This project was approved by the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). No. 2.423.466.

**Informed consent statement:** Patients who accepted to participate in the study signed the Informed Consent Form.

**Conflict-of-interest statement:** The authors declare no conflicts of interest for this article.

**STROBE statement:** The authors have read the STROBE Statement checklist of items, and the

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## Abstract

### *BACKGROUND*

Obesity is a global health problem that is continuing to increase in the young population. In Brazil, the frequency of obesity in 2018 was 19.8%. Several comorbidities are directly associated with obesity, such as non-alcoholic fatty liver disease (NAFLD), which is considered the most common liver disorder in Western countries and affects up to 46% of adults. Bariatric surgery is effective in treating obesity and can improve NAFLD; however, the effect of bariatric surgery on body composition, phase angle (PA), and improving NAFLD needs to be further studied.

### *AIM*

To analyze the PA in the postoperative period of bariatric surgery and to correlate it with changes in body composition and liver disease.

manuscript was prepared and revised according to the STROBE Statement checklist of items.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Brazil

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 30, 2020
**Peer-review started:** June 30, 2020
**First decision:** August 8, 2020
**Revised:** August 24, 2020
**Accepted:** October 12, 2020
**Article in press:** October 12, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Sasaki A
**S-Editor:** Fan JR
**L-Editor:** Filipodia
**P-Editor:** Xing YX

*METHODS*

This study is a retrospective cohort study of the analysis of the medical records of patients undergoing bariatric surgery in a reference center of a teaching hospital in Porto Alegre over a 2-year period. Patients older than 18 years whose record contained all information relevant to the study were included. The data analyzed were body composition and PA through electrical bioimpedance and NAFLD through liver biopsy in the pre- and postoperative period. The level of significance adopted for the statistical analyses was 5%.

*RESULTS*

We evaluated 379 patients with preoperative data. Regarding PA, 169 patients were analyzed, and 33 patients had liver biopsy pre- and postoperatively with NAFLD information. In total, 79.4% were female, with a mean age of 39.1 ± 10.6 years. The average body mass index (BMI) was 45.9 ± 7.5 kg/m². The PA showed a mean of 5.8 ± 0.62° in the preoperative period and a significant reduction in the postoperative period. A postoperative reduction in body composition data (skeletal muscle mass, fat percentage, fat mass, body cell mass, BMI and visceral fat area) was shown as well. Regarding liver disease, all patients presented a reduction in the degrees and stages of liver disease in the postoperative period, and some had no degree of liver disease at all.

*CONCLUSION*

PA decreased after bariatric surgery, with a direct correlation with weight loss and changes in body composition. The decrease in PA was not correlated with the improvement in NAFLD.

**Key Words:** Obesity; Body composition; Bariatric surgery; Phase angle; Non-alcoholic fatty liver disease; Liver disease



**Core Tip:** We retrospectively evaluated 379 patients who underwent bariatric surgery, with non-alcoholic fatty liver disease in the preoperative period; we compared body composition, phase angle (PA) behavior and change in non-alcoholic fatty liver disease (NAFLD) in the pre- and postoperative period. There was an important improvement in body composition/body fat percentage and an improvement in NAFLD after bariatric surgery. Worsening PA was directly correlated with weight loss and skeletal muscle mass.

**Citation:** Teixeira J, Marroni CA, Zubiaurre PR, Henz A, Faina L, Pinheiro LK, Mottin CC, Fernandes SA. Phase angle and non-alcoholic fatty liver disease before and after bariatric surgery. *World J Hepatol* 2020; 12(11): 1004-1019
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1004.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1004

## INTRODUCTION

Obesity is a complex chronic inflammatory disease characterized by excessive accumulation of body fat. It has multiple etiologies, such as biochemical, genetic, behavioral, social, environmental, and nutritional factors and imbalance between food intake and energy expenditure[1-4]. This disease presents its own pathophysiology as well as associated comorbidities[3,5].

According to VIGITEL Brazil 2018[6], the frequency of obese adults was 19.8% in a society that is aging and more ill. Obesity is closely associated with cardiovascular diseases, dyslipidemia, fatty liver, diabetes mellitus (DM) and other endocrine and metabolic disorders[1]. In obese people, a common comorbidity is non-alcoholic fatty liver disease (NAFLD).

NAFLD is defined as accumulation of fat in more than 5% of hepatocytes[7] without secondary cause, such as alcohol consumption (> 20 g for women and > 30 g for men,

daily), use of steatogenic medications, or hereditary disorders[8].

The gold standard for the diagnosis of NAFLD is liver biopsy[9], and its classification encompasses a wide spectrum of histopathological changes, from simple hepatic steatosis, which can evolve from non-alcoholic steatohepatitis (NASH), to cirrhosis and/or hepatocellular carcinoma[10]. Therefore, NAFLD, due to its different staging, may or may not present fibrosis in hepatocytes[11,12]. NAFLD is considered the most common liver disease in Western countries, affecting 90% of morbidly obese patients eligible for bariatric surgery[11].

Because of the difficulty found in the clinical treatment of obesity, bariatric surgery is more efficient as a treatment option for individuals with severe obesity, when compared to non-surgical interventions[13]. Furthermore, surgical treatment shows improvement or remission in NAFLD[14].

According to the Brazilian Society of Bariatric and Metabolic Surgery[15], bariatric surgery combines techniques aimed to treat morbid obesity, severe obesity, and diseases associated with excess body fat or exacerbated by it. Gastric bypass surgery in Y by Roux (BPGYR) or Fobi-Capella surgery is the most commonly performed technique in Brazil and worldwide.

Electrical bioimpedance (BIA) is a method for analyzing body composition and is based on the principle of resistance and reactance that cells impose on the electrical current emitted by the device[16]. The human body is constituted of conductors like water and non-conductors, like body fat[17,18].

Several parameters are measured using BIA, including body water, lean mass, fat mass, and phase angle (PA). BIA assesses nutritional status and can be a good method for prognostic evaluation, as it is practical, fast, non-invasive, and low cost[19]. However, body composition values in patients with dysmorphia (edema, ascites, and morbid obesity), as measured by BIA, may suffer interference. This is why PA has been widely used, since it is not associated with interference[20,21].

Currently, there are segmented, multifrequency BIA devices with greater precision for assessing body composition in morbidly obese patients, as validated by Faria *et al*[22].

PA was originally described by Baumgartner *et al*[23] for the diagnosis of metabolic disorders. It is a parameter applicable in clinical practice because it reliably helps to describe cell vitality and integrality. High values (up to 8°) may indicate body homeostasis, whereas values below 6°, depending on the disease, reflect a poor clinical prognosis, indicating changes in the selective permeability of the cell membrane[23,24].

As already mentioned, PA reflects cellular integrity and functionality by measuring, through an electrical current, the values of resistance and reactance of the membrane of these cells, with skeletal muscle being a conductor of electrical current and the opposite occurring with fat mass[23-25]. In view of this fact, we believe that the body change resulting from bariatric surgery will reflect an improvement in NAFLD and can be measured by PA.

To date, there are not enough studies evaluating morbid obesity, body composition (described by the BIA), and associated comorbidities, such as NAFLD.

The present study aims to analyze the behavior of PA in the postoperative period of bariatric surgery, correlating it with changes in body composition and improvement of liver disease.

## MATERIALS AND METHODS

This is a retrospective cohort study that analyzed the medical records of patients undergoing bariatric surgery in a referral center of a teaching hospital in Porto Alegre. Patients over 18-years-old whose record contained all the information relevant to the study in the electronic or physical medical record were included. Patients who did not contain complete data were excluded. For convenience, the sample was carried out from July 2015 to July 2017. The data obtained were related to the protocol for routine pre- and postoperative care at the service's outpatient clinic.

### *Electrical bioimpedance-PA and body composition*

Data on body composition and weight were measured using BIA in all patients in the week preceding bariatric surgery without prior preparation. For BIA, the patient stood upright on the InBody 770 device from Ottoboni, with an electric current intensity of 80 μA and 50/60 kHz frequency. The PA was obtained through the values of resistance and reactance through the formula: PA = tangent arc (Xc/R) × 180/3.1416, described in the result sheet. The BIA was performed in a second step, and for

comparative postoperative analysis, we analyzed those patients who used the same BIA device 6 mo to 12 mo after bariatric surgery for routine postoperative evaluation.

### Anthropometric data-height and body mass index

Height was measured using a wall Tonelli stadiometer, model E150 A, with the patient standing upright and barefoot, with their feet together, and with their backs positioned against the wall. Body mass index (BMI) was calculated using the equation weight in kilograms, divided by height in meters squared and classified according to the World Health Organization[4]: BMI ≥ 30 kg/m² to 34.9 kg/m² Obesity Grade I, BMI between 35 kg/m² to 39.9 kg/m² Obesity Grade II, and BMI ≥ 40 kg/m² Obesity Grade III. The group of patients with a BMI greater than 50 kg/m² was analyzed separately.

### Bariatric surgery

The bariatric surgery used was the BPGYR with intestinal derivation after monitoring and preparation with a multidisciplinary service team. All patients met the criteria for bariatric surgery. The surgeries were performed by four specialist surgeons, from the same team, trained and with much experience.

### Liver biopsy-NAFLD

Liver biopsies were routinely performed during the bariatric surgery trans operation by the surgeon under direct vision using a Tru-Cut needle at the beginning of the surgical procedure before liver withdrawal. The biopsies were analyzed by the same pathologist at the Hospital's Pathology Laboratory. The classification was made using the criteria of Kleiner *et al*[26], as follows: Absence or presence of NAFLD and/or cirrhosis; steatosis activity (absent, discreet, moderate, accented, and massive–according to grade and location of the injury); ballooning and lobular inflammation; degrees of NASH (1, 2, 3, and 4), and fibrosis stages (1, 2, 3 and 4). The liver biopsies performed in the postoperative period were obtained by the same surgical team in patients who underwent a second intervention (cholecystectomy or appendectomy) in the period from 6 to 12 mo after bariatric surgery and analyzed in the same way as those of the first biopsy.

### Statistical analysis

Quantitative variables are described as mean and standard deviation or median and interquartile range. Categorical variables are described by absolute and relative frequencies.

To compare means before and after bariatric surgery, the t-student test for paired samples was applied. When comparing nominal categorical variables, the McNemar test was used and, for ordinals, the Wilcoxon test. To compare means between genders, the t-student test for independent samples was applied.

In the association between quantitative and ordinal variables, Pearson or Spearman correlation tests were used.

The level of significance adopted was 5% ($P < 0.05$), and the analyses were performed using the SPSS version 21.0 program (Armonk, NY, United States).

The project was elaborated in accordance with resolution 466 of 2012, which regulates the conduct of research in human beings, and submitted to and approved by the Research Ethics Committee under number 2.423.466. Patients who accepted to participate in the study signed the Informed Consent Term.

This study was reviewed by our specialist Biostatistics, Mestre, Ceres Andréia Vieira de Oliveira.

## RESULTS

Of the 727 patients operated on in the period, 379 who had complete preoperative information were allocated. For the analysis of data related to PA, 169 of these patients who underwent pre- and post-evaluation on the same device were allocated. Regarding NAFLD, we analyzed 33 patients who underwent postoperative liver biopsy.

Of the 379 patients, 79.4% were female, with a mean age of 39.1 ± 10.6 years and a BMI of 45.9 ± 7.5 kg/m², classified as Obesity Grade III[4]. It is noteworthy that more than 22% of patients had a BMI greater than 50 kg/m². Full-body PA showed an average of 5.89° ± 0.62°, with a minimum of 4.3° and a maximum of 7.9°. The other characteristics of the sample are shown in Table 1.

**Table 1 Sample characteristics, *n* = 379**

| Variables | *n* = 379 |
|---|---|
| Gender, *n* (%) | |
| Male | 78 (20.6) |
| Female | 301 (79.4) |
| Age in yr, mean ± SD | 39.1 ± 10.6 |
| Weight in kg, mean ± SD | 123.7 ± 24.8 |
| Minimum | 77 |
| Maximum | 235 |
| BMI in kg/m$^2$, mean ± SD | 45.9 ± 7.5 |
| BMI classification, *n* (%) | |
| 30-34.99 kg/m$^2$ | 3 (0.8) |
| 35-39.99 kg/m$^2$ | 82 (21.6) |
| 40-49.99 kg/m$^2$ | 210 (55.4) |
| 50 kg/m$^2$ or more | 84 (22.2) |
| SMM in kg, mean ± SD | 34.1 ± 7.5 |
| BCM in kg, mean ± SD | 39.4 ± 8.3 |
| Fat mass in kg, mean ± SD | 62.6 ± 15.1 |
| % Fat-mean ± SD | 50.7 ± 4.6 |
| Visceral fat area in cm$^2$, mean ± SD | 243.7 ± 31.2 |
| Phase angle °, mean ± SD | |
| Full-body | 5.89 ± 0.62 |
| RA | 5.58 ± 0.62 |
| LA | 5.42 ± 0.65 |
| Tr | 7.97 ± 1.18 |
| RL | 6.14 ± 0.81 |
| LL | 6.07 ± 0.84 |

BCM: Body cell mass; BMI: Body mass index; LA: Left arm; LL: Left leg; RA: Right arm; RL: Right leg; SD: Standard deviation; SMM: Skeletal muscle mass; Tr: Torso.

All patients (*n* = 379) were diagnosed with NAFLD by liver biopsy, and the histological characteristics are shown in Table 2 Regarding the degrees of the disease, 78.1% had NASH and 43% fibrosis, ranging from F1 to F3. No patient had cirrhosis.

The difference in pre- and postoperative body composition (*n* = 379) is found in Table 3, with all items statistically significant ($P < 0.001$).

The associations of body composition variables with PA, stratified by gender (*n* = 379), are shown in Table 4. In males, there was an inverse, statistically significant association between the percentage of fat, fat mass, and weight with PA in most body compartments, except the torso.

In females, there was a negative, statistically significant association between the percentage of fat and PA in all compartments. There was a positive, statistically significant association between skeletal muscle mass (SMM) and body cell mass (BCM) with PA in most body compartments, except the legs.

There was a negative, statistically significant association ($r = -0.483$; $P < 0.001$) between the reduction of full-body PA and the percentage of weight loss, as shown in Figure 1. There was a statistically significant positive association between the reduction of full-body PA with loss of SMM ($r = 0.307$; $P < 0.001$), as shown in Figure 2. The association of the reduction in PA of the whole body was also positive and significant with the loss of fat mass, ($r = 0.280$; $P < 0.001$), MCC ($r = 0.287$; $P < 0.001$), visceral fat area ($r = 0.275$; $P < 0.001$), BMI ($r = 0.413$; $P < 0.001$), and variation in the fat

**Table 2 Sample characteristics for liver disease, *n* = 379**

| Variables | *n* (%) |
|---|---|
| NAFLD | |
| Yes | 379 (100.0) |
| No | 0 (0.0) |
| Hepatic steatosis | |
| Absent | 0 (0.0) |
| Discreet | 159 (42.0) |
| Moderate | 104 (27.4) |
| Accented | 84 (22.2) |
| Massive | 32 (8.4) |
| Ballooning | |
| Absent | 91 (24.0) |
| Discreet | 221 (58.3) |
| Moderate | 18 (4.7) |
| Accented | 49 12.9) |
| Massive | 0 (0.0) |
| Lobular inflammation | |
| Absent | 243 (64.1) |
| Discreet | 100 (26.4) |
| Moderate | 28 (7.4) |
| Accented | 8 (2.1) |
| Massive | 0 (0.0) |
| NASH | |
| Absent | 83 (21.9) |
| Grade 1 | 201 (53.0) |
| Grade 2 | 67 (17.7) |
| Grade 3 | 28 (7.4) |
| Grade 4 | 0 (0.0) |
| Absent | 216 (57.0) |
| Grade 1 | 86 (22.7) |
| Grade 2 | 44 (11.6) |
| Grade 3 | 33 (8.7) |
| Grade 4 | 0 (0.0) |
| Cirrhosis | |
| Yes | 0 (0.0) |
| No | 379 (100.0) |

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

percentage ($r$ = 0.304; $P$ < 0.001), as shown in Figure 3.

Men showed a more marked and significant reduction in the percentage of fat (-16 ± 4.9 *vs* -12.3 ± 5.3; $P$ < 0.001) and visceral fat area (-110.7 ± 58.9 *vs* -90 ± 42.8; $P$ = 0.024) when compared to women.

After bariatric surgery, a significant reduction in PA values ($n$ = 169) was observed in all body compartments ($P$ < 0.001), as described in Table 5.

**Table 3 Variation of body composition before and after bariatric surgery, *n* = 379**

| Variables | Pre | Post | *P* value |
|---|---|---|---|
| | mean ± SD | mean ± SD | |
| BMI in kg/m² | 45.9 ± 7.5 | 31.2 ± 5.0 | < 0.001 |
| SMM in kg | 34.1 ± 7.5 | 29.0 ± 6.4 | < 0.001 |
| BCM in kg | 39.4 ± 8.3 | 33.9 ± 6.9 | < 0.001 |
| Fat mass in kg | 62.6 ± 15.1 | 31.9 ± 11.1 | < 0.001 |
| % Fat | 50.7 ± 4.6 | 37.2 ± 8.1 | < 0.001 |
| Visceral fat area in cm² | 243.7 ± 31.2 | 152.0 ± 51.0 | < 0.001 |

BCM: Body cell mass; BMI: Body mass index; SD: Standard deviation; SMM: Skeletal muscle mass.

**Table 4 Association of body composition variables with phase angle in men and women, using Pearson's correlation coefficient, *n* = 379**

| Variables | Weight in kg | SMM in kg | BCM in kg | Fat mass in kg | % Fat | Visceral fat area in cm² |
|---|---|---|---|---|---|---|
| Male gender, *n* = 78 | | | | | | |
| PA full body | -0.418[c] | -0.118 | -0.101 | -0.469[c] | -0.401[c] | -0.075 |
| PA RA | -0.299[b] | -0.083 | -0.064 | -0.337[b] | -0.287[a] | -0.095 |
| PA LA | -0.286[a] | -0.031 | -0.008 | -0.349[b] | -0.341[b] | -0.136 |
| PA Tr | -0.074 | 0.074 | 0.094 | -0.134 | -0.185 | 0.311[b] |
| PA RL | -0.493[c] | -0.179 | -0.170 | -0.531[c] | -0.421[c] | -0.101 |
| PA LL | -0.509[c] | -0.211 | -0.209 | -0.534[c] | -0.403[c] | -0.066 |
| Female gender, *n* = 301 | | | | | | |
| PA full body | -0.106 | 0.125[a] | 0.122[b] | -0.152[b] | -0.393[c] | -0.098 |
| PA RA | 0.011 | 0.169[b] | 0.148[a] | -0.036 | -0.245[c] | -0.111 |
| PA LA | 0.043 | 0.193[b] | 0.155[b] | -0.009 | -0.226[c] | -0.135[a] |
| PA Tr | 0.024 | 0.120[a] | 0.133[a] | -0.030 | -0.172[b] | -0.058 |
| PA RL | -0.154[b] | 0.083 | 0.068 | -0.182[b] | -0.406[c] | -0.043 |
| PA LL | -0.151[b] | 0.069 | 0.052 | -0.187[b] | -0.388[c] | -0.086 |

[a]*P* < 0.05.
[b]*P* < 0.01.
[c]*P* < 0.001. BCM: Body cell mass; LA: Left arm; LL: Left leg; PA: Phase angle; RA: Right arm; RL: Right leg; SMM: Skeletal muscle mass; Tr: Torso.

There was a statistically significant inverse association between BMI and full-body PA ($P < 0.018$), right leg and left leg ($P < 0.001$), as shown in Table 6. Regarding PA and NAFLD, there was no significant association ($P > 0.05$).

The analysis of the postoperative liver biopsy, compared with the preoperative biopsy of these patients ($n = 33$), showed that all of them had a reduction in the degrees and stages of liver disease, and 18.2% had no degree of liver disease ($P < 0.05$).

The other histological changes, which decreased from 75 to 90%, are described in Table 7. The body composition of this group ($n = 33$) showed that all parameters significantly decreased ($P < 0.001$) and that there was a reduction in PA in all compartments ($P < 0.001$), as described in Table 8.

There was a positive, statistically significant association between BMI (kg/m²) and lobular inflammation ($P < 0.05$), NASH ($P < 0.01$), and fibrosis ($P < 0.05$). The other body composition variables did not correlate with the different histological characteristics of NAFLD.

There was an association between the variations of PA before and after bariatric surgery regarding the degree of lobular inflammation ($r_s = -0.593$; $P = < 0.001$) but not

**Table 5 Variation of phase angle in the pre- and postoperative period of bariatric surgery, *n* = 169**

| Variables | Pre | Post | *P* value |
|---|---|---|---|
| | mean ± SD | mean ± SD | |
| PA full body | 5.92 ± 0.55 | 4.98 ± 0.55 | < 0.001 |
| PA RA | 5.58 ± 0.60 | 4.73 ± 0.59 | < 0.001 |
| PA LA | 5.42 ± 0.63 | 4.54 ± 0.57 | < 0.001 |
| PA Tr | 7.87 ± 1.15 | 6.76 ± 1.29 | < 0.001 |
| PA RL | 6.25 ± 0.66 | 5.19 ± 0.65 | < 0.001 |
| PA LL | 6.18 ± 0.70 | 5.14 ± 0.66 | < 0.001 |

LA: Left arm; LL: Left leg; PA: Phase angle; RA: Right arm; RL: Right leg; SD: Standard deviation; Tr: Torso.

**Table 6 Association of body mass index with phase angle through Pearson correlation, *n* = 169**

| Variables | BMI | |
|---|---|---|
| | *r* | *P* value |
| PA full body | -0.121 | 0.018 |
| PA RA | 0.031 | 0.551 |
| PA LA | 0.057 | 0.265 |
| PA Tr | 0.054 | 0.298 |
| PA RL | -0.262 | < 0.001 |
| PA LL | -0.262 | < 0.001 |

BMI: Body mass index; LA: Left arm; LL: Left leg; PA: Phase angle; *r*: Pearson's correlation coefficient; RA: Right arm; RL: Right leg; Tr: Torso.

steatosis ($r_s$ = 0.305; *P* = 0.095), ballooning ($r_s$ = 0.057; *P* = 0.760), NASH ($r_s$ = -0.197; *P* = 0.288), and fibrosis ($r_s$ = -0.183; *P* = 0.324).

## DISCUSSION

Obese patients, candidates for bariatric surgery diagnosed with NAFLD, have been extensively studied[8,27-31]; and, to date, changes in lifestyle are the only effective forms of treatment for NAFLD. It was established that a loss of 7%-10% of body weight is necessary to present any change[32]. However, in order to guarantee body homeostasis, it is essential that the weight loss of these patients is the highest possible percentage of fat mass and not of SMM, preserving muscle volume and functionality. Therefore, BIA and PA are essential tools for monitoring the body characteristics and health of these patients[19,33].

Most of our patients were women, with a mean age of 39.1 ± 10.6 years and a BMI of 45.9 ± 7.5 kg/m², findings similar to those of Losekann *et al*[9], who in 2013 analyzed 250 patients with liver biopsies performed in the bariatric surgery trans operation, which 80% were women, with a mean age of 36.8 ± 10.2 years and a BMI of 43.6 ± 5.2 kg/m².

BMI is an analytical, non-laboratory method that is easy to apply and reproducible, allowing an indirect assessment of body composition, and is a defining parameter of indication for bariatric surgery.

The mean BMI after surgery decreased to 31.2 ± 5.0 kg/m² (*P* < 0.001), changing from Grade III Obesity to Grade I. The reduction in BMI was significant in both sexes, with no significant difference between them. All body composition parameters had a significant decrease (*P* < 0.001), mainly on fat mass and visceral fat area. In the present study, men compared to women showed a more marked and significant reduction in the percentage of fat and in the area of visceral fat.

Perrone *et al*[34] reported that the decrease in BMI after bariatric surgery in men was

**Table 7 Degrees and staging of non-alcoholic fatty liver disease before and after bariatric surgery, *n* = 33**

| Variables | Pre | Post | *P* value |
|---|---|---|---|
| | *n* (%) | *n* (%) | |
| NAFLD | | | 0.031 |
| Yes | 33 (100) | 27 (81.8) | |
| No | 0 (0.0) | 6 (18.2) | |
| Hepatic steatosis | | | < 0.001 |
| Absent | 0 (0.0) | 6 (18.2) | |
| Discreet | 14 (42.4) | 24 (72.7) | |
| Moderate | 8 (24.2) | 3 (9.1) | |
| Accented | 8 (24.2) | 0 (0.0) | |
| Massive | 3 (9.1) | 0 (0.0) | |
| Ballooning | | | < 0.001 |
| Absent | 5 (15.2) | 29 (87.9) | |
| Discreet | 23 (69.7) | 4 (12.1) | |
| Moderate | 3 (9.1) | 0 (0.0) | |
| Accented | 2 (6.1) | 0 (0.0) | |
| Lobular inflammation | | | 0.003 |
| Absent | 21 (63.6) | 30 (90.9) | |
| Discreet | 7 (21.2) | 3 (9.1) | |
| Moderate | 3 (9.1) | 0 (0.0) | |
| Accented | 2 (6.1) | 0 (0.0) | |
| NASH | | | < 0.001 |
| Absent | 6 (18.2) | 25 (75.8) | |
| Grade 1 | 20 (60.6) | 5 (15.2) | |
| Grade 2 | 4 (12.1) | 3 (9.1) | |
| Grade 3 | 3 (9.1) | 0 (0.0) | |
| Fibrosis | | | 0.033 |
| Absent | 26 (78.8) | 27 (81.8) | |
| Grade 1 | 3 (9.1) | 4 (12.1) | |
| Grade 2 | 0 (0.0) | 0 (0.0) | |
| Grade 3 | 4 (12.1) | 2 (6.1) | |

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

greater than that in women, without influencing the improvement of comorbidities in the long term, because the BMI does not differentiate or qualify weight loss, which should be mostly fat and not SMM. A study by Hartwig *et al*[35], showed similar results in men and women regarding the decrease in the percentage of fat and fat mass in the postoperative period. De Paris *et al*[36] showed a reduction in body composition (weight, fat-free mass, SMM, fat mass, and fat percentage) in the postoperative period of bariatric surgery, results similar to ours.

All of our patients had NAFLD; accentuated or massive steatosis in 30.6%; ballooning in 76%; lobular inflammation in 35.9%; NASH in 78.1%; fibrosis in 43%; and no case of cirrhosis. NAFLD patients are obese[37], with a prevalence of 51% of cases[38]. In patients with obesity undergoing bariatric surgery, the percentage of steatosis ranged from 87.6% to 100%[8,27-31], ballooning from 58.9% to 88%[29,30], lobular inflammation from 23% to 88%[29,31], and fibrosis from 31% to 44.9%[28,29,31], findings

**Table 8 Phase angle and body composition in patients who underwent liver biopsy after bariatric surgery, *n* = 33**

| Variables | Pre | Post | *P* value |
|---|---|---|---|
| | mean ± SD | mean ± SD | |
| BMI in kg/m² | 45.2 ± 6.9 | 31.8 ± 5.4 | < 0.001 |
| SMM in kg | 31.9 ± 6.8 | 27.1 ± 5.3 | < 0.001 |
| BCM in kg | 37.0 ± 7.8 | 32.1 ± 5.9 | < 0.001 |
| Fat mass in kg | 60.7 ± 13.3 | 33.6 ± 11.1 | < 0.001 |
| % Fat | 51.6 ± 3.5 | 39.8 ± 8.2 | < 0.001 |
| Visceral fat area in cm² | 249.3 ± 23.1 | 162.5 ± 52.3 | < 0.001 |
| PA full body ° | 5.93 ± 0.65 | 4.91 ± 0.60 | < 0.001 |
| PA RA ° | 5.55 ± 0.77 | 4.61 ± 0.64 | < 0.001 |
| PA LA ° | 5.44 ± 0.86 | 4.44 ± 0.58 | < 0.001 |
| PA Tr ° | 7.84 ± 1.24 | 6.73 ± 1.03 | < 0.001 |
| PA RL ° | 6.42 ± 0.72 | 5.18 ± 0.68 | < 0.001 |
| PA LL ° | 6.38 ± 0.74 | 5.19 ± 0.66 | < 0.001 |

BCM: Body cell mass; BMI: Body mass index; LA: Left arm; LL: Left leg; PA: Phase angle; RA: Right arm; RL: Right leg; SMM: Skeletal muscle mass; Tr: Torso.

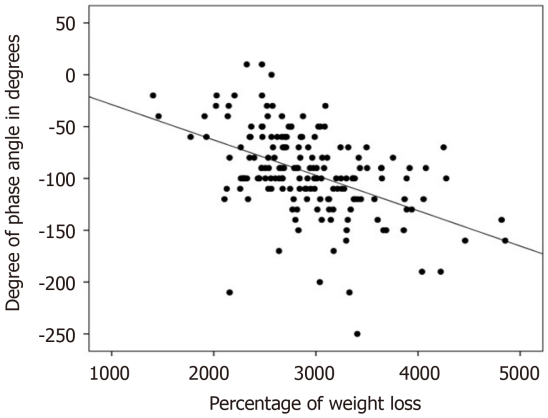


**Figure 1 Association between the percentage of weight loss with the reduction of the phase angle in the postoperative period of bariatric surgery (*n* = 169).**

similar to ours.

Some NAFLD patients present progression from simple steatosis to advanced stages, such as NASH and fibrosis, increasing the risk of cirrhosis and hepatocellular carcinoma. In addition, it is believed that NAFLD is implicated in the pathogenesis of type 2 DM and cardiovascular diseases[32]. These facts are fundamental in the search for the reduction of obesity that bariatric surgery provides.

We found a direct association between BMI and lobular inflammation, NASH, and fibrosis ($P < 0.05$), which is why it is important to monitor evolution of this group of patients.

The assessment of body composition is limited in several clinical conditions; and, therefore, the use of BIA data has gained increasing attention[39]. PA can be used as a biomarker for lean mass and/or for reducing muscle mass[39-41], besides being recognized as a marker of malnutrition[40,41] and predictor of morbidity and mortality in several diseases[18,42].

The PA of healthy individuals can vary between 6° and 7°, according to Bosy-Westphal *et al*[43] and 6.96° according to Barbosa-Silva *et al*[44]. There is no reference value that classifies PA for patients with obesity who have NAFLD. In the studied population, the PA was 5.89 ± 0.62°, lower than the values described above, probably

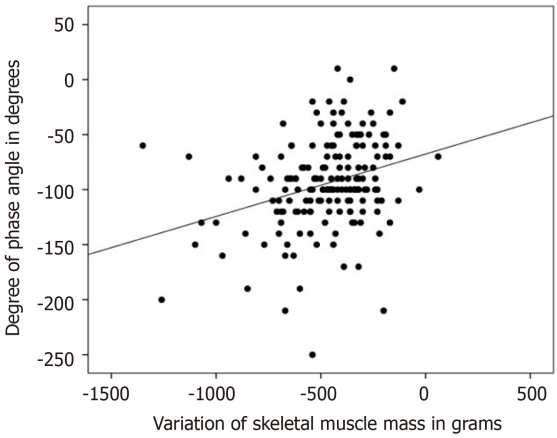


**Figure 2 Association between the variation in skeletal muscle mass with the reduction of phase angle in the postoperative period of bariatric surgery (*n* = 169).**

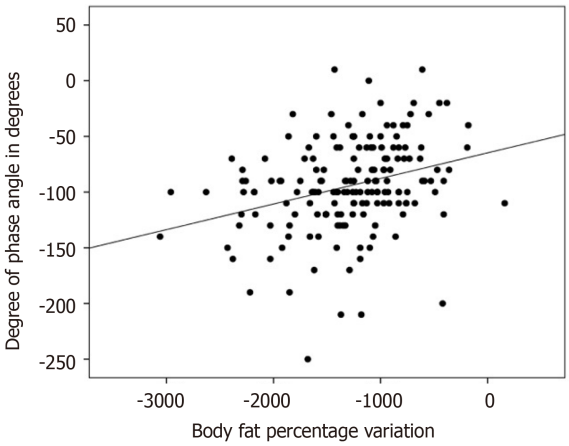


**Figure 3 Association between the reduction in the percentage of body fat with the reduction in phase angle in the postoperative period of bariatric surgery (*n* = 169).**

due to obesity.

In Table 4, we evaluated the associations of PA with the variables of body composition, and we observed in men a significant negative relation with decreased weight, fat mass, and percentage of fat ($P < 0.01$). In women, this relation of PA was with the decrease in the percentage of fat ($P < 0.01$). There was a significant positive relation with SMM and BCM ($P < 0.05$). PA decreased in most variables related to weight loss ($P < 0.05$). Baumgartner *et al*[45], in 1988, observed a statistically significant negative correlation of PA with the percentage of body fat, corroborating the findings of our study. A study by Peres *et al*[46] analyzed 66 patients over 18 years of age with NAFLD, chronic hepatitis, cirrhosis, and hepatocellular carcinoma who had a mean PA of 5.1°, values similar to those found in the present study.

The post-surgical evaluation showed that PA decreased significantly in all evaluated segments ($P < 0.001$), as shown in Table 5, and its correlation with BMI showed a significant difference ($P = 0.018$), as shown in Table 6. These are apparently paradoxical findings, based on knowledge that the highest PA means improvement and the lowest means clinical worsening. We must consider the time of the second assessment, within the first year, as directly related to the decrease. A new evaluation of PA, at a longer time point, already programmed, may show an increase in PA.

Norman *et al*[47] described that BMI is one of the biological factors that influences PA. Furthermore, Llames *et al*[33] found a reduction in PA in individuals with a BMI greater than 35 kg/m². Bosy-Westphal *et al*[43] showed that PA increased with an increase in BMI up to 30 kg/m² and that such physiological behavior can be explained as a reflection of the increase in the number of cells (adipocytes and myocytes), since the reactivity of BIA is dependent on the amount of cell membranes. The same study

showed that with BMI above 40 kg/m², there was an inverse relation between PA and BMI. The explanation is that severe obese individuals have a loss of functionality in the cell membrane, which may contribute to the decrease in PA.

In 2017, Vassilev *et al*[48] in Germany, with 173 patients undergoing bariatric surgery (BPGYR), showed a correlation between PA and weight loss, between 6 and 12 mo postoperatively, and a significant reduction in lean mass with a reduction in PA, results similar to ours. Koehler *et al*[49], with 20 patients undergoing bariatric surgery (BPGYR), showed significant results in reducing PA, at an earlier time, from 3 to 6 mo.

In the present study, there was a negative association between the reduction of full-body PA and the percentage of weight loss ($P < 0.001$), as shown in Figure 1. There was a positive association between the reduction of full-body PA with the variation of SMM ($P < 0.001$), as shown in Figure 2; and there was a positive association between the reduction of PA of the whole body and the percentage variation of fat ($P < 0.001$), as shown in Figure 3. These same associations were observed in the other parameters (loss of fat mass; BCM; visceral fat area, BMI), all with significant value ($P < 0.001$).

In the Vassilev *et al* study[48], the higher the percentage of body fat in the postoperative period, the lower the PA, and the reduction in BCM occurred with a reduction in PA after 9 mo of surgery. Thus, it is clear that the percentage of fat, even after weight loss, continues to have a negative influence on PA. The decrease in PA in the postoperative period is linked to weight loss, which can be a confusing factor when relating the reduction in the percentage of fat and PA, since the present study also associated a reduction in BMI with a decrease in PA.

There was an association between the variations of PA before and after bariatric surgery regarding the degree of lobular inflammation ($r_s = -0.593$; $P = < 0.001$) but not with steatosis ($r_s = 0.305$; $P = 0.095$), ballooning ($r_s = 0.057$; $P = 0.760$), NASH ($r_s = -0.197$; $P = 0.288$), and fibrosis ($r_s = -0.183$; $P = 0.324$).

Regarding the role of PA and NAFLD staging, Peres *et al*[46] found no difference in PA values in patients with different degrees and stages of liver disease; our findings did not show significance associating the decrease in PA with the variation in the degree of steatosis, ballooning, NASH, and fibrosis in the pre- and postoperative period, demonstrating that the improvement of liver disease after bariatric surgery is not related to the worsening of PA.

The comparison of liver histology before and after surgery ($n = 33$) showed a significant improvement in NAFLD, where 18.2% had no degree of liver disease ($P = 0.031$); all parameters analyzed showed a reduction in histological changes, from 75% to 90% ($P < 0.05$), as described in Table 7.

Máttar *et al*[24] analyzed patients undergoing different bariatric surgery techniques and observed significant improvement in steatosis (from 88% to 8%), lobular inflammation (from 23% to 2%), and fibrosis (31% to 13%). In addition, 37% no longer had lobular inflammation, and 20% had no fibrosis, in line with our study. Similar data were found in Cazzo *et al*[50] review in 2017 with patients undergoing bariatric surgery using different surgical techniques[27,30,51-53].

A pioneering study by Silverman *et al*[54] in 1995 observed an improvement in liver disease in the postoperative period of bariatric surgery with the same surgical technique we used, with a 71% reduction in steatosis; 19% absence of steatosis; 76.9% absence of fibrosis, and 7.6% reduction in fibrosis, corroborating with our findings. There was a marked change in body composition in this group ($n = 33$), in all parameters ($P < 0.001$), as well as a reduction in PA ($P < 0.001$), as shown in Table 8. These data reinforce that weight loss is fundamental in the treatment of NAFLD.

## CONCLUSION

The PA decreased after bariatric surgery, with a direct correlation with weight loss and changes in body composition. Grade III obesity became Grade I obesity. The decrease in the PA after bariatric surgery did not correlate with the improvement of NAFLD, even with the marked improvement of NAFLD. The decrease in the PA after performing bariatric surgery correlated with the decrease in the BMI, loss of SMM, and decreases in body fat (in percentages and kilograms), BCM, and visceral fat area.

We believe that PA should increase with more time after bariatric surgery, since the change in the body composition of the operated patient will reflect an improvement in body mass distribution and, consequently, less inflammatory process. An important point is what form of protein supplementation should be used with these patients after surgery in order to minimize the loss of muscle mass and thereby increase PA. With the data presented in this study, we suggest that PA may be a marker of the state of

body composition linked to the functionality of SMM.

## ARTICLE HIGHLIGHTS

### *Research background*

Obesity is a complex chronic inflammatory disease characterized by excessive accumulation of body fat. In obese people, a common comorbidity is non-alcoholic fatty liver disease (NAFLD). NAFLD is considered the most common liver disease in Western countries, affecting 90% of morbidly obese patients eligible for bariatric surgery. To evaluate this patients, bioimpedance (BIA) can be a good method for nutritional and prognostic evaluation, using phase angle (PA).

### *Research motivation*

There are not enough studies to evaluate morbid obesity, its body composition (described by the BIA), and associated comorbidities, such as NAFLD. We believe that the body change resulting from bariatric surgery will reflect an improvement in NAFLD and can be measured by PA, since it reflects cellular integrity and functionality by measuring, through an electrical current, the values of resistance and reactance of the membrane of these cells, with skeletal muscle being a conductor of electrical current and the opposite occurring with fat mass.

### *Research objectives*

The aim of this study was to analyze the behavior of PA in the postoperative period of bariatric surgery, correlating it with changes in body composition and improvement of liver disease.

### *Research methods*

This was a retrospective cohort study that analyzed the medical records of 727 patients undergoing bariatric surgery in a referral center of a teaching hospital in the south of Brazil. For convenience, the sample was carried out from July 2015 to July 2017. The data obtained were related to the protocol for routine pre- and postoperative care at the service's outpatient clinic. Quantitative and categorical variables analyses were performed to assess the association between PA, NAFLD, and body composition before and after bariatric surgery.

### *Research results*

We analyzed 727 patients' medical records, and 379 patients were selected for having all preoperative data. Regarding PA, 169 patients were analyzed, and 33 patients had liver biopsy pre-and postoperatively with NAFLD information. The PA showed a significant reduction in the postoperative period as well as body composition data. Regarding liver disease, all patients presented a reduction in the degrees and stages of liver disease in the postoperative period, and some had no degree of liver disease.

### *Research conclusions*

The PA decreased after bariatric surgery, with a direct correlation with weight loss and changes in body composition, and it did not correlate with the improvement of NAFLD. With the data presented in this study, we suggest that PA may be a marker of the state of body composition linked to the functionality of skeletal muscle mass.

### *Research perspectives*

Performing large scale prospective studies with long-term follow-up are needed to verify if PA increases with more time after bariatric surgery, since the change in the body composition of the operated patient will reflect an improvement in body mass distribution and, consequently, less inflammatory process.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1020-1030

DOI: 10.4254/wjh.v12.i11.1020

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

# Factors associated with 5-year survival of combined hepatocellular and cholangiocarcinoma

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**Author contributions:** Sempokuya T contributed to study design, data collection, statistical analysis; Sempokuya T, Wien E and Pattison R contributed to the literature review, manuscript drafting, and editing; Ma J contributed to study design and statistical analysis; Wong L contributed to study supervision, manuscript drafting and editing; and all of the authors approved the final version of the manuscript.

**Institutional review board statement:** Since we utilized publicly available de-identified database, review by our institutional review board was not required.

**Informed consent statement:** Informed consent was not required to conduct this study.

**Conflict-of-interest statement:** LW is a speaker bureau for Eisai. Other authors do not have any conflict of interest.

**Data sharing statement:** All the data used in this analysis is

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## Abstract

### *BACKGROUND*

Combined hepatocellular and cholangiocarcinoma (HCC/CC) is a rare primary hepatic malignancy which carries a poor prognosis due to its aggressive nature. Few centers have enough cases to draw definitive conclusions and there is limited understanding of prognosis. Given the rarity of HCC/CC, an analysis of large national cancer database was needed to obtain larger number of HCC/CC cases.

### *AIM*

To identify associated factors for 5-year survival of HCC/CC.

### *METHODS*

We conducted a retrospective study of The Surveillance, Epidemiology, and End Results (SEER) database obtained from SEER*Stat 8.3.6 software. Previously defined histology code 8180 for the International Classification of Disease for Oncology, 3rd edition was used to identify HCC/CC cases from 2004 to 2015. We collected demographics, American Joint Committee on Cancer (AJCC) stage, treatment, tumor size, and survival data. These data were converted to categorical variables. The Shapiro-Wilk normality test was used to assess normal distribution. Mann-Whitney *U* test was used to compare continuous variables without normal distribution, and *t*-test was used to compare continuous variables with a normal distribution. The Kaplan-Meier survival curve analyzed 5-year survival.

Univariate and multivariate logistic regression model was used to analyze factors associated with 5-year survival. Multivariate Cox proportional hazard regression was done on 5-year survival. We defined $P < 0.05$ was statistically significant.

***RESULTS***

We identified 497 patients with the following characteristics: Mean age 62.4 years (SD: 11.3), 149 (30.0%) were female, racial distribution was: 276 (55.5%) white, 53 (10.7%) black, 84 (16.9%) Asian and Pacific Islander (API), 77 (15.5%) Hispanic, and 7 (1.4%) others or unknown. Stage I/II disease occurred in 41.5% and tumor size < 50 mm was seen in 35.6% of patients. Twenty-four (4.8%) received locoregional therapy (LRT), 119 (23.9%) underwent resection, and 50 (10.1%) underwent liver transplantation. The overall median survival was 6 mo [Interquartile range (IQR): 1-22]. After multivariate logistic regression, tumor size < 50 mm [Odds ratios (OR): 2.415, $P = 0.05$], resection (OR: 12.849, $P < 0.01$), and transplant (OR: 27.129, $P < 0.01$) showed significance for 5-year survival. Age > 60, sex, race, AJCC stages, metastasis, and LRT were not significant. However, API *vs* white showed significant OR of 2.793 (CI: 1.120-6.967). Cox proportional hazard regression showed AJCC stages, tumor size < 50 mm, LRT, resection, and transplant showed significant hazard ratio.

***CONCLUSION***

HCC/CC patients with tumor size < 50 mm, resection, and transplant were associated with an increase in 5-year survival. API showed advantageous OR and hazard ratios over white, black.

**Key Words:** Combined hepatocellular and cholangiocarcinoma; Surveillance, Epidemiology, and End Results database; Survival; Race; Hepatobiliary cancer; Transplant



**Core Tip:** Combined hepatocellular and cholangiocarcinoma (HCC/CC) is a rare primary hepatic malignancy which carries a poor prognosis due to its aggressive nature. Few centers have enough cases to draw definitive conclusions and there is limited understanding of prognosis. This analysis of Surveillance, Epidemiology, and End Results database comprised of 497 patients. HCC/CC patients with tumor size < 50 mm, resection, and transplant were associated with an increase in 5-year survival. Asian and Pacific Islander (API) showed advantageous odds ratios and hazard ratios over white, black. Elucidation of better prognosis on API are needed in the future studies.

**Citation:** Sempokuya T, Wien EA, Pattison RJ, Ma J, Wong LL. Factors associated with 5-year survival of combined hepatocellular and cholangiocarcinoma. *World J Hepatol* 2020; 12(11): 1020-1030
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1020.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1020

## INTRODUCTION

Malignancies of the liver are broadly grouped into hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). Combined hepatocellular and cholangiocarcinoma (HCC/CC) is a rare primary hepatic malignancy which has a distinct phenotype, shares characteristics of both HCC and ICC. HCC/CC carries a particularly poor prognosis due to its aggressive nature. HCC/CC has an estimated incidence between 1% and 14.2%[1-4]; however, this is likely an underestimation due to diagnostic inaccuracy. HCC/CC shows phenotypic characteristics of both HCC and CC with malignant differentiation of hepatocytes and biliary epithelial cells. While HCC/CC is thought to originate from a common hepatic stem cell[5,6], there has been much debate on whether HCC/CC shares more commonalities with HCC or CC[1,7-9].

available from SEER database.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** April 15, 2020
**Peer-review started:** April 15, 2020
**First decision:** June 4, 2020
**Revised:** July 25, 2020
**Accepted:** October 12, 2020
**Article in press:** October 12, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Fujino Y, Messina C
**S-Editor:** Wang JL
**L-Editor:** A
**P-Editor:** Li X

Current knowledge suggests HCC/CC is a unique entity with a spectrum of clinical features between those of HCC and CC[10]. In addition to its aggressive nature, clinical and pathologic heterogeneity results in reduced survival compared to HCC or CC alone. Furthermore, treatment of HCC/CC is challenging as there are no broadly accepted guidelines other than recommendation for resection and possible liver transplantation in patients afflicted with this condition[11,12].

Because HCC/CC is a rare malignancy, there are no large studies or randomized clinical trials to compare diagnostic or treatment modalities. The Liver Imaging Reporting and Data System is a widely used criteria for imaging diagnosis of HCC[13], but its performance in differentiating HCC/CC from HCC by magnetic resonance imaging is much less reliable[14]. Prognosis of HCC/CC after resection compared to HCC or CC alone is controversial. While Zhang *et al*[15] showed better early survival of HCC/CC compared to ICC and worse outcome than HCC alone, this study only included 15 HCC/CC patients. On the other hand, Song *et al*[16] showed HCC/CC had a significantly shorter recurrence free survival after resection compare to ICC, 0.9 years and 1.3 years, respectively. Prognostic indicators beyond this are mostly unknown. The role and indications of liver transplantation in HCC/CC also remain equivocal at this time[12,17]. For HCC, downstaging can be employed for patients to be eligible for liver resection or transplantation with favorable outcomes. However, no established protocol is available for HCC/CC.

Despite the diagnostic and prognostic challenges, distinguishing this unique entity is crucial to its optimal management and outcomes. Few centers have enough cases to draw definitive conclusions, and there is a limited understanding of prognosis. This study attempts to further understand and identify factors associated with 5-year survival in patient with HCC/CC.

## MATERIALS AND METHODS

### Study design

Population data from the Surveillance, Epidemiology, and End Results (SEER) database published by the National Cancer Institute were obtained through Surveillance Research Program, National Cancer Institute SEER*Stat software (seer.cancer.gov/seerstat/) version <8.3.6>[18]. SEER Registries are population-based registries that report cancer incidence, characteristics, treatment and, mortality on select U.S. states since 1973. Approximately 34.6 % of all cancer cases in the U.S. population are covered[19]. This study was conducted after complying with the SEER Research Data Use Agreement. As we utilized a publicly available, de-identified database, approval from an Institutional Review Board was not required to conduct this study.

### Patients

We collected data on patients with a diagnosis of HCC/CC between 2004 to 2015 with the previously defined International Classification of Diseases for Oncology, 3rd Edition the histology code of 8180[4]. Variables collected included age at diagnosis, year at diagnosis, sex, race (Whites, blacks, Hispanics, Asians or Pacific Islanders (API), or others), marital status, stage by the American Joint Committee on Cancer (AJCC) Staging Manual, 6th edition[20], SEER Staging, presence of metastasis, state and county of residence, tumor sizes, treatment modality of primary site, and survival (mo). SEER data utilized in this study was based on information from 18 U.S. states and regions available to conduct survival analysis including: Alaska Native Tumor Registry, California (San Francisco-Oakland, San Jose-Monterey, Los Angeles, Greater California), Connecticut, Georgia (Atlanta, Greater Georgia, Rural Georgia), Hawaii, Iowa, Kentucky, Louisiana, Michigan (Detroit), New Jersey, New Mexico, Utah and Washington (Seattle-Puget Sound) (More details available at https://seer.cancer.gov/registries/terms.html).

### Statistical analysis

We performed statistical analysis with R version 3.4.1 (The R foundation for Statistical Computing, Vienna, Austria), EZR version 1.36 (Division of Hematology, Saitama Medical Center, Jichi Medical University, Japan)[21], and SAS version 9.4 (SAS Institute Inc., Cary, NC, United States). $\chi^2$ test was used to compare categorical variables. The Shapiro-Wilk normality test was used to assess normal distribution. Mann-Whitney *U* test was used to compare continuous variables without normal distribution, and *t*-test was used to compare continuous variables with a normal distribution. The Kaplan-

Meier survival curve with log-rank test was used to estimate overall survival probability and compare 5-year survival curves for risk factor groups. Continuous variables were converted to categorical variables for logistic regression models. A univariate and multivariate logistic regression model was used to analyze factors associated with 5-year survival. Exclusion of patients with unknown or other race and surgical status was done on this regression model due to small population size. Risk factor variables included in the logistic regression mode were sex (male and female), age (< 60 and ≥ 60-years old), race (White, black, Hispanic, and API), AJCC stages (I/II, III/IV, and unknown), metastasis (distant metastasis, none/unknown), tumor size (< 50 and ≥ 50 mm), surgical status [Locoregional therapy (LRT), resection, and transplant]. All of these variables were included in the multivariate model. The primary outcome variable of interest was 5-year overall survival, defined as the time from HCC diagnosis to death from any cause, with censoring if the patients were still alive after 5-years of follow-up. Hazard ratios (HR) for overall survival evaluated using multivariate Cox proportional hazard regressions mode by using the same variable as logistic regression. Logistic regression model allows prediction of variables with survival status and Cox proportional hazard regression model enables analysis of time dependent variables related to survival. $P < 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Ma J from Department of Biostatistics, College of Public Health, University of Nebraska Medical Center.

## RESULTS

We identified 497 patients with the following characteristics: Mean age 62.4 years (SD: 11.3), 270 (54.3%) were age > 60 years old, and 149 (30.0%) were female. Racial distribution was as follows: 276 (55.5%) white, 53 (10.7%) black, 84 (16.9%) API, 77 (15.5%) Hispanic, and 7 (1.4%) others or unknown. Stage I/II disease occurred in 206 (41.5%), 128 (25.8%) had metastasis at the time of diagnosis, and tumor size < 50 mm was seen in 177 (35.6%) of patients. Twenty-four (4.8%) received LRT, 119 (23.9%) underwent resection, and 50 (10.1%) underwent liver transplantation. Detailed baseline characteristics are shown on Table 1. The overall median survival was 6 mo [Interquartile range (IQR): 1-22] (Figure 1). Age at diagnosis and survival months did not show a normal distribution by the Shapiro-Wilk normality test; therefore, comparison was made by the Mann-Whitney *U* test. There were significant differences for age at diagnosis, age > 60 years old, AJCC stages, SEER stages (localized, regional extension, lymph nodes involvement, distant metastasis), proportion of Stage I/II, positive lymph nodes, metastasis at the diagnosis, tumor size < 30 mm, < 40 mm, and < 50 mm, resection, and liver transplantation (all $P < 0.01$). There was no significant difference between 5-year survivors and non-5-year survivors for sex, race, state of residence, marital status, tumor size < 20 mm, LRT.

We compared racial differences in 12-mo, 36-mo, and 60-mo survival among white, black, Hispanics, API and others by $\chi^2$ test and there were no statistically significant differences. Among same groups, gender, age > 60 years-old, size > 50 mm, LRT, resection, or transplant did not show significant differences. We then compared API with non-Asians. API had higher resection rate 35.7% compared to 21.1% in non-Asians ($P < 0.01$) and higher rate of 60 mo survival 15.4% compared to 8.0% ($P = 0.05$). Gender, age > 60 years-old, rate of stage I or II disease, LRT, transplant, and size > 50 mm did not show significant difference.

After excluding 8 patients with other or unknown race (7) and surgery status (1), we conducted logistic regression model. Univariate analysis (Table 2) showed age > 60, stage I/II *vs* unknown, I/II *vs* III/IV, metastasis, tumor size < 50 mm, resection, and transplant were significant predictors (all $P < 0.01$). Sex, race, stage III/IV *vs* unknown, and LRT were not significant factors. After multivariate logistic regression (Table 2), tumor size < 50 mm [Odds ratio (OR): 2.415, $P = 0.05$], resection (OR: 12.849, $P < 0.01$), and transplant (OR: 27.129, $P < 0.01$) were statistically significant for 5-year survival. Age > 60, sex, race, AJCC stage, metastasis, and LRT were not significant. However, API *vs* white showed significant OR of 2.793 (CI: 1.120-6.967). It is important to note that 12 API and 18 Hispanic patients had untraced survival status.

Cox proportional hazard regression showed AJCC stage, tumor size < 50 mm, LRT, resection, and transplant showed significance (Table 3). Age > 60, sex, race, and metastasis did not show significant HR. Although overall race did not show significance, API showed HR of 0.654 (CI: 0.452-0.948) over black, and HR of 0.727 (CI: 0.555-0.952) over white. There was no difference for Hispanic over API, black, or

**Table 1 Baseline characteristics, *n* (%)**

| | Overall | 5-year survivor | Non-5-year survivor | *P* value |
|---|---|---|---|---|
| Median age (IQR) | 62 (56-69) | 57 (51.25-63.75) | 62 (56-70) | < 0.01 |
| Sex (%Male) | 348 (70.0) | 36 (78.3) | 312 (69.2) | 0.27 |
| Race | | | | 0.23 |
| Whites | 276 (55.5) | 23 (50.0) | 253 (56.1) | |
| Blacks | 53 (10.7) | 2 (4.3) | 51 (11.3) | |
| Hispanics | 77 (15.5) | 8 (17.4) | 69 (15.3) | |
| API | 84 (16.9) | 13 (28.3) | 71 (15.7) | |
| Others | 7 (1.4) | 0 (0) | 7 (1.6) | |
| AJCC stages | | | | < 0.01 |
| I | 116 (23.3) | 19 (41.3) | 97 (21.5) | |
| II | 90 (18.1) | 19 (41.3) | 71 (15.7) | |
| III | 78 (15.7) | 5 (10.9) | 73 (16.2) | |
| IV | 139 (28.0) | 1 (2.2) | 138 (30.6) | |
| Unknown | 74 (14.9) | 2 (4.3) | 72 (16.0) | |
| Metastasis | 128 (25.8) | 1 (2.2) | 127 (28.2) | < 0.01 |
| Tumor size < 50 mm | 177 (35.6) | 34 (91.9) | 143 (56.5) | < 0.01 |
| Treatment | | | | |
| LRT | 24 (4.8) | 2 (4.3) | 22 (4.9) | 1.00 |
| Resection | 119 (23.9) | 21 (45.7) | 98 (21.8) | < 0.01 |
| Transplant | 50 (10.6) | 20 (43.5) | 30 (6.7) | < 0.01 |
| Median survival months (IQR) | 6 (1-22) | 96.5 (83.25-129.5) | 5 (1-16) | < 0.01 |

IQR: Interquartile range; API: Asians and Pacific Islanders; AJCC: American Joint Committee on Cancer; LRT: Locoregional therapy.

white, and black over white.

## DISCUSSION

HCC/CC is a rare, aggressive variant with features of both HCC and CC and few centers have enough cases to understand how to effectively treat this. It is not clear if the treatments typically used for HCC will be effective. Before we offer specific therapies, we need to better understand the natural history of this disease so we can target our efforts appropriately. This study showed that of the clinical factors, tumor size < 50 mm, resection and transplant were predictors of 5-year survival. However, Age > 60, sex, race, AJCC stages, metastasis, and LRT were not associated with significant odds of 5-year survival. Treatment with liver transplantation or liver resection were associated with 5-year survival but transplantation had a higher odds-ratio. In addition, although overall race was not significant, API showed significant CI for OR over white, and HR over white and black.

Our study highlighted that API patients had a higher chance of 5-year survival compared to white and black. The reasons for this were not completely clear and may be related to the underlying chronic liver disease or access to care. The high prevalence of hepatitis B in Asia may account for some of these differences. A Chinese study suggested that hepatitis B was a strong risk factor for developing HCC/CC and while this is similar in both HCC and CC alone, there was no association between underlying hepatitis C and HCC/CC[22]. A previous SEER study on HCC showed that Asians had a higher proportion of localized HCC compared to advanced HCC[23]. This may suggest that the high prevalence of hepatitis B in Asians may have prompted HCC surveillance and earlier detection. Furthermore, hepatitis B infections may lead

**Table 2 Univariate and multivariate logistic regression for 5-year survival**

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | OR [95%CI] | *P* value | OR [95%CI] | *P* value |
| Age > 60 yr old | 0.372 [0.195-0.708][1] | < 0.01 | 0.502 [0.231-1.088] | 0.08 |
| Sex (Male) | 1.604 [0.774-3.323] | 0.20 | 1.264 [0.537-2.975] | 0.59 |
| Race | | 0.13 | | 0.07 |
| Black | 0.432 [0.099-1.888] | | 0.483 [0.095-2.444] | |
| Hispanic | 1.275 [0.547-2.976] | | 2.043 [0.744-5.613] | |
| API | 2.014 [0.971-4.176] | | 2.793 [1.120-6.967][1] | |
| AJCC stages | | | | 0.41 |
| Stage I/II *vs* unknown | 8.143 [1.913-34.660][1] | < 0.01 | 1.048 [0.185-5.935] | |
| Stage III/IV *vs* unknown | 1.024 [0.202-5.186] | 0.06 | 0.498 [0.074-3.328] | |
| Stage I /II *vs* III/IV | 7.954 [3.284-19.264][1] | < 0.01 | | |
| Metastasis | 0.057 [0.008-0.416][1] | < 0.01 | 0.602 [0.059-6.165] | 0.67 |
| Tumor size < 50 mm | 6.098 [3.067-12.200][1] | < 0.01 | 2.415 [1.010-5.780][1] | 0.05 |
| LRT | 0.884 [0.201-3.886] | 0.87 | 4.622 [0.671-31.856] | 0.12 |
| Resection | 3.017 [1.620-5.620][1] | < 0.01 | 12.849 [3.359-49.142][1] | < 0.01 |
| Transplant | 10.769 [5.398-21.485][1] | < 0.01 | 28.129 [6.639-119.187][1] | < 0.01 |

[1]Significant confidence interval.

White as a comparison group for race. AJCC: American Joint Committee on Cancer; API: Asians and Pacific Islanders; LRT: Locoregional therapy; OR: Odds ratio.

to primary liver cancers in the absence of cirrhosis which may have allowed for more aggressive attempts at surgical resection in Asians. Unfortunately, the SEER data does not have information on underlying disease or whether HCC/ICC was found with surveillance.

Our study also demonstrated that API had a survival advantage over whites and blacks but did not have a difference in survival compared to Hispanics. However, a slightly higher proportion of API and Hispanics had no survival information. Previous studies have shown a higher incidence of HCC in Hispanics compared to Asians and Hispanics were more likely to have underlying non-alcoholic fatty liver disease and chronic hepatitis C virus infections[24]. Ha *et al*[25] suggested that blacks and Hispanics were less likely to receive curative therapy for HCC due to the advanced stage at presentation of HCC. Similar observations in racial and socioeconomic disparities were found in Hispanic patients with CC[26,27]. While all of these observations suggest a worse outcome for Hispanics with HCC or CC, ours is the first to describe a non-inferior prognosis for Hispanics with the combined HCC/CC variant.

The burden of tumor likely affects overall prognosis and our study showed that tumors less than 5 cm were associated with better 5-year survival. Several other small studies have also suggested that tumor size > 5 cm was associated with a poor overall survival[15,28]. Multiple tumors and microvascular invasion were other factors associated with worse outcome after surgery[15]. However, there were several additional studies that did not support specific tumor characteristics as being prognostic in survival (Table 4). While our study demonstrated that tumor size affected long term survival, this was likely because patients with smaller tumors were more suitable candidates for surgery. Transplantation in the U.S. requires meeting Milan criteria or undergoing downstaging with LRT to meet Milan criteria and these presumably affected candidacy in our cohort.

The prognosis for HCC/CC is generally poor and our study showed that the median survival for HCC/CC was only 6 mo. Treatment with resection or transplant were associated with 5-year survival, however previous studies are divisive on which treatment is superior or how these treatments compare to patients with HCC. Itoh *et al*[29] compared long-term outcomes after living donor transplantation between 8 HCC/CC and 170 HCC patients and did not demonstrate a difference between overall

**Table 3 Multivariate Cox proportional hazard regression for 5-year survival**

| | Hazard ratio | 95%CI | *P* value |
|---|---|---|---|
| Age > 60 years old | 0.862 | 0.708-1.050 | 0.14 |
| Sex (Male) | 1.071 | 0.863-1.328 | 0.54 |
| Race | | | 0.07 |
| API *vs* black | 0.654 | 0.452-0.948[1] | |
| API *vs* Hispanic | 0.838 | 0.595-1.180 | |
| API *vs* white | 0.727 | 0.555-0.952[1] | |
| Black *vs* Hispanic | 1.280 | 0.884-1.852 | |
| Black *vs* white | 1.111 | 0.819-1.506 | |
| Hispanic *vs* white | 0.868 | 0.660-1.140 | |
| AJCC stages | | | < 0.01 |
| Stage I/II *vs* unknown | 0.547 | 0.390-0.768[1] | |
| Stage III/IV *vs* unknown | 0.709 | 0.509-0.988[1] | |
| Stage I/II *vs* III/IV | 0.772 | 0.571-1.042 | |
| Metastasis | 1.229 | 0.918-1.645 | 0.17 |
| Tumor size < 50 mm | 0.704 | 0.545-0.908[1] | < 0.01 |
| LRT | 1.782 | 1.134-2.801[1] | 0.01 |
| Resection | 2.770 | 2.137-3.590[1] | < 0.01 |
| Transplant | 4.247 | 2.809-6.542[1] | < 0.01 |

[1]Significant confidence interval. AJCC: American Joint Committee on Cancer; API: Asians and Pacific Islanders; LRT: Locoregional therapy.

**Table 4 Summary of previous studies**

| Ref. | Country | Number of HCC/CC patients | 1-year survival (%) | 3-year survival (%) | Factors predictive of survival |
|---|---|---|---|---|---|
| Park *et al*[32], 2013 | South Korea | Hepatic resection (*n* = 10) | 20 | 20 | Age, sex, TACE and T stage by univariate analysis, but none multivariate analysis |
| Antwi *et al*[33], 2018 | United States | Liver transplant (*n* = 19) | 84 | 74 | Response to neoadjuvant LRT |
| Groeschl *et al*[31], 2013 | United States | Hepatic resection (*n* = 35); Liver transplant (*n* = 19) | Resection: 71; Transplant: 89 | Resection: 46; Transplant: 48 | NA |
| Itoh *et al*[29], 2015 | Japan | Living donor transplant (*n* = 8) | 87.5 | 72.9 | NA |
| Li *et al*[30], 2018 | Meta-analysis | Hepatic resection (*n* = 1390); Liver transplant (*n* = 301) | Resection: 79; Transplant: 85 | Resection: 63; Transplant: 63 | Vascular invasion, lymph node involvement, tumor size > 5 cm and advanced stage |

HCC/CC: Combined hepatocellular and cholangiocarcinoma; TACE: Transarterial chemoembolization; LRT: Locoregional therapy; NA: Not available.

and disease free survival. A meta-analysis of 1691 patients (42 studies) with HCC/CC suggested that there was no significant difference for 5-year overall survival after liver resection or transplantation[30]. However, Groeschl *et al*[31] compared the outcome between 3378 HCC and 54 HCC/CC patients and showed that both transplant and resection demonstrated a survival benefit in HCC/CC, but this benefit was inferior to transplant for HCC. They questioned the use of liver transplant in the HCC/CC variant.

Unfortunately, only a limited number of patients qualify for surgical treatment, and this is likely contributing to a poor median and 5-year survival rate. While surgical treatment can improve survival, the use of locoregional therapy or systemic therapies for this variant is not clear. While a small study did show improvement in survival

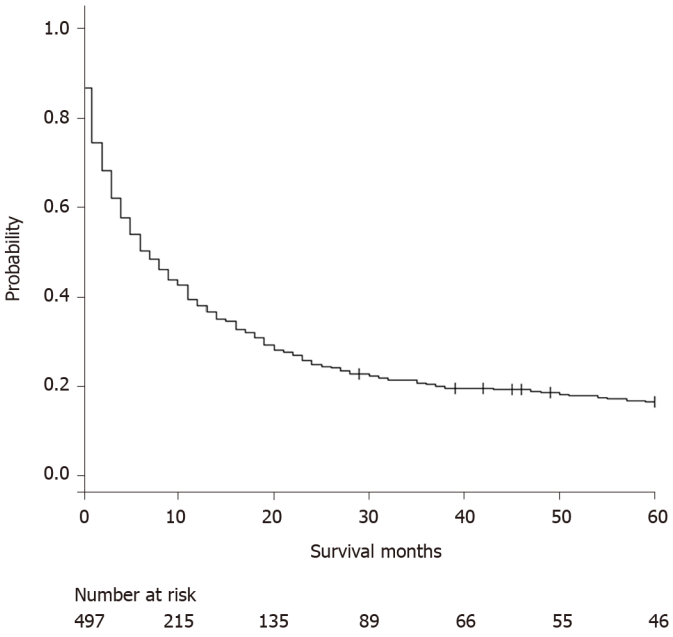


**Figure 1 Overall survival shown by Kaplan-Meir curve**. Median survival was 7 mo with 95%CI of 5-9 mo.

from HCC/CC with transarterial chemoembolization[32], our study did not demonstrate a 5-year survival benefit in patients who received locoregional therapy. HCC/CC patients who respond to pre transplant locoregional therapy may have better post-liver transplantation 3-year overall survival[33]. However, unlike HCC, there are no large studies with established pre-transplant therapies for patients with HCC/ICC. The A.L.A.N. score, which is calculated with baseline actual neutrophil count, lymphocytes-monocytes ratio, albumin, and neutrophil-lymphocytes ratio developed by a group in the U.K., may provide prognostic information for patients with advanced biliary cancer who received the first-line chemotherapy[34].

The role of lymph node involvement in HCC/ICC may be contributing to the outcome. A previous report noted that up to 70% of HCC/CC cases demonstrate lymph node metastasis, similar to the frequency in CC cases[35,36]. Lymph node dissection is not generally done for HCC but is recommended for moderate and high-risk CC. Unfortunately, the SEER data did not have information as to whether a node dissection was performed so we cannot draw definite conclusions on the role for this in patients with the HCC/CC variant.

This study is limited in that it was based on a large database from multiple institutions, and may be subject to reporting bias and coding errors. Data from the SEER did not report the underlying chronic liver disease, laboratory studies to assess hepatic function, and calculate CHILD Pugh score or detailed information on tumor characteristics, which would be important in determining resectability and transplant candidacy and thus impact on 5-year survival. Due to the nature of the SEER database, it is not possible to know if patients received adjuvant chemotherapy after surgical treatment, which may improve recurrence-free survival if combined HCC/CC has a higher CC component[37].

It would have also been helpful to know if patients had their tumor found with surveillance or whether they were symptomatic as this would help identify any disparity in access to care, but this information is also unknown. In spite of these limitations, the strength of this study is that it included a large number of patients with a very rare variant. Individual institutions would never have enough cases of combined HCC/CC to have the statistical power to show differences in the factors analyzed.

## CONCLUSION

Management of HCC/CC variant is difficult. The trend toward radiologic diagnosis of HCC may be facilitating misdiagnosis of this variant and delaying recognition until after the resected liver specimen has been examined. It is unclear if we should be

treating HCC/ICC using similar protocols as HCC or if we should be adding adjuvant therapies to address nodal involvement of the CC component or perhaps some different approach altogether. We demonstrated that selection of tumors smaller than 5 cm and treatment with liver resection and transplant seem to be best associated with long term survival. While this study can help identify prognostic factors, further studies will be necessary to explain racial/ethnic differences, the effect of underlying chronic liver disease and the role of locoregional and systemic therapies in this rare variant.

## ARTICLE HIGHLIGHTS

### *Research background*

Combined hepatocellular and cholangiocarcinoma (HCC/CC) is a rare primary hepatic malignancy which carries a poor prognosis due to its aggressive nature. Few centers have enough cases to draw definitive conclusions and there is limited understanding of prognosis.

### *Research motivation*

As there has not been a randomized clinical trial done on this topic to elucidate the best treatment modality on HCC/CC, there is a need to better characterize the prognosis of this disease.

### *Research objectives*

In this retrospective study, we attempted to identify associated factors for 5-year survival.

### *Research methods*

We conducted a retrospective study of The Surveillance, Epidemiology, and End Results database to identify HCC/CC cases from 2004 to 2015. We collected demographics, American Joint Committee on Cancer (AJCC) stage, treatment, tumor size, and survival data. Mann-Whitney *U* test was used to compare continuous variables without normal distribution, and *t*-test was used to compare continuous variables with a normal distribution. The Kaplan-Meier survival curve analyzed Five-year survival. These data were converted to categorical variables. Univariate and multivariate logistic regression model was used to analyze factors associated with 5-year survival. Multivariate Cox proportional hazard regression was done on 5-year survival.

### *Research results*

We identified 497 patients with the following characteristics: Mean age 62.4 years, 149 (30.0%) were female, racial distributions were 276 (55.5%) white, 53 (10.7%) black, 84 (16.9%) Asian and Pacific Islander (API), 77 (15.5%) Hispanic, and 7 (1.4%) others or unknown. Stage I/II disease occurred in 41.5% and tumor size < 50 mm was seen in 35.6% of patients. The overall median survival was 6 mo. After multivariate logistic regression, tumor size < 50 mm [odds ratio (OR): 2.415, $P = 0.05$], resection (OR: 12.849, $P < 0.01$), and transplant (OR: 27.129, $P < 0.01$) showed significance for 5-year survival. Age > 60, sex, race, AJCC stages, metastasis, and LRT were not significant. However, API *vs* white showed significant OR of 2.793 (CI: 1.120-6.967). Cox proportional hazard regression showed AJCC stages, tumor size < 50 mm, LRT, resection, and transplant showed significant hazard ratio.

### *Research conclusions*

HCC/CC patients with tumor size < 50 mm, resection, and transplant were associated with an increase in 5-year survival. API showed advantageous OR and hazard ratios over white, black.

### *Research perspectives*

Prognosis and possible treatment modality for HCC/CC is different from hepatocellular carcinoma or cholangiocarcinoma alone. As we depend heavily on imaging diagnosis of hepatocellular carcinoma, this study may suggest the importance of role of biopsy to confirm correct diagnosis.

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*World J Hepatol* 2020 November 27; 12(11): 1031-1045

DOI: 10.4254/wjh.v12.i11.1031

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

# Circulating miR-21-5p level has limited prognostic value in patients with hepatocellular carcinoma and is influenced by renal function

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**Author contributions:** Franck M performed the experiments; Schütte K and Malfertheiner P provided the clinical material; Franck M, Thon C, and Link A carried out the analysis and interpretation of the data and drafting of the manuscript; Link A created the study concept and design and is the guarantor of the study; All authors edited and approved the final version of the manuscript.

**Institutional review board statement:** The study was reviewed and approved by the ethical board of the Otto-von-Guericke University (Study number 99/10).

**Informed consent statement:** The study was performed according to the principle of the Declaration of Helsinki. The study was approved by Institutional Review Board of Otto-von-Guericke University Magdeburg No. 99/10. All patients provided written informed consent.

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## Abstract

### *BACKGROUND*

MicroRNAs (miRNAs) have been suggested as biomarkers for malignant diseases including hepatocellular carcinoma (HCC). Specifically, hsa-miR-21-5p (miR-21) is among the most frequently deregulated miRNA in cancer. The diagnostic and prognostic value of miR-21 has been demonstrated in HCC tissue, mostly in the Asian population. Although the impact of various factors has been recently reported for circulating hsa-miR-122-5p (miR-122), at present only limited knowledge is available for miR-21.

### *AIM*

To evaluate the value of miR-21 for the assessment of prognosis in HCC patients and to delineate the influence of clinical and preanalytical factors on miR-21 level in sera.

### *METHODS*

Patients with confirmed HCC from our European cohort with predominantly alcohol-associated liver damage were included in the study. All subjects were characterized according to their clinical and laboratory work-up and overall survival data were obtained. Quantitative real-time polymerase chain reaction was performed for miR-21 and spiked-in cel-miR-39-3p. The results were compared to previously reported miR-122 data.

**Conflict-of-interest statement:** Authors declare that they have no potential conflicts of interest.

**Data sharing statement:** Technical appendix and dataset available from the corresponding author: alexander.link@med.ovgu.de. Participants gave informed consent for data analysis and publication. Certain restriction may apply in accordance to patient´s consent.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Germany

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 11, 2020
**Peer-review started:** June 12, 2020
**First decision:** July 30, 2020
**Revised:** August 15, 2020
**Accepted:** September 18, 2020
**Article in press:** September 18, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Rodrigues P
**S-Editor:** Zhang L
**L-Editor:** Filipodia
**P-Editor:** Wang LL

*RESULTS*

Survival of HCC patients was comparable between patients with low and high serum miR-21 concentration. No association was observed between miR-21 level in sera and Child-Pugh score, Barcelona Clinic Liver Cancer staging system, or etiology of HCC/liver disease. Age, gender, or pretreatment had no association with miR-21 level. A positive correlation was observed between miR-21 and aspartate aminotransferase ($r$ = 0.2854, $P$ = 0.0061), serum miR-122 ($r$ = 0.2624, $P$ = 0.0120), and the International Normalized Ratio ($r$ = 0.2065, $P$ = 0.0496). Negative correlation of miR-21 with serum creatinine ($r$ = -0.2215, $P$ = 0.0348) suggests renal function as a potential influencing factor in miR-21 biogenesis in blood.

*CONCLUSION*

The results from this work do not support clinically relevant prognostic value of circulating miR-21 in HCC patients in real-life settings. Following systematic evaluation, we identified renal function and aspartate aminotransferase as potential factors that may affect miR-21 concentration in blood. This knowledge should be considered in future miRNA-based biomarker studies not only for HCC but also for other diseases.

**Key Words:** Hepatocellular cancer; Hepatocellular carcinoma; MicroRNA; Prognosis; miR-21-5p; Renal function



**Core Tip:** In our previous work, we identified renal function, hemoglobin, and liver injury as potential factors that may impact circulating microRNA expression. miR-21-5p is the most frequently deregulated miRNA in various types of cancer. Several reports have proposed miR-21-5p as a prognostic biomarker in hepatocellular carcinoma. In this study, serum miR-21-5p values were not associated with prognosis of hepatocellular carcinoma in a European cohort of patients with predominantly alcohol-related liver injury. In a similar fashion as previously reported for miR-122-5p, changes in circulating miR-21-5p level correlated with renal function and liver injury. This observation shows that caution should be taken in interpreting circulating miR-21-5p level and its biomarker potential.

**Citation:** Franck M, Thon C, Schütte K, Malfertheiner P, Link A. Circulating miR-21-5p level has limited prognostic value in patients with hepatocellular carcinoma and is influenced by renal function. *World J Hepatol* 2020; 12(11): 1031-1045
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1031.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1031

## INTRODUCTION

There is a substantial effort to identify disease-specific biomarkers. Increasing evidence from intense research in the past several years strongly suggests that microRNAs (miRNAs) may become a valuable tool for the early identification and assessment of disease prognosis or treatment prediction. However, prior potential implementation of novel biomarkers including miRNAs in clinical settings, there is a great need for studies that deal with possible influencing factors[1]. At present, only limited knowledge is available regarding the translational utility of miRNAs as biomarkers in the real-life setting.

Hepatocellular carcinoma (HCC) is one of the most common cancers, even though the factors responsible for disease development are very heterogeneous[2,3]. Prognosis of patients with HCC is dependent on multiple factors including tumor biology, but is also strongly related to residual liver and kidney function. Therefore, HCC is probably the disease that may be best suited for evaluation of various influencing factors in biomarker research. There are several prognostic assessment models and staging systems for liver diseases and/or HCC available including Child-Pugh score[4,5], Model of End-Stage Liver Disease score[6], the Cancer of the Liver Italian Program score[7],

Okuda staging system[8], and the Barcelona Clinic Liver Cancer (BCLC) staging system[9] and many of those include multiple variables related to liver and/or renal function.

There is a great need for novel diagnostic, prognostic, and predictive biomarkers for HCC patients that would help to refine the forecast and subsequently individualize treatment decisions. MiRNA analyses of different solid cancers suggest the extensive involvement of miRNAs in cancer pathogenesis[10]. Multiple miRNAs have been examined in HCC previously, but substantial heterogeneity of data has hampered its utility[11]. We recently re-evaluated the prognostic value of hsa-miR-122-5p (miR-122) in a European cohort of well-characterized patients[1]. According to our data, circulating miR-122 was associated with the overall survival (OS) of HCC patients only in a subgroup of patients. We and others have identified multiple co-existing factors that may influence circulating miR-122 levels such as liver injury, hemoglobin concentration, or kidney function, but knowledge of other miRNAs in HCC is limited.

Undoubtedly, hsa-miR-21-5p (miR-21) is among the most frequently deregulated miRNA in human cancers[10-12]. In-depth evidence gathered over the past several years clearly demonstrates the pro-oncogenic function of miR-21 in carcinogenesis[13,14]. For instance, an *in vivo* study used an miR-21-overexpression mouse model where miR-21 overexpression led to a pre-B malignant lymphoid-like phenotype, indicating that miR-21 is an oncogenic miRNA[15].

In HCC, miR-21 promotes lipid accumulation in the liver and carcinogenesis through the Hbp1-p53-Srebp1c pathway, and therefore represents a potential link between non-alcoholic fatty liver disease and HCC[16]. It may play a role in mediating sorafenib resistance of HCC[17]. In a mouse model, treatment with a miR-21 inhibitor led to inhibition of tumor growth implicating miR-21 as potential therapeutic target in HCC[18]. Overexpression of miR-21 in HCC tumors was uniformly described[19-21] and is associated with poor OS and disease-free survival of HCC patients[19,20].

Higher miR-21 blood level was described in patients with HCC compared to healthy donors[22-24], but the opposite observation has also been reported[25]. Several studies have reported discordant results on circulating miR-21, particularly in patients with chronic hepatitis B virus (HBV) infection and HCC[22,26,27]. Diagnostic value of miR-21 in HCC has mostly been evaluated in HBV-dominant cohorts[22,23,27]. However, a recently published meta-analysis suggested that high ubiquitous miR-21 expression may be a limiting factor in the diagnostic performance of circulating miR-21[28].

In this study, we evaluated the value of serum miR-21 as a prognostic marker in a European cohort with mainly alcohol-induced HCC. Furthermore, we identified and explored the potential clinical conditions and laboratory co-factors that may influence performance of miR-21 as a biomarker in HCC and potentially in other conditions.

## MATERIALS AND METHODS

### Study cohort

In total, the sera of 91 patients with HCC were available for analysis. The samples were collected between January 2009 and April 2011 as previously described[1]. Diagnosis of HCC was confirmed either histologically or *via* non-invasive criteria based on typical imaging using computer tomography and magnetic resonance imaging. The study was performed according to the World Medical Association "Declaration of Helsinki–Ethical Principles for medical research involving human subjects," and was approved by the local Institutional Review Board of Otto-von-Guericke University Magdeburg (No. 99/10). Written informed consent was obtained from patients prior inclusion in the primary study. All patients were well characterized according to laboratory parameters, concomitant liver disease, and HCC including Child-Pugh score and BCLC staging as previously reported[1].

### Extraction of RNA and miRNA expression analysis

Following centrifugation, all serum samples were stored at -80 °C prior to further use. We performed extraction of RNA (including miRNA) according to a previously described and established protocol using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany)[29]. Briefly, 700 μL QIAzol Lysis Reagent were initially mixed with 100 μL serum. During this step, we added 5 μL of a 5 nM cel-miR-39 solution for internal normalization. The quality of RNA was examined using spectrophotometry. Following washing steps, RNA was finally eluted in 30 μL RNase-free water and stored at -80 °C until further use. Quantification of cel-miR-39-3p (assay ID: 000200) and hsa-miR-122-5p (assay ID: 002245) expression were assessed using the TaqMan miRNA assay and

TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, United States). Hsa-miR-21-5p was assessed with internally validated SYBR green method as previously described[30]. RNA (20 ng) was transcribed and used for the quantitative PCR (qPCR). The analyses were performed on the BioRad CFX Cycler System (BioRad, Hercules, CA, United States) in duplicate and the Ct or threshold cycle value was used for normalization and subsequent relative quantification.

### Statistical analysis

For statistical analysis, we used GraphPad Prism Version 7.0 (GraphPad Software, San Diego, CA, United States). Two-sided *P* values ≤ 0.05 were defined as significant. Based on the normality of distribution of miRNA values (2-deltaCt [miR-21/cel-miR-39]), we used nonparametric tests such as Spearman's rank correlation coefficient, Mann-Whitney test, Kruskal-Wallis test, and Dunn's post-hoc test. Chi-square ($\chi^2$) test and Fisher's exact test were used for contingency testing accordingly. Additionally, the unpaired *t*-test was used for analysis of laboratory parameters as appropriate. The data are presented as boxplots with whiskers for minimum and maximum. The survival time was defined as the time between inclusion in our study (date of blood withdrawal) and death or last documented contact. Kaplan-Meier survival curves and nonparametric log-rank test were used to evaluate survival differences. Median (50% percentile) was used to define the groups with high and low serum miR-21 expression, miR-122, alpha-fetoprotein (AFP) expression.

## RESULTS

The analysis of miR-21 in serum showed high variability between samples (mean Ct value: 29.61 ± 0.78, [min: 27.38, max: 31.22, range: 3.84]). First, we divided the cohort in two groups with miR-21-high and miR-21-low serum levels based on the median as shown in Table 1. Most parameters showed no statistically significant difference between the groups including gender, age, etiology of HCC, BCLC stage, or treatment status.

### Circulating miR-21 in relation to staging and etiology of liver disease

To evaluate the role of disease stage or etiology of the concomitant liver disease, we performed subgroup testing. Circulating miR-21 in patients with HCC were similar between patients with different Child-Pugh scores ($P$ = 0.7991; Figure 1A), BCLC stages ($P$ = 0.3947; Figure 1B) or was interdependent of etiologies of underlying liver disease ($P$ = 0.6331; Figure 1C) suggesting that other factors than HCC or liver disease may contribute to miR-21 variation.

### Circulating miR-21 and parameters of liver and kidney injury

To evaluate if concomitant conditions including liver injury, liver function or kidney injury may affect miR-21 level, we performed comparison of various laboratory parameters and serum miR-21. As shown in Figure 2A, there was a non-significant trend for a positive correlation between miR-21 and alanine aminotransferase (ALAT) ($r$ = 0.1709, $P$ = 0.1053). In much stronger fashion, we observed a statistically significant positive correlation between miR-21 and aspartate aminotransferase (ASAT) ($r$ = 0.2854, $P$ = 0.0061; Figure 2B). Correspondingly, patients with higher miR-21 had also significantly higher ASAT levels ($P$ = 0.0307; Table 1). Even though, miR-21 showed no correlation with AFP ($r$ = 0.1025, $P$ = 0.3337; Figure 2C), the miR-21-high group was associated with higher AFP levels ($P$ = 0.0469; Table 1). MiR-21 levels were furthermore associated with the International Normalized Ratio ($r$ = 0.2065, $P$ = 0.0496; Figure 2D) and patients with high miR-21 had higher International Normalized Ratio accordingly ($P$ = 0.0486; Table 1). Subsequent analysis of other parameters of the liver function such as bilirubin or albumin revealed not association with miR-21 (Tables 1 and 2). Most importantly from the clinical perspective, we observed a negative correlation between miR-21 level and creatinine level ($r$ = -0.2215, $P$ = 0.0348; Figure 2E), suggesting that renal function may impact miR-21 level in blood. Tables 1 and 2 provide a detailed overview on other factors that have been evaluated in the study with no significant difference.

### Circulating miR-21 and survival analysis

The survival data of our cohort have been previously validated and reported[1]. To evaluate the prognostic value of miR-21, we divided our study population based on the 50$^{th}$ percentile of miR-21 into patients with high and low level. We did not find any

**Table 1 Clinical and laboratory characteristics of hepatocellular carcinoma patients and hsa-miR-21-5p expression**

| | | All patients | miR-21 low[1] | miR-21 high[2] | *P* value |
|---|---|---|---|---|---|
| Patient number: | | 91 | 47 | 44 | |
| Gender (*n*, %): | Women | 17 (18.7%) | 7 (14.9%) | 10 (22.7%) | 0.4231 |
| | Men | 74 (81.3%) | 40 (85.1%) | 34 (77.3%) | |
| Age in yr | mean ± SD | 67.91 ± 8.98 | 69.11 ± 8.84 | 66.64 ± 9.05 | 0.1912 |
| Etiology (*n*, %): | Alcohol abuse | 41 (45.1%) | 22 (46.8%) | 19 (43.2%) | 0.7560 |
| | Viral hepatitis | 12 (13.2%) | 5 (10.6%) | 7 (15.9%) | |
| | NASH | 13 (14.3%) | 8 (17.0%) | 5 (11.4%) | |
| | Rare or other cause[3] | 25 (27.5%) | 12 (25.5%) | 13 (29.5%) | |
| BCLC stage (*n*, %): | A | 16 (17.6%) | 7 (14.9%) | 9 (20.5%) | 0.7787 |
| | B | 37 (40.7%) | 20 (42.6%) | 17 (38.6%) | |
| | C + D | 38 (41.8%) | 20 (42.6%) | 18 (40.9%) | |
| Child-Pugh score (*n*, %): | No liver cirrhosis | 16 (17.6%) | 8 (17.0%) | 8 (18.2%) | 0.7443 |
| | A | 45 (49.5%) | 25 (53.2%) | 20 (45.5%) | |
| | B + C | 30 (33.0%) | 14 (29.8%) | 16 (36.4%) | |
| Treatment (*n*, %)[4]: | Therapy naïve | 26 (28.6%) | 11 (23.4%) | 15 (34.1%) | 0.3535 |
| | Pretreated | 65 (71.4%) | 36 (76.6%) | 29 (65.9%) | |
| Ascites (*n*, %): | No ascites | 58 (63.7%) | 30 (63.8%) | 28 (63.6%) | 0.9999 |
| | Ascites present | 33 (36.3%) | 17 (36.2%) | 16 (36.4%) | |
| Bilirubin (μmol/L): | mean ± SD | 23.38 ± 31.73 | 24.27 ± 40.47 | 22.43 ± 18.78 | 0.7833 |
| AFP (ng/mL): | mean ± SD | 4593 ± 20162 | 540 ± 1533 | 8922 ± 28481 | 0.0469 |
| INR[5]: | mean ± SD | 1.076 ± 0.2127 | 1.034 ± 0.1450 | 1.122 ± 0.2611 | 0.0486 |
| Platelets (Gpt/L): | mean ± SD | 195.6 ± 118.7 | 182.7 ± 80.5 | 209.3 ± 149.0 | 0.2870 |
| ALAT (μmol/Ls): | mean ± SD | 0.7457 ± 0.4460 | 0.6955 ± 0.3425 | 0.7993 ± 0.5340 | 0.2697 |
| ASAT (μmol/Ls): | mean ± SD | 1.267 ± 1.041 | 1.040 ± 0.597 | 1.510 ± 1.330 | **0.0307** |
| Hemoglobin (mmol/L): | mean ± SD | 7.811 ± 1.254 | 7.655 ± 1.122 | 7.977 ± 1.375 | 0.2230 |
| Creatinine (μmol/L): | mean ± SD | 92.92 ± 67.95 | 102.3 ± 88.46 | 82.91 ± 33.08 | 0.1751 |
| Albumin (g/L): | mean ± SD | 36.29 ± 6.539 | 36.47 ± 5.668 | 36.10 ± 7.420 | 0.7938 |

[1]miR-21 50% percentile.
[2]miR-21 > 50% percentile.
[3]Including hemochromatosis.
[4]Patients with different kind of pretreatments (for example hepatic resection, transarterial chemoembolization, selective internal radiation therapy and sorafenib) were included.
[5]For 30 patients we obtained no exact laboratory value of the International Normalized Ratio (only < 1.5). For these patients, we calculated the International Normalized Ratio based on the Quick. *P* values were calculated with unpaired *t*-test, Fisher's exact test, and $\chi^2$ test as appropriate. AFP: Alpha-fetoprotein; ALAT: Alanine aminotransferase; ASAT: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer; INR: International Normalized Ratio; NASH: Non-alcoholic steatohepatitis; SD: Standard deviation.

significant difference in survival between these groups ($P$ = 0.2697; Figure 3A). Furthermore, no survival difference was found between the groups if the cohort was divided into three groups according to the 25$^{th}$, 33$^{th}$, and 75$^{th}$ percentile (data not shown). Taking into account the differences in tumor biology, we performed subgroup analysis for different BCLC stages. Neither separate analysis for combined BCLC A and B ($P$ = 0.1428; Figure 3B) nor BCLC C and D ($P$ = 0.4955; Figure 3C) were associated with survival differences. Having shown an association to creatinine, we further explored the prognostic value in subjects with normal and impaired renal function, but no prognostic difference was observed based on miR-21 level in patients with normal or pathological creatinine ($P$ = 0.2564, Figure 4A and $P$ = 0.8378,

**Table 2 Correlation analyses of hsa-miR-21-5p and clinical or laboratory parameters**

| Parameter | $2^{-\Delta Ct}$ miR-21 / cel-miR-39 | | |
|---|---|---|---|
| | Spearman *r* | Confidence interval | *P* value |
| Age in yr | -0.1147 | -0.3188 to 0.09956 | 0.2789 |
| Creatinine (μmol/L) | -0.2215 | -0.4139 to -0.01013 | 0.0348 |
| Bilirubin (μmol/L) | 0.1484 | -0.06547 to 0.3493 | 0.1603 |
| ALAT (μmol/Ls) | 0.1709 | -0.0425 to 0.3694 | 0.1053 |
| ASAT (μmol/Ls) | 0.2854 | 0.07828 to 0.4689 | 0.0061 |
| Albumin (g/L) | -0.01562 | -0.2267 to 0.1969 | 0.8832 |
| AFP (ng/mL) | 0.1025 | -0.1118 to 0.3077 | 0.3337 |
| Hemoglobin (mmol/L) | 0.1377 | -0.07638 to 0.3396 | 0.1931 |
| INR[1] | 0.2065 | -0.005615 to 0.4008 | 0.0496 |
| Platelets (Gpt/L) | 0.09185 | -0.1224 to 0.2979 | 0.3865 |
| $2^{-\Delta Ct}$ miR-122 / cel-miR-39 | 0.2624 | 0.05357 to 0.4493 | 0.0120 |

[1]For 30 patients, we obtained no exact laboratory value of the International Normalized Ratio (only < 1.5). For these patients, we calculated the International Normalized Ratio based on the Quick. ALAT: Alanine aminotransferase; ASAT: Aspartate aminotransferase; AFP: Alpha-fetoprotein; INR: International Normalized Ratio; miR-21: Hsa-miR-21-5p.

Figure 4B, respectively). Higher miR-21 levels were not associated with a worse prognosis in patients with normal ALAT ($P$ = 0.8094, Figure 4C), while a slight trend for worse OS was observed in patients with pathological ALAT ($P$ = 0.0800, Figure 4D). In a similar way, we found no significant difference in OS in patients with normal ASAT but a non-significant trend in patients with pathological ASAT ($P$ = 0.8423, Figure 4E, $P$ = 0.1170, Figure 4F, respectively).

### *Circulating miR-21, miR-122 and AFP*

Next, we evaluated the link between serum miR-21 and serum miR-122 levels. There was a positive correlation between miR-21 and miR-122 ($r$ = 0.2624, $P$ = 0.0120; Figure 5A), which suggests that circulating miRNAs may behave in a similar manner dependent on certain conditions like liver disease or renal function. We investigated if the combination of serum miR-21 and miR-122 may improve prognostic value in patients with HCC. After dividing our study group according to the median of miR-21 and miR-122 level, we formed three groups (patients with low miR-21 and low miR-122, patients with either low miR-21 or low miR-122, patients with high miR-21 and high miR-122); however, no significant difference in OS was observed ($P$ = 0.3377; Figure 5B).

In a similar fashion, we investigated the association of serum miR-21 in correlation with AFP on OS. In HCC patients, higher AFP levels were associated with shorter OS in subjects with miR-21 Low ($P$ = 0.0237; Figure 5C) but only with a trend for shorter OS in miR-21 high group ($P$ = 0.0917; Figure 5D). In subgroup analysis, AFP was indeed associated with a worse prognosis, but the impact was independent of miR-21 level (data not shown).

## DISCUSSION

Data on translational value of serum miR-21 in HCC in real-life settings are heterogeneous and only limited evidence is available for the European population. In this work, we systematically analyzed the prognostic value of serum miR-21 in our well-characterized European cohort of HCC patients, and evaluated the potential influence of various factors on its level in serum. The results of our study do not support the prognostic role of miR-21 in HCC. MiR-21 was neither associated with worse OS nor with etiology or tumor stage. Most interestingly, circulating miR-21 levels correlated significantly with ASAT, as surrogate for liver injury, and with creatinine, as a surrogate for renal function.

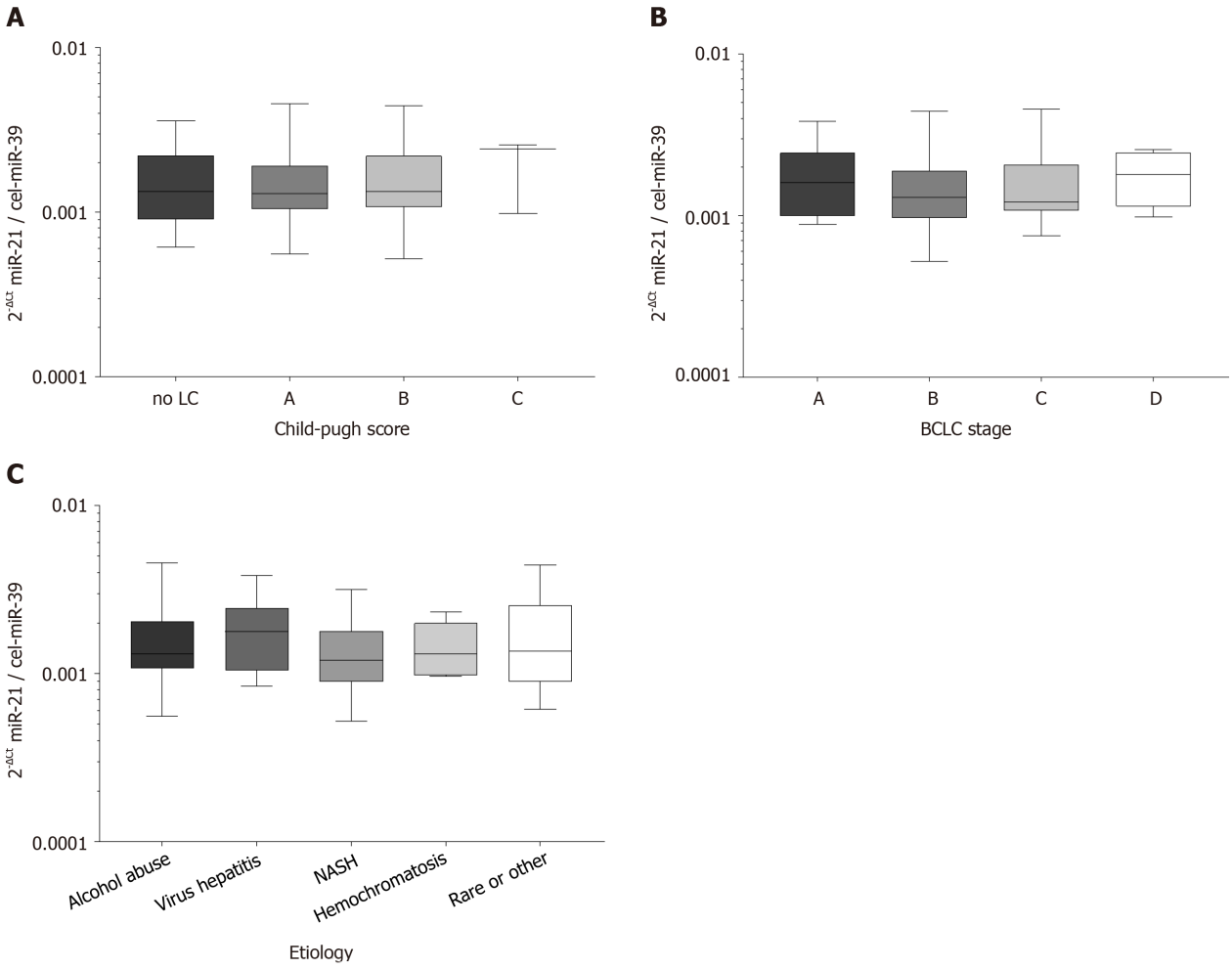


**Figure 1 Serum hsa-miR-21-5p level in correlation to Child-Pugh score, Barcelona Clinic Liver Cancer stage, and underlying etiology.** A: Serum hsa-miR-21-5p (miR-21) level and Child-Pugh score: No liver cirrhosis ($n$ = 16), Child-Pugh A ($n$ = 45), Child-Pugh B ($n$ = 27), Child-Pugh C ($n$ = 3), $P$ = 0.7991; B: Serum miR-21 and Barcelona Clinic Liver Cancer (BCLC) staging system: Stage A ($n$ = 16), stage B ($n$ = 37), stage C ($n$ = 32), stage D ($n$ = 6), $P$ = 0.3947; and C: Serum miR-21 and underlying etiology of the hepatocellular carcinoma: Alcohol abuse ($n$ = 41), viral hepatitis ($n$ = 12), non-alcoholic steatohepatitis (NASH) ($n$ = 13), hemochromatosis ($n$ = 6), rare or other ($n$ = 19), $P$ = 0.6331. Kruskal-Wallis test and post-hoc Dunn's test were used for statistical analysis. MiR-21: Hsa-miR-21-5p.

The prognostic value of circulating miR-21 has been studied in several studies. The overview of the studies is provided in Table 3. Among those, four studies reported that high miR-21 was associated with a worse prognosis[18,24,31,32], while one study[25], in concordance with our data, did not support prognostic role of miR-21. Another study demonstrated significantly better liver transplantation-free survival in HCC patients with higher plasma miR-21 levels[33]. As mentioned above, the available data are mostly based on the evidence from Asian populations and mainly include HBV/hepatitis C virus-induced HCC, while our work is among the first to evaluate the survival in patients from European origin. One may speculate if etiology of HCC may be responsible for the differences in the study outcome; however, our subgroup analysis did not reveal any difference in this regards. From another point of view, differences in tumor stage may also influence miR-21 level. Our cohort involved a large proportion of patients with intermediate or advanced HCC (BCLC B and C). Nevertheless, the samples size in our work may be too low to precisely delineate the interaction and larger studies with possibility to perform multivariate adjustments for liver and renal injury in view of tumor stage and etiology of the disease will be needed.

AFP is among the most valuable prognostic markers in HCC. Wang *et al*[19] reported a positive correlation between miR-21 in tissues and serum AFP[19]. Tomimaru *et al*[24] and Liu *et al*[32] reported a similar correlation between circulating miR-21 in the blood and AFP[24,32]. In concordance with the data form Zhang *et al*[18], although our study demonstrated higher AFP values in patients with high serum miR-21 levels, it still did not reveal any correlation among the parameters.

**Table 3 Comparison of studies with a statement about the prognostic value of blood hsa-miR-21-5p in patients with hepatocellular carcinoma**

| Characteristics | Qi *et al*[25] | Tomimaru *et al*[24] | Liu *et al*[32] | Wang *et al*[31] | Cho *et al*[33] | Zhang *et al*[18] | Current data |
|---|---|---|---|---|---|---|---|
| High miR-21 and prognosis | ↔ | Trend for worse prognosis | Worse prognosis | Worse prognosis | Better liver transplant-free survival | Worse prognosis | ↔ |
| Group allocation: Cut-off-value | NA | Cut-off based on ROC analyzes | 50$^{th}$ percentile | 50$^{th}$ percentile | 50$^{th}$ percentile | NA | 50$^{th}$ percentile |
| Origin of the cohort | Asia | Asia | Asia | Asia | Asia | Asia | Europe |
| Predominant etiology | HBV | HCV/HBV | HBV | HBV | HBV | NA | Mainly alcohol abuse |
| TNM reported | Yes | Yes | | Yes | Yes | Yes | |
| BCLC reported | | Yes | Yes | | | | Yes |
| Child-Pugh score reported | Yes | Yes | Yes | | Yes | | Yes |
| Sampling | Before surgery | Before surgery | Before TACE | No exact information, surgical resection | Before surgery or RFA | Pre- and postoperative serum samples | Therapy naïve and pretreated |
| Patients | 70 | 126 | 136 | 97 | 120 | 46 | 91 |
| Specimen | Serum | Plasma | Serum | Serum | Plasma | Serum | Serum |
| qPCR method | TaqMan® | TaqMan® | SYBRGreen | SYBR Green | TaqMan® | Unknown | TaqMan® SYBRGreen |
| Normalization | miR-16 | miR-16 | Quanto EC1, Quanto EC2 | cel-miR-39 | MiR-16 | RNU6b | cel-miR-39 |
| Publishing year | 2011 | 2012 | 2014 | 2015 | 2017 | 2019 | 2020 |

BCLC: Barcelona Clinic Liver Cancer; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NA: Non-available; NASH: Non-alcoholic steatohepatitis; qPCR: Quantitative PCR; RFA: Radiofrequency ablation; ROC: Receiver operating characteristic; TACE: Transarterial chemoembolization; TNM: Tumor-Node-Metastasis Classification of Malignant Tumors.

An open question remains regarding the source of circulating miR-21 in HCC patients. Since miR-21 is present at a relatively high level in serum, it is hard to consider it a specific surrogate for the HCC tumor load. Nevertheless, several reports have suggested that circulating miR-21 may originate from tumor tissue. Studies have shown a reduction of miR-21 in blood after surgery[18,22,24,26]. Furthermore, concordant data have been reported for serum miR-21 and associated tissue levels[18,22,24], hypothesizing HCC tumor tissue as a potential source of miR-21 in HCC patients. At the same time, another hypothesis suggests that elevated circulating miR-21 may be triggered by non-specific liver damage. Based on our observation, miR-21 correlated with ASAT, which at least indirectly supports the hypothesis of liver injury. Additionally, miR-21 correlated significantly with serum miR-122 as highly abundant liver-specific miRNA[34,35]. Non-specific release of miR-122 from liver tissue has also been previously discussed[36,37]. Another study explored the influence of necroinflammation in patients with hepatitis C virus with and without HCC[38]. The authors demonstrated similar values of serum miR-21 between patients with and without HCC, suggesting that higher miR-21 level may be caused by non-specific liver injury rather than tumor-induced miR-21 release. Although our results do not link miR-21 with liver cirrhosis or liver function, Karakatsanis *et al*[20] showed that miR-21 was associated with cirrhosis[20], and Wang *et al*[39] correlated exosomal miR-21 with presence of liver cirrhosis[39].

Renal function seems to be a crucial confounding factor for miRNA analysis in blood not only for miR-122[1] as previously reported, but also for miR-21. On one hand, upregulation of miR-21 in kidney plays a role in acute kidney injury as a defense mechanism to reduce cell death. On the other hand, overexpression of miR-21, caused by massive cell death, leads to renal inflammation and fibrosis[40]. Renal injury in animal studies is associated with increased miR-21 expression in tissue and lower levels in blood[41]. In human, miR-21 was lower in subjects with cardiac surgery-

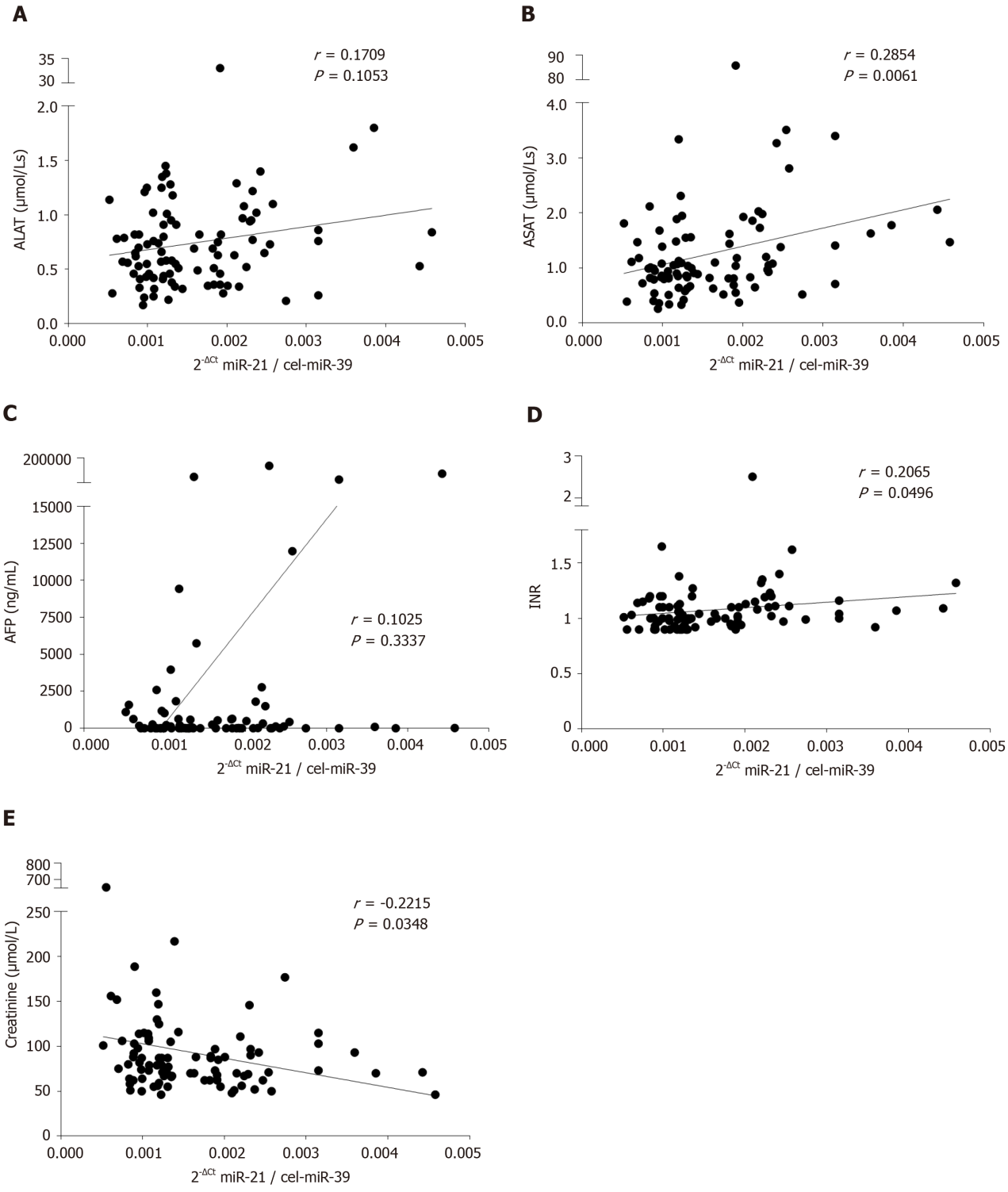


**Figure 2 Correlation analysis of serum hsa-miR-21-5p and common laboratory parameters.** Correlation between serum hsa-miR-21-5p and A: Alanine aminotransferase (ALAT); B: Aspartate aminotransferase (ASAT); C: Alpha-fetoprotein (AFP); D: International Normalized Ratio (INR); and E: Creatinine. For 30 patients only laboratory quick value was available and reverse calculation of International Normalized Ratio for values below < 1.5 were performed. Spearman's correlation coefficient was used for statistical analysis. MiR-21: Hsa-miR-21-5p.

associated acute kidney injury[42]. Our data strongly support the suggestion that renal function may affect circulating miRNAs as both miR-21 and miR-122 have shown a clear correlation with creatinine values. Taking into account the fact that miR-21 has been suggested as a potential biomarker for a large number of diseases including cancer[11,43] it will be necessary in the future to consider those variations in ongoing studies. In particular, miR-21 has been suggested as a diagnostic marker for malignant diseases including gastric, colorectal cancer, and HCC. Because of the miR-21 variation in our cohort, we speculate that the diagnostic value of miR-21 may be also affected by

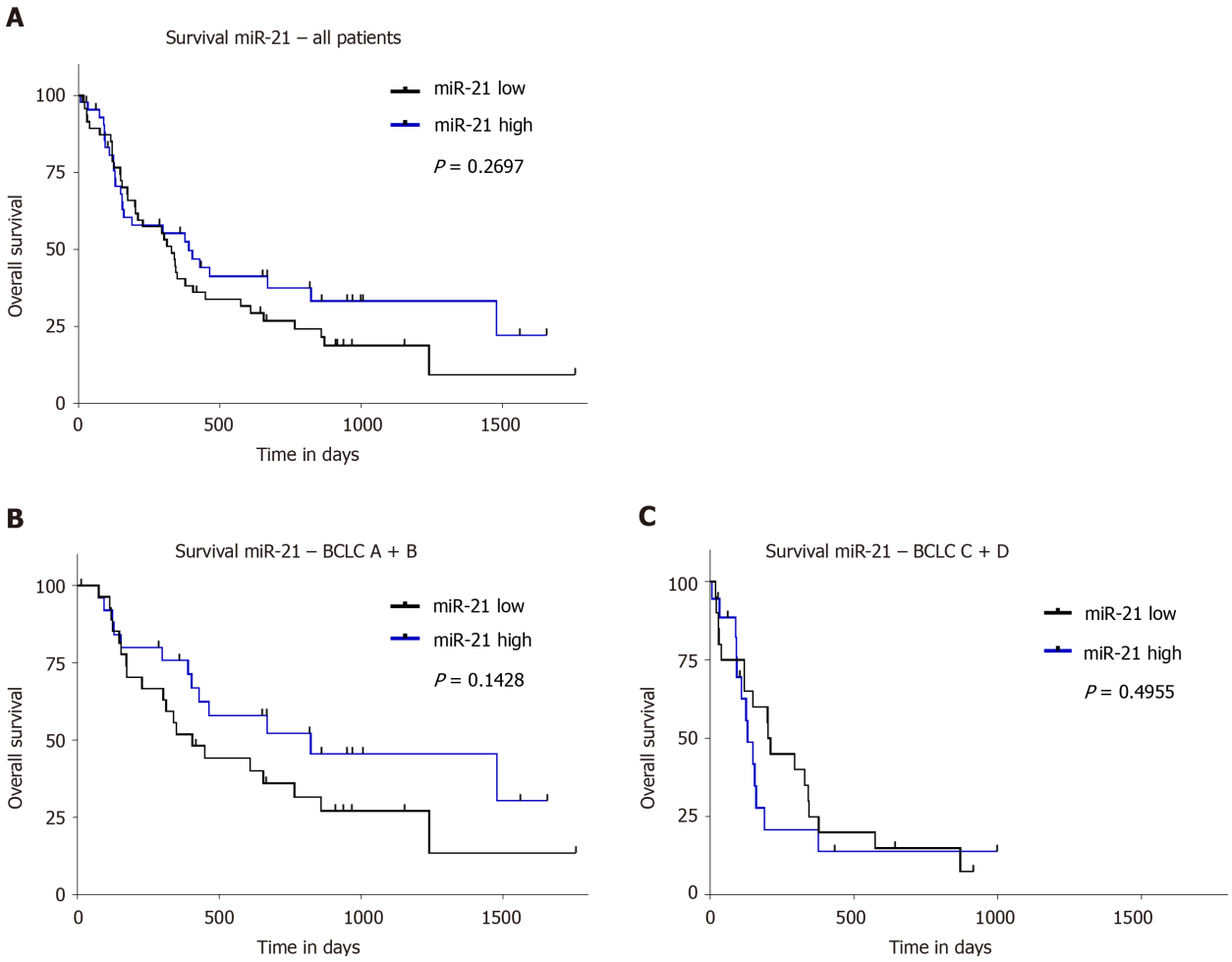


**Figure 3 Overall survival analysis in relation to hsa-miR-21-5p level.** A: All patients were divided into two groups: (1) Low: < 50$^{th}$ percentile ($n$ = 47) and (2) High: > 50$^{th}$ percentile ($n$ = 44), two patients exhibit exactly the median expression of serum hsa-miR-21-5p, that is why, the group division is not exactly symmetric; B: Overall survival analysis in patients with Barcelona Clinic Liver Cancer staging system A + B (divided into two groups: (1) Low: < 50$^{th}$ percentile [$n$ = 27] and (2) High: > 50$^{th}$ percentile [$n$ = 26]); C: Survival analysis in patients with Barcelona Clinic Liver Cancer staging system C + D (divided into two groups: (1) Low: < 50$^{th}$ percentile [$n$ = 20] and (2) High: > 50$^{th}$ percentile [$n$ = 18]). Nonparametric log-rank test was used for statistical analysis. MiR-21: Hsa-miR-21-5p.

the renal and liver function. In-depth systematic data including miRNA-profiling are encouraged to address this topic in detail.

## CONCLUSION

In summary, the results of this study do not support the role of circulating miR-21 as prognostic biomarker for patients with HCC in our European cohort. A positive correlation between miR-21 and ASAT and negative correlation with creatinine strongly emphasizes that non-specific liver injury and renal function may influence the sensitivity and specificity of miRNAs as biomarkers, and that those factors need to be carefully assessed in future studies.

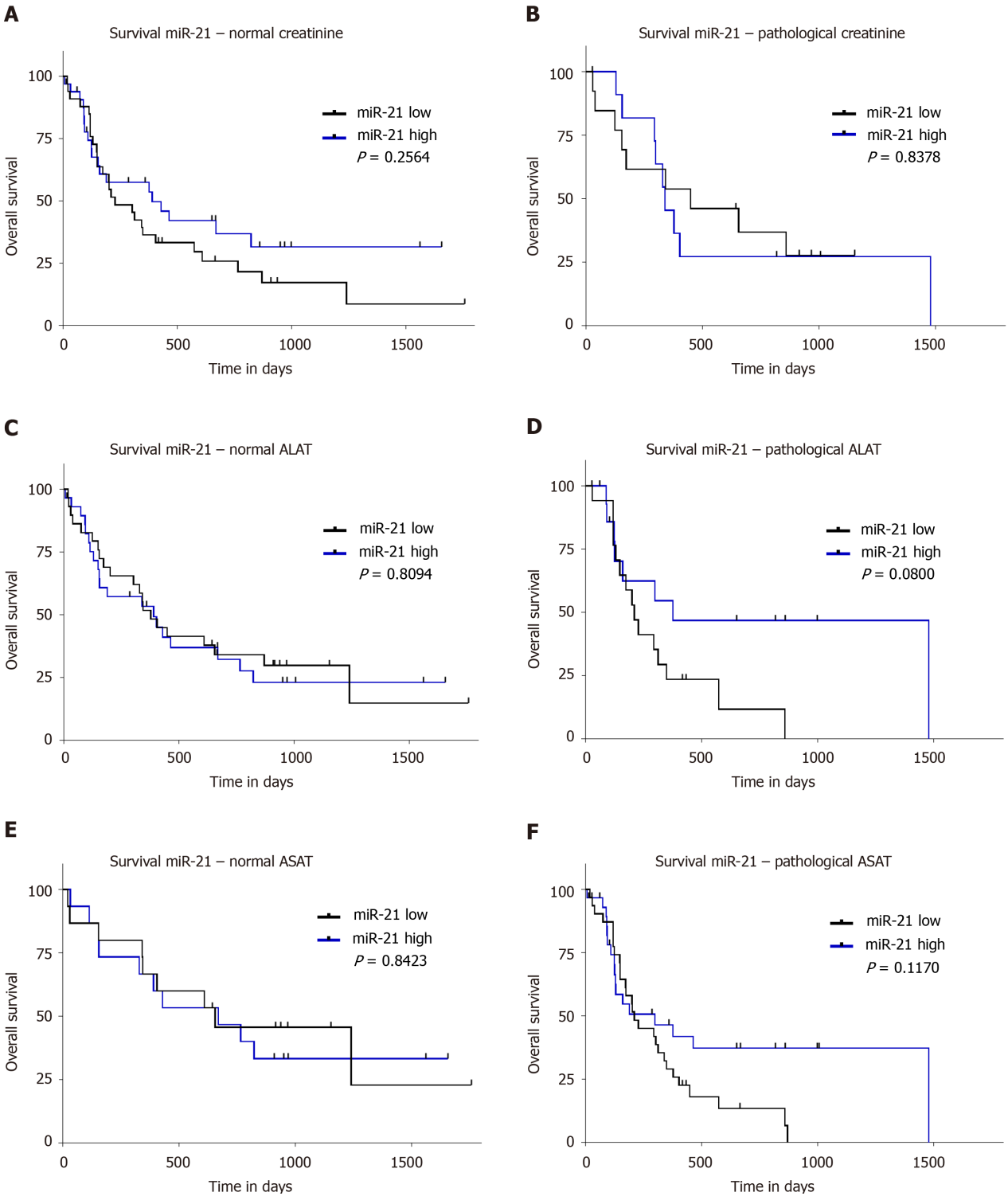


**Figure 4 Overall survival analysis in subgroups of patients in relation to hsa-miR-21-5p level.** A: Survival analysis in patients with normal creatinine (divided into two groups: (1) Low: < 50$^{th}$ percentile ($n$ = 33) and (2) High: > 50$^{th}$ percentile [$n$ = 33]); B: Survival analysis in patients with pathological creatinine (hsa-miR-21-5p [miR-21] low $n$ = 13; miR-21 high $n$ = 12); C: Survival analysis in patients with normal alanine aminotransferase (miR-21 low $n$ = 29; miR-21 high $n$ = 29); D: Survival analysis in patients with pathological alanine aminotransferase (ALAT) (miR-21 low $n$ = 17, miR-21 high $n$ = 16); E: Survival analysis in patients with normal aspartate aminotransferase (ASAT) (miR-21 low $n$ = 15; miR-21 high $n$ = 15); and F: Survival analysis in patients with pathological ASAT (miR-21 low $n$ = 31; miR-21 high $n$ = 30). Nonparametric log-rank test was used for statistical analysis. MiR-21: Hsa-miR-21-5p.

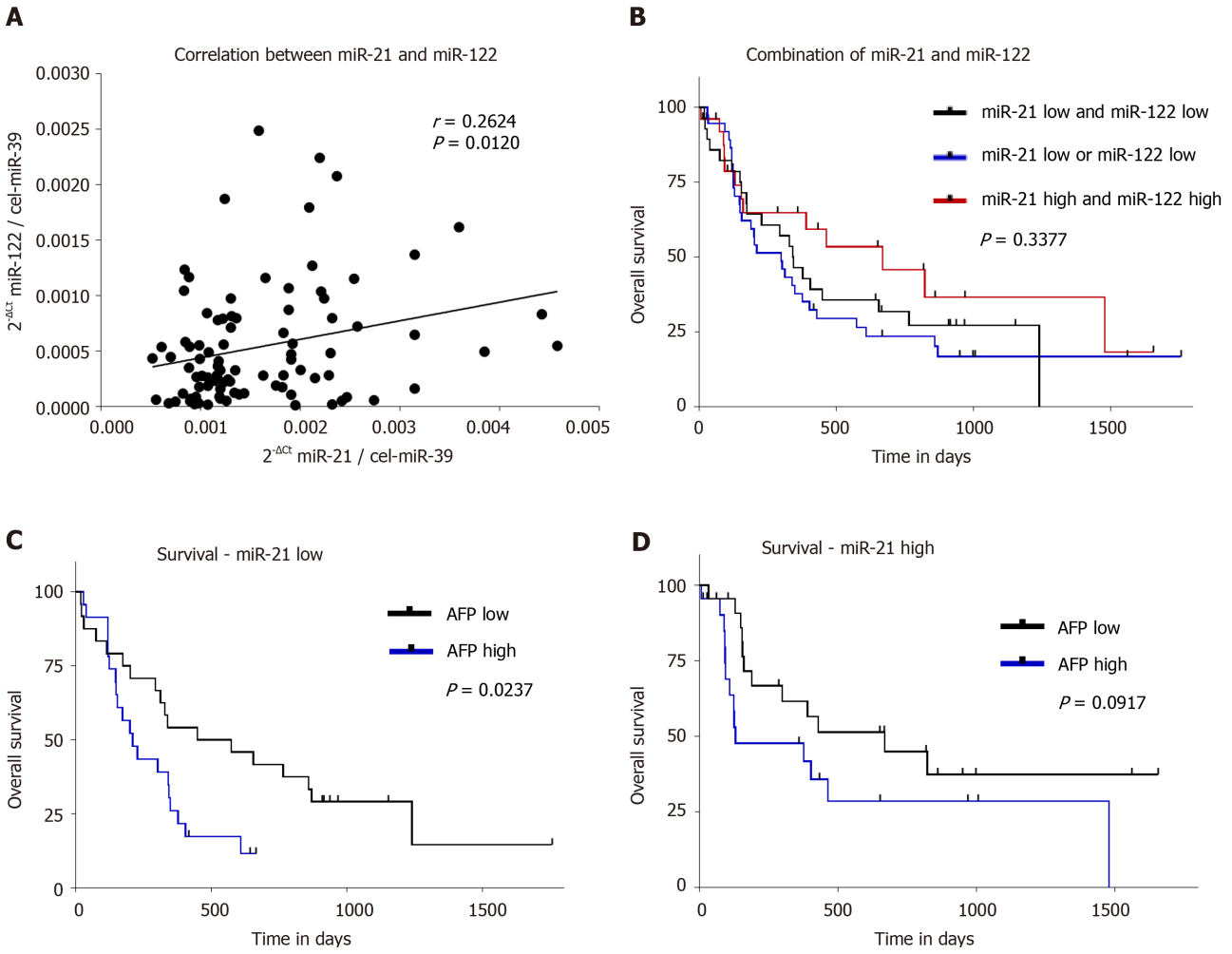


**Figure 5 Overall survival analysis in hepatocellular carcinoma patients in relation to serum hsa-miR-21-5p, hsa-miR-122-5p, and alpha-fetoprotein levels.** A: Correlation between serum hsa-miR-21-5p (miR-21) and serum hsa-miR-122-5p (miR-122); B: All patients were divided into three groups: Both miR-21 and miR-122 low (50$^{th}$ percentile, $n$ = 28); one of miR-21 or miR-122 low (50$^{th}$ percentile, $n$ = 37); both miR-21 and miR-122 high (> 50$^{th}$ percentile, $n$ = 26); C: Analysis of overall survival of alpha-fetoprotein (AFP) low ($n$ = 24) *vs* AFP high ($n$ = 23) in patients with low miR-21 levels; and D: Analysis of overall survival of AFP low ($n$ = 22) *vs* AFP high ($n$ = 22) in patients with high miR-21 levels. Spearman correlation and nonparametric log-rank test were used for statistical analysis. MiR-21: Hsa-miR-21-5p; MiR-122: Hsa-miR-122-5p.

## ARTICLE HIGHLIGHTS

### *Research background*

MiR-21-5p (miR-21) is one of the most frequently deregulated microRNAs (miRNAs) in tissue and in body fluids in different diseases and most importantly in cancer. Overexpression and prognostic value of tissue miR-21 in hepatocellular carcinoma (HCC) has been previously suggested, but the data are heterogeneous. Greatest evidence in support of miR-21 as a biomarker originate from Asian population, highlighting the need for additional scientific effort and search for potential confounding factors including etiological background of HCC.

### *Research motivation*

Data on prognostic value of serum miR-21 in patients with mainly alcohol abuse-induced HCC as well as the knowledge of potential influencing factors and underlying mechanism are still limited.

### *Research objectives*

We evaluated the prognostic value of serum miR-21 in a European cohort of HCC patients with mainly alcohol-associated liver disease in real-life settings. We also explored the potential confounding clinical or laboratory influencing factors on the serum miR-21 levels.

### Research methods

Circulating miR-21 level were analyzed in 91 sera samples from well-characterized patients with HCC. Clinical and laboratory parameters (including alpha-fetoprotein), miR-122 as well as the overall survival (OS) were examined.

### Research results

We observed no association between serum miR-21 and OS, Child-Pugh scores, or BCLC staging data. Significant correlation between serum miR-21 and aspartate aminotransferase, International Normalized Ratio, creatinine, and hsa-miR-122 suggested the potential influence of liver and renal function on circulating miR-21 levels.

### Research conclusions

Our data do not support prognostic role of miR-21 in HCC patients. An association of miR-21 with surrogate markers of liver injury and renal function strongly support the need for better understanding of influencing factors in miRNA biogenesis.

### Research perspectives

Systematic profiling studies that provide overall data assessment of miRNAs in view of potential influencing factors in blood are needed prior clinical implementation of miRNAs as clinical biomarkers.

## ACKNOWLEDGEMENTS

The authors would like to thank Ursula Stolz (from the GI Research Laboratory of the Department of Gastroenterology, Hepatology and Infectious Diseases) for technical support.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1046-1054

DOI: 10.4254/wjh.v12.i11.1046

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

**Retrospective Study**

# Real impact of tumor marker AFP and PIVKA-II in detecting very small hepatocellular carcinoma (≤ 2 cm, Barcelona stage 0) - assessment with large number of cases

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**Author contributions:** Tarao K summarized the data and wrote the paper; Nozaki A, Komatsu H, Komatsu T, Tanaka K, Chuma M, Numata K and Maeda S were responsible for patient follow-up; Taguri M conducted the statistical analyses.

**Supported by** the Kanagawa Association of Medical and Dental Practitioners.

**Institutional review board statement:** The study was reviewed and approved by the Yokohama Municipal Citizen's Hospital Institutional Review Board (Approval No. 19-02-03).

**Informed consent statement:** This study is a retrospective observational study and does not require consent form.

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## Abstract

*BACKGROUND*

In hepatocellular carcinoma (HCC), detection and treatment prior to growth beyond 2 cm are relevant as a larger tumor size is more frequently associated with microvascular invasion and/or satellites.

*AIM*

To examine the impact of the tumor marker alpha-fetoprotein (AFP) or PIVKA-II in detecting very small HCC nodules (≤ 2 cm in maximum diameter, Barcelona stage 0) in the large number of very small HCC. The difference in the behavior of

**Conflict-of-interest statement:** Tanaka K has received research funding from Bristol-Myers Squibb and Abb Vie; Nozaki A has received research funding from Gilead Sciences; Tarao K, Komatsu H, Komatsu T, Taguri M, Chuma M, Numata K and Maeda S, declare that they have no conflict of interest.

**Data sharing statement:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.



**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Japan

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 23, 2020
**Peer-review started:** June 23, 2020
**First decision:** July 30, 2020
**Revised:** August 10, 2020
**Accepted:** September 2, 2020
**Article in press:** September 2, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Wang GY, Zhong JH
**S-Editor:** Zhang L

these tumor markers in HCC development was also examined.

*METHODS*
A total of 933 patients with single-nodule HCC were examined. They were subdivided into 394 patients with HCC nodules ≤ 2 cm in maximum diameter and 539 patients whose nodules were > 2 cm. The rates of patients whose AFP and PIVKA-II showed normal values were examined.

*RESULTS*
The positive ratio of the marker PIVKA-II was significantly different ($P < 0.0001$) between patients with nodules ≤ 2 cm in diameter and those with nodules > 2 cm, but there was no significant difference in AFP ($P = 0.4254$). In the patients whose tumor was ≤ 2 cm, 50.5% showed normal levels in AFP and 68.8% showed normal levels in PIVKA-II. In 36.4% of those patients, both AFP and PIVKA-II showed normal levels. The PIVKA-II-positive ratio was markedly increased with an increase in the tumor size. In contrast, the positivity in AFP was increased gradually and slowly.

*CONCLUSION*
In the surveillance of very small HCC nodules (≤ 2 cm in diameter, Barcelona clinical stage 0) the tumor markers AFP and PIVKA-II are not so useful.

**Key Words:** Hepatocellular carcinoma; AFP; PIVKA-II; Barcelona clinical stage; Tumor markers



**Core Tip:** In hepatocellular carcinoma, detection and treatment prior to nodule growth of 2 cm (Barcelona stage 0) are relevant as a larger tumor size is more frequently associated with microvascular invasion and/or satellites. We surveyed the real impact of the tumor markers alpha-fetoprotein (AFP) or PIVKA-II in detecting very small hepatocellular carcinoma with a large number of cases (≤ 2 cm in diameter 394 cases) and found in AFP that 50.5% and in PIVKA-II that 68.8% showed normal levels. Moreover, 36.4% of the patients showed normal levels in both AFP and PIVKA-II. In the surveillance of very small hepatocellular carcinoma nodules, the tumor markers are not so useful.

**Citation:** Tarao K, Nozaki A, Komatsu H, Komatsu T, Taguri M, Tanaka K, Chuma M, Numata K, Maeda S. Real impact of tumor marker AFP and PIVKA-II in detecting very small hepatocellular carcinoma (≤ 2 cm, Barcelona stage 0) - assessment with large number of cases. *World J Hepatol* 2020; 12(11): 1046-1054
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1046.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1046

## INTRODUCTION

For the detection of hepatocellular carcinoma (HCC) from various liver diseases, especially liver cirrhosis, surveillance with the tumor markers, alpha-fetoprotein (AFP) and PIVKA-II, or detection with the imaging modalities, ultrasonography (US) or magnetic resonance imaging (MRI) [computed tomography (CT)], is usually performed.

Detection and treatment prior to growth beyond 2 cm are relevant as a larger tumor size is more frequently associated with microvascular invasion and/or satellites, which are major predictors of recurrence after initial effective treatment[1]. The same tendency was observed by Stravitz *et al*[2], and they reported that the early detection of HCC improves the prognosis.

Therefore, we must identify minute HCC nodules (≤ 2 cm in diameter) in the surveillance of HCC. Previous reports concerning the tumor markers AFP and PIVKA-II in very small HCCs included a relatively small number of cases. In this retrospective

**L-Editor:** Filipodia
**P-Editor:** Wang LYT

analysis, we examined the precise levels of these markers in a large number of very small HCC cases (< 2 cm in diameter, 394 cases) and whether the tumor marker AFP or PIVKA-II is useful to find very small HCC (≤ 2 cm in maximum diameter, Barcelona clinic liver cancer staging 0)[3,4]. Also, we examined the difference in the behavior of these tumor markers in relation to the tumor size of HCC nodules.

## MATERIALS AND METHODS

### Study population

This was a retrospective study that included 933 patients with single HCC nodules who entered the following three hospitals in Yokohama City for the first time, between January 2008 and January 2019: Gastroenterological Center, Yokohama City University Medical Center, Department of Gastroenterology, Yokohama Municipal Citizen's Hospital, Department of Clinical Research, National Hospital Organization Yokohama Medical Center. HCCs were diagnosed chiefly by dynamic CT and abdominal angiography, which showed early enhancement and early wash out. This work was performed in accordance with the Declaration of Helsinki.

Previously diagnosed HCC was excluded from the protocol. This study was performed after approval by the respective institutional review boards.

The patients were classified according to etiologies of liver diseases: 72 with hepatitis B (presence of hepatitis B surface antigen in serum), 540 with hepatitis C (presence of hepatitis C antibody in serum), 10 with primary biliary cholangitis, five with autoimmune hepatitis, 70 with alcoholic liver diseases, and others (Table 1).

### Measurement of PIVKA-II and AFP

Samples were collected before the treatment for HCC. Concentrations of PIVKA-II and AFP in serum samples were determined by the chemiluminescent enzyme immunoassay in all three hospitals, and the cutoff values for PIVKA-II and AFP were 40 mAU/mL and 10 ng/mL, respectively, in every hospital. For PIVKA-II and AFP, ≤ 40 mAU/mL and ≤ 10 ng/mL were set as normal values, respectively.

### HCC detection

The diagnosis of HCC was confirmed by US, MRI, CT, enhanced dynamic CT, and abdominal angiography. All patients underwent abdominal angiography to confirm the single nodules. The maximum diameter of the HCC nodules was scaled by US or MRI.

Helical dynamic CT and abdominal angiography were performed in almost all patients except the patients with hypersensitivity to iodine and with advanced kidney disease. In the helical dynamic CT, an intravenous bolus injection of contrast material and sequential scanning were performed, and intense homogenous arterial-phase (early enhancement) and early washout were thought to be characteristic of HCC[5-7]. Abdominal angiography was also performed to exclude the benign nodular lesions and to exclude the HCC patients with macrovascular invasion.

The patients with macrovascular invasion or extrahepatic metastasis were excluded. In the hepatectomy performed patients, final decision of HCC was made by pathological diagnosis and cases of benign nodules were excluded.

### Statistical analysis

For the comparisons of test-positive proportions between > 2 cm and ≤ 2 cm tumors, we conducted chi-squared tests for AFP and PIVKA-II, respectively. To understand the relationships between the tumor size and test-positive proportions for AFP and PIVKA-II, we applied logistic regression models using the tumor size as an independent variable and test results (positive or not) as the dependent variable. All reported $P$ values correspond to two-sided tests, and $P < 0.05$ was considered significant. All analyses were performed with Statistics Analysis System, version 9.4 (Statistics Analysis System Institute, Cary, NC, United States).

## RESULTS

The clinical characteristics of the patients are summarized in Table 1. Our study included 933 HCC patients with a single nodule. In total, 622 patients were male, and 311 patients were female. The average age was 72.0 ± 9.6 years. Concerning the tumor

**Table 1 Background of the hepatocellular carcinoma patients**

| | | Maximum diameter of nodules | |
|---|---|---|---|
| | | ≤ 20 mm | > 20 mm |
| Number of patients | | 394 | 539 |
| Sex | Male | 230 (58.4%) | 392 (72.7%) |
| | Female | 164 (41.6%) | 147 (22.3%) |
| Age in yr | | 71.3 ± 8.8 | 72.5 ± 10.1 |
| Etiology | HBV | 33 (8.4%) | 39 (7.2%) |
| | HCV | 274 (69.5%) | 266 (49.4%) |
| | PBC | 4 (1.0%) | 6 (1.1%) |
| | Alcohol | 17 (4.3%) | 53 (9.8%) |
| | NASH | 4 (1.0%) | 8 (1.5%) |
| | Autoimmune hepatitis | 3 (0.8%) | 2 (0.4%) |
| | Unknown | 23 (5.8%) | 62 (11.5%) |

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

size at diagnosis, 394 patients had HCC nodules ≤ 2 cm in maximum diameter, and 539 patients had nodules larger than 2 cm in maximum diameter.

The positive rates of AFP and PIVKA-II in patients whose tumor was ≤ 2 cm and those whose tumor size was more than 2 cm are shown in Table 2. The level of PIVKA-II showed a significant difference ($P < 0.0001$), but there was no difference in AFP ($P = 0.4254$).

Table 3 shows the rates of patients whose AFP and PIVKA-II exhibited normal values in those with a maximum tumor size of ≤ 2 cm. In AFP, 50.5% showed normal levels, and in PIVKA-II, 68.8% showed normal levels. A more important finding was that, in 36.4% of the patients, both AFP and PIVKA-II showed normal levels.

Table 4 shows the treatment methods of all HCC patients. In the very small HCC patients (≤ 2 cm), the radiofrequency ablation group occupied the majority. In the relatively large HCC group (> 2 cm), treatment by transcatheter arterial chemoembolization was the most frequent, followed by hepatectomy and radiofrequency ablation.

Figure 1 shows the relationship between the tumor size and PIVKA-II and AFP positivity. The PIVKA-II positive ratio was markedly increased with an increase in tumor size. In contrast, the positivity in AFP was increased gradually and slowly.

Figure 2 shows the correlation between the tumor size and PIVKA-II levels. The correlation ratio was 0.5691 ($P < 0.0001$).

Figure 3 shows the correlation between the tumor size and AFP levels. The correlation ratio was 0.1895 ($P < 0.0001$).

## DISCUSSION

Although the early detection of HCC with imaging modalities has been developed in recent years, tumor markers are still commonly used in HCC detection and follow-up.

We demonstrated in this study that 36.4% of the patients whose maximum diameter of HCC nodules was equal to or less than 2.0 cm (Barcelona stage 0) showed normal levels of both AFP and PIVKA-II. In support of our results, it was reported that about 30% of HCC patients show false-negative results regarding tumor markers, especially in its early stage[4-10]. Moreover, it was demonstrated that AFP has a sensitivity of about 68% in the diagnosis of HCC, but the sensitivity decreased to about 59% in its early stage[11,12].

In recent years, Huang *et al*[13] demonstrated that PIVKA-II combined with AFP showed a better diagnostic ability than AFP alone for HCC diagnosis. However, our study confirmed the limitation in detecting HCC in patients with very small single HCC nodules (≤ 2 cm) (Barcelona clinic liver cancer staging 0)[3,4], even in combination with AFP and PIVKA-II.

**Table 2 Positive rates of alpha-fetoprotein and PIVKA-II in patients whose tumor size was ≤ 2 cm and those whose tumor size was more than 2 cm**

| | | Maximum diameter of HCC nodules | | *P* value[1] |
|---|---|---|---|---|
| | | ≤ 2 cm, *n* = 394 | > 2 cm, *n* = 539 | |
| AFP | (+) | 195 (49.5%) | 281 (52.1%) | 0.4254 |
| | (-) | 199 (50.5%) | 258 (47.9%) | |
| PIVKA | (+) | 123 (31.2%) | 385 (71.4%) | < 0.0001 |
| | (-) | 271 (68.8%) | 154 (28.6%) | |

[1]$\chi^2$ tests. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

**Table 3 The rates of patients whose alpha-fetoprotein and PIVKA-II showed normal values in those whose maximum tumor size was ≤ 2 cm, *n* = 394 cases**

| HCC tumor marker | No. of cases (%) |
|---|---|
| AFP, normal cases | 199 (50.5) |
| PIVKA-II, normal cases | 271 (68.8) |
| Both AFP and PIVKA-II, normal cases | 142 (36.4) |

AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

**Table 4 Treatment methods of hepatocellular carcinoma patients**

| Therapy | No of treated patients | |
|---|---|---|
| | Group | |
| | ≤ 2 cm, *n* = 394 | > 2 cm, *n* = 539 |
| Hepatectomy | 45 | 110 |
| RFA | 223 | 107 |
| TACE | 56 | 136 |
| TACE + RFA | 6 | 32 |
| TAI | 2 | 10 |
| Chemotherapy | 9 | 21 |
| BSC | 10 | 60 |
| Others | 13 | 63 |

BSC: Best supportive care; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; TAI: Transcatheter arterial infusion.

We demonstrated that more than a third of the patients with minute HCC nodules (≤ 2 cm in diameter) were dropped from surveillance using the tumor markers AFP and PIVKA-II alone. Based on the results, we must depend on imaging modalities such as US or MRI (CT) for the surveillance of minute HCC.

Colli *et al*[14] conducted a systemic review on this issue and found that pooled estimates of 14 US studies were 60.5% (95% confidence interval (CI): 44-76) for sensitivity[13-29], and that nine MRI studies were 80.6% (95%CI: 70-91) for sensitivity[27,30-37]. The difference in sensitivity between US and MRI may be due to the fact that MRI is less influenced by the operator's technique and patient's body type.

More recently, Kim *et al*[38] compared MRI and US in a cohort of 407 patients with cirrhosis who underwent 1100 surveillance examinations and found that MRI had a sensitivity of 83.7% (95%CI: 69.7%-92.2%) for early HCC detection, which was significantly higher than US (25.6%, 95%CI: 14.8%-49.4%). Thus, we must follow-up

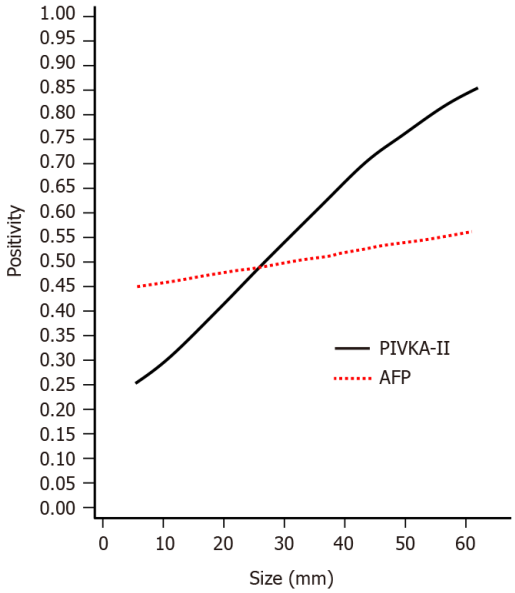


**Figure 1 Relationship between tumor size and PIVKA-II and alpha-fetoprotein positivity.** AFP: Alpha-fetoprotein.

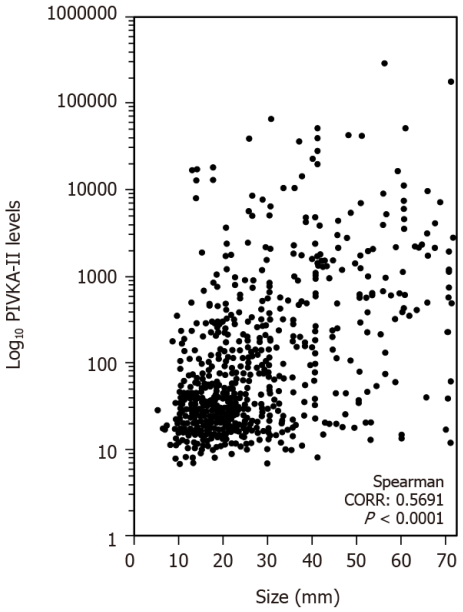


**Figure 2 Correlation between tumor size and PIVKA-II levels.**

patients with liver disease, especially liver cirrhosis, at regular intervals (at least every 6 mo) with MRI to detect very small HCC (diameter ≤ 2 cm).

Furthermore, we demonstrated that the PIVKA-II positive ratio was markedly increased with an increase in tumor size. In support of this phenomenon, previous studies established the correlation between the PIVKA-II level and tumor size[16], and that PIVKA-II maintains the growth of HCC[17]. Moreover, Ma *et al*[18] reported direct clinical evidence of the correlation between PIVKA-II and cell proliferation.

## CONCLUSION

More than one third of the patients with very small HCC nodule (≤ 2 cm in diameter, Barcelona stage 0) were dropped from the surveillance using the tumor markers AFP and PIVKA-II. So, we must survey patients with liver diseases by MRI at regular

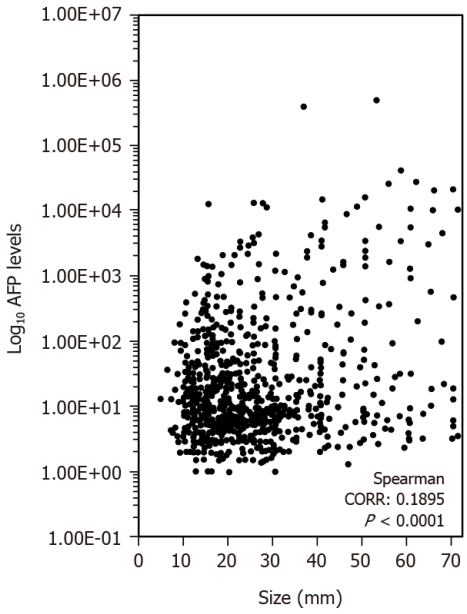


**Figure 3 Correlation between tumor size and alpha-fetoprotein levels.** AFP: Alpha-fetoprotein.

intervals to detect very small HCC nodules.

## ARTICLE HIGHLIGHTS

### *Research background*

In hepatocellular carcinoma (HCC), detection and treatment prior to growth of 2 cm are relevant as a larger tumor size is more frequently associated with microvascular invasion and/or satellites. However, we often experience cases whose tumor size was ≤ 2 cm and who showed normal values in both AFP and PIVKA-II.

### *Research motivation*

Previous reports concerning the tumor markers AFP or PIVKA-II in very small HCC included relatively small number of cases, and a larger study is necessary in order to elucidate the precise levels of these markers.

### *Research objectives*

In the present study, we surveyed the levels of AFP and PIVKA-II in a large number of very small HCC cases (≤ 2 cm in diameter, 394 cases).

### *Research methods*

We analyzed 933 patients with single HCC nodules and surveyed the limitation of these tumor markers in the surveillance of very small HCC (≤ 2 cm, Barcelona stage 0, 394 cases).

### *Research results*

It was found in patients with very small HCC (≤ 2 cm in diameter) that AFP and PIVKA-II levels were normal in 50.5% and 68.8%, respectively. Moreover, 36.4% of the patients showed normal levels of both AFP and PIVKA-II. We examined the difference in behavior of these tumor markers in relation to the size of HCC nodules and found that PIVKA-II positive ratio was markedly increased with an increase in tumor size, whereas the positivity in AFP was increased gradually and slowly.

### *Research conclusions*

More than one third of the patients with very small HCC nodule (≤ 2 cm in diameter, Barcelona stage 0) were dropped from the surveillance using the tumor markers AFP and PIVKA-II.

### *Research perspectives*

We propose that for detecting very small HCC nodules, we must survey patients with liver diseases by imaging modalities, especially by magnetic resonance imaging.

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World Journal of Hepatology

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*World J Hepatol* 2020 November 27; 12(11): 1055-1066

DOI: 10.4254/wjh.v12.i11.1055

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

# Non-invasive splenic parameters of portal hypertension: Assessment and utility

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**Author contributions:** Ahmad AK collected data, performed statistical analyses, wrote the manuscript with support from other authors; Atzori S performed the experiments and statistical analyses; Maurice J critically appraised study design; Taylor-Robinson SD and Lim AKP designed, supervised and implemented the research; all authors discussed the results and contributed to the final manuscript.

**Institutional review board statement:** This study was performed in accordance with the 1975 Declaration of Helsinki, with approvals from the Research Ethics Committee and the Joint Research and Compliance Office of Imperial College London and Imperial College Healthcare NHS Trust.

**Informed consent statement:** Patients were required to give informed consent to the study in the form of written consent.

**Conflict-of-interest statement:** This work was supported by Philips

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## Abstract

### *BACKGROUND*

Portal hypertension is a major complication of cirrhosis that is associated with significant morbidity and mortality. The present gold-standard method to risk stratify and observe cirrhosis patients with portal hypertension is hepatic venous pressure gradient measurement or esophagogastroduodenoscopy. However, these methods are invasive, carry a risk of complications and are associated with significant patient discomfort. Therefore, non-invasive splenic parameters are of clinical interest as potential useful markers in determining the presence of portal hypertension. However, diagnostic accuracy and reproducibility remains unvalidated.

### *AIM*

To assess the diagnostic accuracy of spleen stiffness, area and diameter in predicting the presence of portal hypertension.

### *METHODS*

Of 50 patients with varying liver disease pathologies were prospectively recruited from the St. Mary's Hospital Liver Unit in London; 25 with evidence of portal hypertension and 25 with no evidence of portal hypertension. Liver stiffness, spleen stiffness, spleen diameter and spleen area were measured using the Philips Affiniti 70 elastography point quantification point shear wave elastography system. The aspartate aminotransferase-to-platelet-ratio-index (APRI) score was also calculated. Performance measures, univariate and multivariate logistic

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**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United Kingdom

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** July 16, 2020
**Peer-review started:** July 16, 2020
**First decision:** August 9, 2020
**Revised:** August 22, 2020
**Accepted:** October 9, 2020
**Article in press:** October 9, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Giuffrè M
**S-Editor:** Fan JR
**L-Editor:** A
**P-Editor:** Wang LYT

regression were used to evaluate demographic, clinical and elastography variables. Interclass correlation coefficient was used to determine the reproducibility of splenic area and diameter.

*RESULTS*

On univariate and individual performance, platelet count [area under the receiver operating characteristic (AUROC) 0.846, *P* value < 0.001], spleen area (AUROC 0.828, *P* value = 0.002) and APRI score (AUROC 0.827, *P* value < 0.001) were the most accurate variables in identifying the presence of portal hypertension. On multivariate logistic regression models constructed, the combination of spleen area greater than 57.90 $cm^2$ and platelet count less than 126 × $10^9$ had 63.2% sensitivity and 100% specificity, 100% positive predictive value and 100% negative predictive value. An alternative combination of spleen stiffness greater than 29.99 kPa and platelet count less than 126 × $10^9$ had 88% sensitivity, 75% specificity, 78.6% positive predictive value and 85.7% negative predictive value. An interclass correlation coefficient value of 0.98 (95%CI: 0.94-0.99, *P* value < 0.001) and 0.96 (95%CI: 0.91-0.99, *P* value < 0.001) were determined for inter-operator variability for spleen area and diameter respectively.

*CONCLUSION*

Spleen area, spleen stiffness and platelet count may be useful markers to assess the presence of portal hypertension in patients of various etiologies.

**Key Words:** Portal hypertension; Esophageal varices; Point shear wave elastography; Spleen stiffness; Spleen area; Non-invasive



**Core Tip:** Non-invasive splenic parameters are useful surrogate markers of portal hypertension (PH). A combination of spleen diameter, spleen area, liver stiffness and aspartate aminotransferase-to-platelet-ratio-index (APRI) score is able to predict the presence of PH. The APRI score has a similar diagnostic accuracy to combination index.

**Citation:** Ahmad AK, Atzori S, Maurice J, Taylor-Robinson SD, Lim AKP. Non-invasive splenic parameters of portal hypertension: Assessment and utility. *World J Hepatol* 2020; 12(11): 1055-1066
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1055.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1055

## INTRODUCTION

Portal hypertension (PH) is a major complication of cirrhosis[1]. Esophageal varices (EV) are present in 40% of compensated advanced chronic liver disease (cACLD) patients and in 70% of decompensated cirrhosis patients. Strategies to identify individuals with clinically significant portal hypertension (CSPH) is vital to reduce morbidity and mortality[2].

The hepatic venous pressure gradient measurement (HVPG) and esophago-gastroduodenoscopy (EGD) form the backbone of diagnosis and surveillance of EV[3]. However, both methods are invasive and carry a risk of complications[1]. Therefore, non-invasive and safe methods of diagnosis and surveillance of PH are of great clinical interest.

Current guidelines propose that non-invasive methods of assessment of liver fibrosis can predict the incidence of cirrhosis-induced PH manifestations[4]. The Baveno VI guidelines suggest cirrhosis patients with a liver stiffness measurement < 20 kPa and a platelet count > 150000/μL can avoid screening endoscopy[3]. Nevertheless, while 20% of EGDs are spared, new algorithms are still required, as up to 40% of EGDs continue unnecessarily[5].

Ultrasound elastography techniques are based on the principle that tissue elastic

properties can be distorted using shear waves to measure stiffness. Spleen stiffness measurements using ultrasound elastography have shown an association with CSPH as the spleen undergoes parenchymal remodelling and fibrogenesis, due to blood pooling in PH[5-7]. Interestingly, evidence on patients with chronic hepatitis C infection also suggests that spleen stiffness is dependent on inflammation present in the liver that directly contributes to the pathogenic mechanisms underlying PH[8,9].

Transient elastography (TE) is the most validated ultrasound elastography technique and shows a sensitivity ≥ 90% in detecting patients with CSPH[6,10]. Nevertheless, limitations exist due to its lack of 2D imaging guidance and attenuation of wave propagation in obesity and ascites[7].

Point shear wave elastography (p-SWE), often referred to as acoustic radiation force impulse, overcomes these issues by providing integrated 2D-ultrasound imaging which can be used in patients who are obese or have ascites[7]. Despite several meta-analyses on spleen stiffness measurements, it remains unclear whether TE or p-SWE has greater diagnostic accuracy[11,12]. Furthermore, although it is well established that spleen stiffness and combination variables such as liver stiffness-spleen diameter to platelet ratio (LSPS) score are superior to liver stiffness for detection of EV[13], little is known whether a combination of splenic parameters can improve diagnostic accuracy[14]. Finally, although two main p-SWE techniques exist–the elastography point quantification (ElastPQ®) and Virtual Touch Quantification (VTQ®)–fewer studies have looked at the performance of ElastPQ due to its novelty.

We aimed to assess whether spleen stiffness measurement, spleen area and spleen diameter can independently predict CSPH, or in combination with other biochemical or elastography parameters; and assess reproducibility of splenic area and diameter measurements.

## MATERIALS AND METHODS

### *Study design*

Patients with varying liver disease etiology were prospectively recruited as part of an ongoing comparative imaging study (REC: 15/EE/0420). All subjects had evidence of chronic liver disease (CLD), were over the age of 18 and provided informed consent. Exclusion criteria included pregnancy, lack of liver disease pathology, transjugular portosystemic shunt (TIPSS) insertion or presence of hepatocellular carcinoma (HCC).

The primary analyses were conducted after all patients were recruited. The patients were divided into the following groups: Evidence of CSPH (group 1) and no evidence of CSPH (group 2). CSPH was defined either as presence of EV or portal hypertensive gastropathy (PHG) during an EGD or if patients had invasive procedures where the HVPG pressure ≥ 10 mmHg. Ultrasound elastography measurements must have been undertaken within a maximum of one year of EGD or HVPG measurements.

### *Ultrasound and elastography*

All patients had to be fasted for up to 6h prior to scans. Participants were placed supine with arms abducted away from the ultrasound probes. The Philips Affiniti 70 (ElastPQ) (Philips Medical Systems, Seattle, WA, United States) was used to record liver stiffness measurement and spleen stiffness measurement for each patient. Ten measurements were taken from the liver and ten measurements from the spleen. Liver elastography measurements were taken from the right lobe of the liver 2.4 cm (± 1 cm) from the liver capsule. Spleen elastography measurements were taken from the middle aspect of the spleen with homogeneous elasticity with the exclusion of big vessels. The median stiffness and IQR values were recorded. Spleen area and diameter were calculated from 2D images obtained.

Clinical and biological parameters including body mass index (BMI), skin to liver capsule distance, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), platelet count, prothrombin time, albumin, bilirubin and international normalized ratio (INR) were obtained for all patients at time of recruitment. APRI score was calculated as: AST (IU/L)/PLT ($\times 10^9$/L)[15]. Parameters determining presence of PH such as HVPG measurements or EGD findings were recorded. Cirrhosis was defined either by histological findings at biopsy or if decompensation had occurred.

### *Spleen area and spleen diameter measurements*

Spleen area and diameter measurements were calculated using maximum spleen diameter and borders that included the splenic hilum in the transverse plane with the

area (Figure 1). Measurements were repeated 4 mo later by two authors independently using a random sample of 19 study patients to calculate inter-operator variabilities.

### Statistical analyses

Descriptive statistics were carried out to compare groups 1 and 2. Ultrasound measurements, BMI and laboratory results were analysed by univariate and multivariate analyses. Correlations between variables were examined using Pearson's correlation coefficient and *P* values determined using ANOVA. A multivariate logistic regression model was built using a stepwise selection to determine the association of spleen area and platelet count and spleen stiffness and platelet count with the presence of CSPH. It was ensured that the data fulfilled all necessary criteria prior to application of the logistic regression analysis. Diagnostic accuracy was assessed using receiver operating characteristic (ROC) curves. Youden's index was used to determine the cut-off values for each parameter. *P* values for ROC curves were identified based on Wilcoxon's test. As subjects were random patients and operators were fixed, a one-way random interclass correlation coefficient (ICC) model on absolute agreement to determine inter-operator variability for spleen area and diameter was carried out. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 24.0.

### Ethics

This study was performed in accordance with the 1975 Declaration of Helsinki. Written and informed consent was obtained from all patients.

## RESULTS

Fifty four of 155 patients recruited had an EGD/HVPG measurement taken within one year of ElastPQ measurements. Four patients were excluded as summarized in Figure 2. A total of 50 patients (mean age 57.86, 62.0% male) were included in final analysis: 25 with evidence of CSPH (group 1) (mean age 60.44, 60.0% male) and 25 with no evidence of CSPH (group 2) (mean age 55.28, 64.0% male). The median time difference between ultrasound elastography measurements and EGD/HVPG was 4 mo.

Baseline clinical and biochemical characteristics for all patients included in statistical analysis are summarized in Table 1. Patients with diagnosis of cirrhosis were found in both groups *n* = 25 in group 1; *n* = 11 in group 2) with majority classified as Child-Pugh A (*n* = 18, 36.0%). The most common primary etiology in group 1 was alcoholic liver disease *n* = 7, 28.0%), while non-alcoholic fatty liver disease (*n* = 8, 32.0%) was more common in group 2.

### Univariate analysis

We hypothesized that clinical parameters associated with CLD may predict the presence of CSPH. The clinical parameters tested were BMI, ALT, AST, GGT, ALP, bilirubin, platelet count, albumin, prothrombin time, APRI score, liver stiffness, spleen stiffness, spleen area and spleen diameter. The univariate analysis showed that bilirubin, platelet count, albumin, prothrombin time, APRI score, liver stiffness, spleen area and diameter correlated with the presence of CSPH (Table 2). The best individual predictor of CSPH was platelet count (AUROC 0.846, *P* value < 0.001), followed by spleen area (AUROC 0.828, *P* value = 0.002) and APRI score (AUROC 0.827, *P* value < 0.001). No statistically significant discrimination was found between liver stiffness measured by the ElastPQ and CSPH (AUROC 0.657, *P* value = 0.061).

### Multivariate analysis

A multiple logistic regression model showed that two combinations independently predict CSPH (Table 3). The combination with the greatest diagnostic accuracy revealed that patients with a combination of spleen area > 57.9 $cm^2$ and platelet count < 126 × $10^9$ produced an estimated area under receiver operating characteristic (AUROC) curve of 0.876 (*P* value < 0.001), with sensitivity of 63.2%, specificity of 100%, positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 61.1%. An alternative combination of spleen stiffness > 29.99 kPa and platelet count < 126 × $10^9$ displayed a similar diagnostic accuracy with an estimated AUROC of 0.855 (*P* value < 0.001) and sensitivity of 88%, specificity of 75%, PPV of 78.6% and NPV of 85.7%. AUROC curves are displayed in Figure 3.

**Table 1 Baseline characteristics of the patient population**

| Parameter | Mean (standard deviation) or *n* | | |
|---|---|---|---|
| | Groups 1 and 2 (*n* = 50) | Group 1 (*n* = 25) | Group 2 (*n* = 25) |
| Age | 57.86 (12.22) | 60.44 (9.89) | 55.28 (13.90) |
| Sex (M:F) | 31:19 | 15:10 | 16:9 |
| BMI | 29.42 (7.61) | 28.59 (5.76) | 30.31 (9.26) |
| Skin to liver capsule Distance (cm) | 2.43 (0.99) | 2.46 (1.01) | 2.40 (0.99) |
| METAVIR score | 3.13 (1.276) | 3.74 (0.752) | 2.52 (1.410) |
| ALD | 12 | 7 | 5 |
| NAFLD | 12 | 4 | 8 |
| ALD and NAFLD | 4 | 3 | 1 |
| HCV | 2 | 2 | 0 |
| AIH | 3 | 0 | 3 |
| Miscellaneous | 17 | 9 | 9 |
| Total bilirubin (μmol/L) | 28.00 (44.728) | 39.92 (60.139) | 16.08 (13.108) |
| ALP (IU/L) | 127.33 (66.902) | 144.56 (75.900) | 109.38 (51.679) |
| GGT (IU/L) | 147.68 (192.683) | 160.96 (166.671) | 134.96 (217.570) |
| ALT (IU/L) | 60.84 (12.219) | 48.52 (29.427) | 73.16 (83.490) |
| AST (IU/L) | 62.50 (49.043) | 64.88 (32.720) | 60.12 (61.872) |
| Albumin (g/L) | 34.10 (6.129) | 30.88 (5.761) | 37.32 (4.679) |
| Platelet count ($\times 10^9$) | 151.42 (73.016) | 112.16 (60.023) | 190.68 (63.804) |
| Prothrombin time (sec) | 12.572 (1.9886) | 13.470 (2.2077) | 11.540 (1.0007) |
| INR | 1.184 (0.1742) | 1.260 (0.1871) | 1.108 (0.1222) |
| EGD (PH present) | 25 | 25 | 0 |
| HVPG (PH present) | 5 | 5 | 0 |

Group 1: Population with evidence of portal hypertension; Group 2: Population with no evidence of portal hypertension. BMI: Body mass index; ALD: Aldosterone; NAFLD: Non-alcoholic fatty liver disease; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio; EGD: Esophago-gastroduodenoscopy; HVPG: Hepatic venous pressure gradient; PH: Portal hypertension.

### *Inter-observer variability for spleen area and spleen diameter measurements*

An estimated single measures one-way random ICC for inter-operator variability for splenic area generated a value of 0.98 (95%CI: 0.94-0.99, *P* value < 0.001). Similarly, splenic diameter generated a value of 0.96 (95%CI: 0.91-0.99, *P* value < 0.001). Table 4 outlines the inter-operator ICC values for spleen area and diameter.

## DISCUSSION

The present study aimed to assess the performance of non-invasive splenic parameters using a new generation p-SWE machine–the ElastPQ–in identifying the presence of CSPH. We demonstrated that spleen stiffness (AUROC 0.712), spleen area (AUROC 0.828) and splenic diameter (AUROC 0.804) may predict the presence of CSPH in patients with mixed underlying etiologies. Adding platelet count to either spleen area (AUROC 0.875) or spleen stiffness (AUROC 0.855) increased diagnostic accuracy. Splenic area and diameter showed little inter-operator variability.

Our findings that spleen stiffness measured by p-SWE has a good diagnostic accuracy (cut-off > 29.99, AUROC 0.712) in identifying patients with CSPH is supported by other studies in the literature[16-18]. However, these studies report varying diagnostic threshold values and performance (AUROC 0.970-0.688)[16-18]. Differences

**Table 2 Univariate analysis showing association of clinical parameters with clinically significant portal hypertension**

| Variable | Odds ratio | Cut-off | *P* value | Estimated AUROC curve | *P* value of AUROC |
|---|---|---|---|---|---|
| BMI | 0.969 | > 26.42 | 0.425 | 0.506 | 0.951 |
| ALT (IU/L) | 0.993 | > 94.00 | 0.147 | 0.514 | 0.869 |
| AST (IU/L) | 1.002 | > 64.00 | 0.728 | 0.652 | 0.065 |
| GGT (IU/L) | 1.001 | > 85.00 | 0.639 | 0.643 | 0.094 |
| ALP (IU/L) | 1.001 | > 96.00 | 0.050 | 0.678 | 0.033 |
| Bilirubin | 1.037 | > 11.00 | 0.013[a] | 0.722 | 0.007[b] |
| Platelet count ($\times 10^9$) | 0.979 | < 126.00 | < 0.001[c] | 0.846 | < 0.001 |
| Albumin (g/L) | 0.786 | < 33.00 | < 0.001[c] | 0.820 | < 0.001[c] |
| Prothrombin time (sec) | 2.721 | > 12.20 | < 0.001[c] | 0.800 | < 0.001[c] |
| APRI score | 2.030 | > 0.81 | 0.008[b] | 0.827 | < 0.001[c] |
| ElastPQ median liver stiffness (kPa) | 1.048 | > 10.16 | 0.021[a] | 0.657 | 0.061 |
| ElastPQ median spleen stiffness (kPa) | 1.007 | > 29.99 | 0.368 | 0.712 | 0.010[b] |
| ElastPQ spleen area ($cm^2$) | 1.075 | > 57.90 | < 0.001[c] | 0.828 | 0.002[c] |
| ElastPQ spleen diameter (cm) | 1.651 | > 13.90 | 0.002[c] | 0.804 | 0.007[b] |

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.005$. AUROC: Area under the receiver operating characteristic; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transpeptidase; ALP: Alkaline phosphatase; APRI: AST-to-platelet-ratio-index; ElastPQ: Elastography point quantification.

**Table 3 Diagnostic performance of combination variables as predictors of clinically significant portal hypertension**

| Variables | AUROC curve | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) |
|---|---|---|---|---|---|
| ElastPQ median spleen area (> 57.90 $cm^2$) and Platelet count (< 126 × $10^9$) | 0.875 | 63.2 | 100.0 | 100.0 | 61.1 |
| ElastPQ median spleen stiffness (> 29.99 kPa) and platelet count (< 126 × $10^9$) | 0.855 | 88.0 | 75.00 | 78.6 | 85.7 |

AUROC: Area under the receiver operating characteristic; ElastPQ: Elastography point quantification.

**Table 4 One-way random intraclass coefficient values for inter-operator variability**

| | Spleen area | Spleen diameter |
|---|---|---|
| Number of operators | 2 | 2 |
| Number of measurements | 19 | 19 |
| Mean | 0.98 | 0.96 |
| 95% confidence interval | 0.94-0.99 | 0.91-0.99 |

between studies may be explained by varying methodologies employed, as well as use of different p-SWE techniques. Nevertheless a recent study, which adopted a similar methodology to our own, identified a cut off of < 31 kPa to rule out the presence of EV of any grade which resonates with our findings[19].

Interestingly, our study did not identify liver stiffness as a predictor of CSPH, which differs from findings of recent studies[16,18,20] and suggestions made by the Baveno VI Guidelines[3]. But, contrasting findings are not uncommon, as differences between

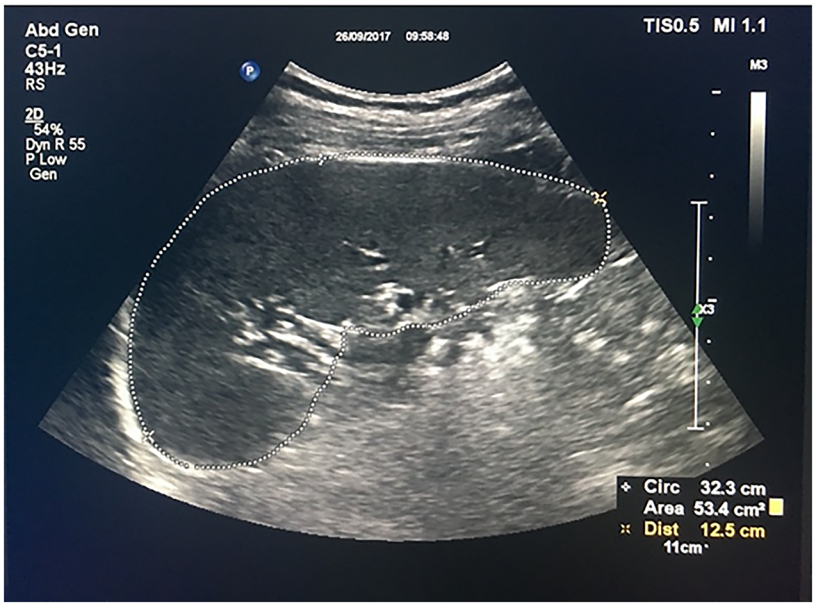


**Figure 1 Spleen area and spleen diameter measurements using the elastography point quantification.**

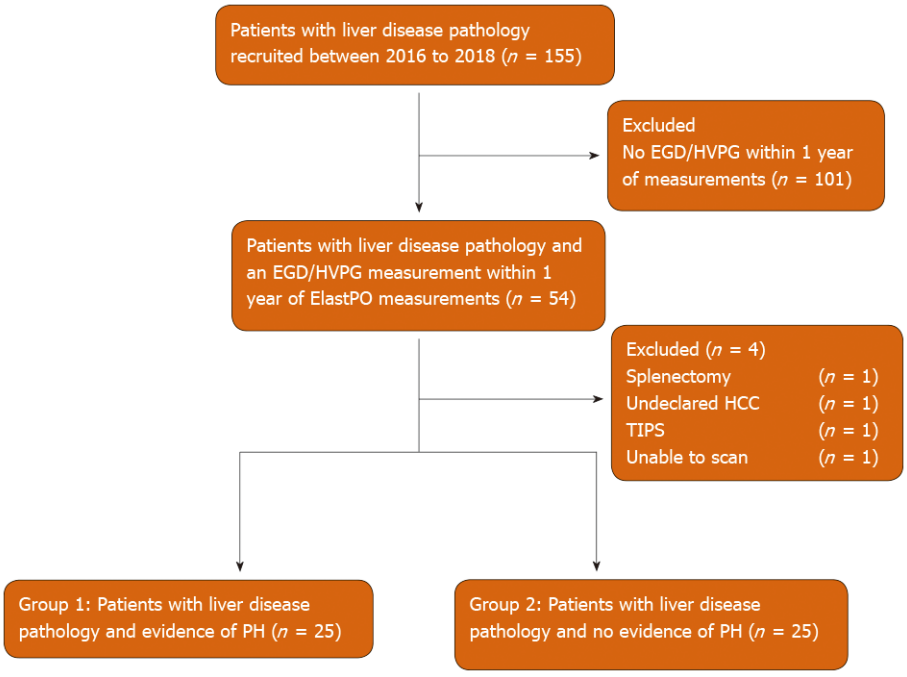


**Figure 2 Flow chart displaying the selection of patients included in the final statistical analysis.** EGD: Esophago-gastroduodenoscopy; HVPG: Hepatic venous pressure gradient; ElastPQ: Elastography point quantification; HCC: Hepatocellular carcinoma; TIPS: Transjugular intrahepatic portosystemic shunt; PH: Portal hypertension.

studies within the literature are also seen. A possible explanation for this may lie in the heterogeneity of populations in our study and between studies in the literature. Furthermore, studies comparing the ElastPQ technique to VTQ have shown significantly lower liver stiffness values, which may provide an added explanation for discrepancies seen[21]. Given the novelty of the ElastPQ, research focus has remained on its ability to detect fibrosis in comparison to other elastography techniques such as TE and VTQ[21-23]. As a result, there is limited data on the ability of ElastPQ to predict the presence of CSPH.

Splenic area and diameter demonstrated a modest ability to diagnose the presence of CSPH. Previous studies have explored spleen size by consideration of splenic diameter[22], which has shown to have acceptable reproducibility in the context of

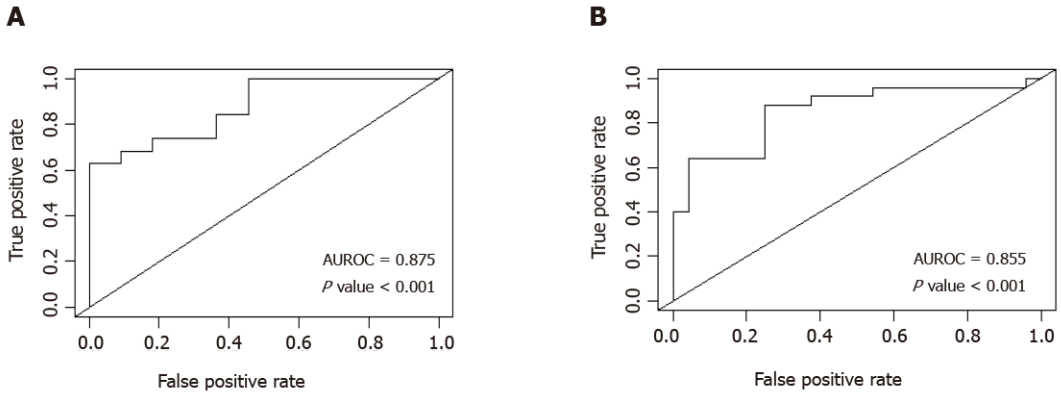


**Figure 3 Comparative area under the receiver operating characteristic curve analyses of combination variables in predicting the presence or absence of clinically significant portal hypertension.** A: Spleen area (> 57.90 kPa) and platelet count (< 126.0 × $10^9$); B: Spleen stiffness (> 29.99 kPa) and platelet count (< 126.0 × $10^9$). AUROC: Area under the receiver operating characteristic.

platelet count/spleen diameter ratio. However, to our knowledge, there has only been one other study which has considered spleen area as a potential non-invasive diagnostic parameter. In this study, Giuffrè *et al*[19] reported similar findings with a median splenic area of 59.2 $cm^2$ and diameter of 13.1 cm in its cohort of 210 patients[19]. Given the excellent reproducibility seen in our study and confirmation of similar findings in one other study, spleen area may be a useful adjunct in predicting CSPH. Further research with an external cohort is needed to validate our findings.

Perhaps one of the most striking results from our study is the diagnostic accuracy of the APRI score. A cut-off value of > 0.81 generated an AUROC of 0.827 in predicting the presence of CSPH. This was comparable to diagnostic performance of spleen stiffness and spleen area. Nevertheless, these findings differ to those in the literature, which has described lower sensitivities in higher cut-off values[24-26]. Only one study by Salzl *et al*[16] demonstrated a similar diagnostic performance (AUROC 0.805), but the cut-off value (1.90) remained higher than seen in our cohort[16]. Differences between studies may be reflected by the smaller sample size and varied etiology within our study population. Of note, the study by Giuffrè *et al*[19], which had a similar study population, demonstrated APRI to be a statistically significant determinant of CSPH with a similar median of 0.70[19]. Although highly applicable due to its non-invasive nature, the APRI score is affected by inflammatory processes such as acute hepatitis, which can generate false positive results that are not seen with p-SWE[4]. We propose that the APRI score may be a useful tool in the follow-up of CLD patients in primary care while p-SWE may fare better in secondary practice.

Since the introduction of liver stiffness measurements by TE, combinations of liver stiffness with spleen size have been carried out. The most common of these is the LSPS score[27-29]. However, despite spleen stiffness being increasingly recognized as a better predictor of CSPH[13], few studies have been carried out combining spleen stiffness measurements to other markers of CLD. Our study showed that the combination of spleen stiffness measurements and platelet counts has a high diagnostic index (AUROC 0.855). Although an exact model has not been replicated, a similar model applying spleen stiffness measurements measured by TE and the Baveno guidelines VI has shown promising results[30]. Colecchia *et al*[30] utilised a combined model where a cut-off of ≤ 46 kPa for spleen stiffness and < 20 kPa for liver stiffness measurements by TE, and a platelet count > 150000/$mm^3$ could effectively rule out CSPH in cACLD patients[30]. A different study by Bota *et al*[31] used a different combination index of liver stiffness and spleen stiffness measured by VTQ, and presence of ascites which generated an AUROC of 0.721[31]. Finally, Giuffrè *et al*[19] was perhaps the most comparable of all the studies mentioned as his team used the ElastPQ model to develop the spleen stiffness probability index[19]. All of the findings above support the premise that a combination of non-invasive parameters may be a better diagnostic indicator than a single parameter alone. No study in the literature has considered addition of spleen area to combination variables. Further studies are needed to validate our proposed spleen area and platelet count combination and determine which set of non-invasive parameters generate the best accuracy.

### Strengths and limitations

Both the use of a novel p-SWE machine (ElastPQ) and investigation of spleen area describe a unique approach in our study compared to others carried out in the field. To our knowledge, this is one of the first studies to assess the role of spleen stiffness, spleen area and splenic diameter measurements in predicting CSPH using the ElastPQ. As a result, our study took into consideration inter-operator variability of splenic area and diameter, which supported its potential use in clinical practice. The prospective recruitment of patients with mixed etiologies described a population representative in clinical practice, but in view of the novelty of p-SWE it would be worth determining the effect of specific etiologies on splenic measurements.

This study has some limitations, the most pertinent of which being that we only assessed for presence or absence of PH, rather than degree of PH. Furthermore, an interval gap of one year between spleen stiffness measurements and EGD/HVPG readings may represent a consistent bias within our study due to the considerable length of time between readings. However, it could be argued that the correlation between CSPH and spleen stiffness may be better if there were a shorter time interval proposed. We did not exclude patients taking pharmacological treatment for PH from the original protocol as it was suspected that non-selective beta blockers and banding of varices would be unlikely to affect splenic measurements[32]. However, the most recent data on cirrhotic patients with high risk varices suggests that taking non-selective beta blockers can affect splenic stiffness[33,34]. Nevertheless these studies were undertaken using Fibroscan® and VTQ (Siemens Acuson S2000TM) ultrasound systems and so, further information is still needed in order to confirm that similar findings are present with the ElastPQ.

Although IQR measurements were taken, the validity of spleen stiffness and liver stiffness could not be determined as quality criteria has not yet been established for this technique[4]. However, Pawluś *et al*[35] conducted a small study in which he measured the spleen stiffness of 59 healthy volunteers using p-SWE, which has provided a reference point of 16.6 ± 2.5 kPa as the normal range with good reproducibility of measurement results[35]. Given the similar methodologies, this has supported our findings despite the small sample size in this study. Ultimately, further studies are needed to validate our findings, but our multivariate models suggest that findings are likely to correlate with CSPH in larger cohorts.

### Clinical implications

p-SWE is a non-invasive, rapid tool that carries minimal complications. It is painless, better tolerated than current gold-standard techniques and is more applicable than TE. Furthermore, this technique can be implemented on regular ultrasound machines and performed during routine screening for HCC in cirrhosis, which is likely to be cost-effective and less time-consuming.

## CONCLUSION

Combinations of spleen area and platelet count, or spleen stiffness and platelet count as measured by the ElastPQ may be safe and effective methods to diagnose CSPH. Currently, this non-invasive technique cannot replace gold-standard as further studies are needed to create validation criteria and assess the diagnostic accuracy of non-invasive parameters in patients with differing degrees of PH.

## ARTICLE HIGHLIGHTS

### Research background

Portal hypertension is a major complication of cirrhosis with a significant morbidity and mortality associated with it. Many of those with advanced chronic liver disease have esophageal varices and so, many patients undergo the gold-standard invasive procedures of performing an esophago-gastroduodenoscopy (EGD) or having the hepatic venous pressure gradient measurement taken through interventional radiology. However, both of these methods are invasive and carry a risk of complications.

### Research motivation

Current guidelines propose that non-invasive methods can predict the incidence of clinically significant portal hypertension (CSPH). The latest guidelines suggest cirrhosis patients with a liver stiffness measurement < 20 kPa and a platelet count > 150000/μL can avoid screening endoscopy. Nevertheless, new algorithms are still required, as up to 40% of EGDs continue unnecessarily.

### Research objectives

The aim of this study was to assess whether spleen stiffness measurement, spleen area and spleen diameter can independently predict CSPH, or in combination with other biochemical or elastography parameters. We also aimed to assess reproducibility of splenic area and diameter measurements.

### Research methods

This was a single-centre prospective cohort study where a total of 50 patients were split into two groups and included in a retrospective analysis: 25 with evidence of CSPH (group 1) and 25 with no evidence of CSPH (group 2). The Philips EPIQ7 [elastography point quantification (ElastPQ)] (Philips Medical Systems, Seattle, United States) was used to record liver stiffness, spleen stiffness, spleen area and spleen diameter measurements for each patient. Univariate, multivariate and one-way random interclass correlation coefficient analyses were performed to assess the diagnostic accuracy of splenic parameters.

### Research results

Body mass index, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, bilirubin, platelet count, albumin, prothrombin time, aspartate aminotransferase-to-platelet-ratio-index (APRI) score, liver stiffness, spleen stiffness, spleen area and spleen diameter were assessed in their ability to predict the presence of CSPH. A univariate analysis showed the best individual predictor of CSPH was platelet count [area under the receiver operating characteristic (AUROC) 0.846, *P* value < 0.001], followed by spleen area (AUROC 0.828, *P* value = 0.002) and APRI score (AUROC 0.827, *P* value < 0.001). A multiple logistic regression model revealed that two combinations independently predict CSPH. The combination with the greatest diagnostic accuracy included a combination of spleen area > 57.9 $cm^2$ and platelet count < 126 × $10^9$ which had 63.2% sensitivity, 100% specificity, 100% positive predictive value (PPV), 61.1% negative predictive value (NPV) (AUROC 0.876, *P* value < 0.001). An alternative combination of spleen stiffness >29.99 kPa and platelet count < 126 × $10^9$ displayed a similar diagnostic accuracy with 88% sensitivity, 75% specificity, 78.6% PPV, 85.7% NPV (AUROC 0.855, *P* value < 0.001). Spleen area and spleen diameter demonstrated little inter-operator variability as measured by a one-way random interclass correlation coefficient (spleen area: 0.98, *P* value < 0.001; spleen diameter: 0.96, *P* value < 0.001).

### Research conclusions

Combinations of spleen area and platelet count, or spleen stiffness and platelet count as measured by the ElastPQ may be safe and effective methods to diagnose CSPH. At present this cannot replace the gold standard.

### Research perspectives

Performing large scale prospective studies with long-term follow-up and are needed to validate our findings.

## ACKNOWLEDGEMENTS

We recognise Hoogenboom T and Patel N for their help and advice.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1067-1075

DOI: 10.4254/wjh.v12.i11.1067

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Retrospective Study

# Outcome of gastric antral vascular ectasia and related anemia after orthotopic liver transplantation

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**Author contributions:** Emhmed Ali S and Dela Cruz AC provided the conception and designed the study; Emhmed Ali S, Benrajab K and Dela Cruz AC analyzed and interpreted the data and wrote the manuscript; all authors have read and approved the final manuscript.

**Institutional review board statement:** The study was reviewed and approved by the University of Kentucky Institutional Review Board (Approval No 54669).

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

**Conflict-of-interest statement:** Authors have no relevant relationships or conflict of interest to disclose.

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

**Open-Access:** This article is an

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## Abstract

*BACKGROUND*

Gastric antral vascular ectasia (GAVE) is a significant complication of cirrhosis. Numerous medical, surgical, and endoscopic treatment modalities have been proposed with varied satisfactory results. In a few small studies, GAVE and associated anemia have resolved after orthotopic liver transplantation (OLT).

*AIM*

To assess the impact of OLT on the resolution of GAVE and related anemia.

*METHODS*

We retrospectively reviewed clinical records of adult patients with GAVE who underwent OLT between September 2012 and September 2019. Demographics and other relevant clinical findings were collected, including hemoglobin levels and upper endoscopy findings before and after OLT. The primary outcome was the resolution of GAVE and its related anemia after OLT.

*RESULTS*

Sixteen patients were identified. Mean pre-OLT Hgb was 7.7 g/dL and mean 12 mo post-OLT Hgb was 11.9 g/dL, ($P$ = 0.001). Anemia improved (defined as Hgb increased by 2g) in 87.5% of patients within 6 to 12 mo after OLT and resolved completely in half of the patients. Post-OLT esophagogastroduodenoscopy was performed in 10 patients, and GAVE was found to have resolved entirely in 6 of those patients (60%).

*CONCLUSION*

**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

**Received:** July 27, 2020
**Peer-review started:** July 27, 2020
**First decision:** September 21, 2020
**Revised:** October 4, 2020
**Accepted:** October 26, 2020
**Article in press:** October 26, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Coelho J, Manrai M
**S-Editor:** Zhang H
**L-Editor:** A
**P-Editor:** Xing YX

Although GAVE and associated anemia completely resolved in the majority of our patients after OLT, GAVE persisted in a few patients after transplant. Further studies in a large group of patients are necessary to understand the causality of disease and to better understand the factors associated with the persistence of GAVE post-transplant.

**Key Words:** Liver cirrhosis; Iron deficiency anemia; End-stage liver disease; Gastroscopy; Gastrointestinal hemorrhage; Liver transplantation



**Core Tip:** In this retrospective study, cirrhotic patients who had gastric antral vascular ectasia (GAVE) with anemia and underwent orthotopic liver transplant (OLT) between September 2012 and September 2019 were reviewed to evaluate the impact of OLT on resolution of GAVE and associated anemia. A total of 296 patients underwent OLT during the study period; sixteen patients had GAVE. Anemia improved in the majority of patients in 6 to 12 mo post-OLT, and of the 10 patients who had a post-OLT esophagogastroduodenoscopy, GAVE was found to have completely resolved in 6 of those patients. We concluded that GAVE and associated anemia completely resolved in the majority of our patients post-OLT.

**Citation:** Emhmed Ali S, Benrajab KM, Dela Cruz AC. Outcome of gastric antral vascular ectasia and related anemia after orthotopic liver transplantation. *World J Hepatol* 2020; 12(11): 1067-1075
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1067.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1067

## INTRODUCTION

Gastric antral vascular ectasia (GAVE) is frequently found in patients with cirrhosis. The estimated prevalence in cirrhotic patients with upper gastrointestinal (GI) bleeding is around 6%, and in cirrhotic patients without apparent upper GI bleeding is about 12%[1]. Another study found that GAVE was present in 1 in 40 of cirrhotic patients who underwent screening esophagogastroduodenoscopy (EGD) during liver transplant evaluation[2]. The clinical presentation extends from occult hemorrhage to transfusion-dependent chronic iron deficiency anemia.

Diagnosed endoscopically, GAVE lesions are located primarily in the antrum of the stomach[3] and appear as red spots that are either organized in stripes that project radially from the pylorus ("watermelon stomach")[4,5], or organized in a diffuse punctate ("honeycomb") pattern[6]. The exact etiology of GAVE remains unclear, although several hypotheses have been proposed, including mechanical stress, hemodynamic alterations, and humoral and autoimmune factors[7-9]. Numerous medical, surgical, and endoscopic treatment modalities such as endoscopic band ligation, endoscopic thermal therapy with argon plasma coagulation (APC), and radiofrequency ablation have been proposed with varying satisfactory results[10-15]. In a few small studies, GAVE and associated anemia have resolved after orthotopic liver transplantation (OLT)[2,16,17].

The aim of this study was to further investigate the influence of OLT on the resolution of GAVE and associated anemia.

## MATERIALS AND METHODS

A retrospective chart review was conducted of all adult patients with diagnosis of cirrhosis and GAVE who underwent OLT at the University of Kentucky Medical Center (UKMC) between September 2012 and September 2019. Patient demographics; body mass index (BMI); co-morbidities; Model of End-stage Liver Disease-sodium (MELD-Na) score at the time of GAVE diagnosis; etiology of cirrhosis; use of proton

pump inhibitors (PPI); GAVE presentation; type of endoscopic treatment utilized; lowest hemoglobin (Hgb) pre-OLT; hemoglobin at 3 mo, 6 mo, 9 mo, and 12 mo post-OLT; and post-OLT EGD findings were collected.

All the data mentioned above were collected from the patient's electronic medical records and stored in a password-protected excel sheet for analysis. The diagnosis of GAVE was obtained from the endoscopy reports. A single endoscopist/hepatologist independently reviewed all endoscopic images to confirm report findings. The primary outcome was the resolution of GAVE and associated anemia post-OLT.

This study was approved by the UKMC Institutional Review Board. No organs from executed prisoners were used.

## RESULTS

Of 296 patients who underwent OLT during the study period, 16 (5.4%) had diagnosis of GAVE.

The individual patient cases are summarized in Table 1. All Caucasians, with the majority being female (62.5%) with a mean age of 56.5 years and mean BMI of 31.6. The average MELD-Na score at the time of GAVE diagnosis was 20.4. The most common etiology of cirrhosis was Non-alcoholic steatohepatitis (NASH, 44%).

Twelve patients (75%) had concomitant portal hypertensive gastropathy, and 13 (81%) had chronic kidney disease (defined as a GFR < 60 mL/min/1.73 $m^2$ for > 3 mo).

Thirteen patients (81%) were on a PPI. All patients presented with anemia, with 50% have at least one episode of overt bleeding requiring a blood transfusion prior to transplant. Five of those without overt bleed had iron deficiency anemia. Half of the patients were treated endoscopically, with APC being the most common treatment (31%), and 25 % of patients underwent banding.

The pattern of GAVE varied in our patient cohort: Six patients had diffuse punctate GAVE (Figure 1), five patients had linear/striped pattern (Figure 2), two had patchy mild punctate GAVE, two patients had nodular polypoid GAVE characterized by polypoid lesions with exudate (Figure 3), and one had nodular striped GAVE (Figure 4). The patterns evolved in two patients, with the initial EGD showing a linear pattern and subsequent EGD showing a nodular/polypoid GAVE.

Mean pre-OLT Hgb was 7.7 g/dL compared to 11.9 g/dL at 12 mo post-OLT ($P$ = 0.001). Anemia improved in 87.5 % (defined as Hgb increase by 2g) of patients ($n$ = 14) & resolved entirely in 50 % of patients within 6 to 12 mo after OLT.

Only two patients required blood transfusion after three months post-transplant. One patient was five months, and the other patient was three years post-OLT; however, neither had evidence of bleeding.

Post-OLT EGD was performed in 10 patients. The mean time between OLT and post-OLT EGD was 27.9 mo. Among the 10 patients who underwent post-OLT EGD, GAVE was found to have resolved entirely in six patients (60%).

It is unclear why GAVE persisted in four out of ten patients who underwent post-OLT EGD. The first patient had psoriatic arthropathy. The second patient had GAVE sixteen years after transplant and had underlying polycythemia vera and chronic splenic thrombosis post-transplant with varices. The third patient had recurrent biopsy-proven cirrhosis. The fourth patient had persistent nodular GAVE, which required two endoscopic band ligations at two and ten weeks post-OLT with an endoscopic resolution of GAVE and associated anemia at six months post-OLT. The pattern of post-transplant GAVE varied in these four patients. Two patients had polypoid GAVE, one patient had linear GAVE, which later evolved into polypoid GAVE, and one patient had patchy mild punctate GAVE.

## DISCUSSION

Gastric antral vascular ectasia is a common finding in patients with cirrhosis and often presents as chronic anemia. The endoscopic pathognomonic feature of GAVE is columns of red tortuous ectatic vessels along the folds of the antrum[18]. Histologically, GAVE is characterized by mucosal vascular ectasia, spindle cell proliferation, fibrohyalinosis, and fibrin thrombi[19]. It is a disease with focal distribution[20]. Therefore, a negative biopsy does not rule out GAVE[20].

Although GAVE has been historically described as a "watermelon stomach," the diffuse pattern of GAVE endoscopically was found to be more frequent in cirrhotic patients in one study[5]. In contrast, the classic watermelon form was found to be more

**Table 1 Clinical characteristics of patients with gastric antral vascular ectasia who underwent orthotopic liver transplantation**

| Patient No. | Age | Gender | BMI | Etiology of liver disease | MELD-Na score at GAVE diagnosis | Pre-OLT Hgb (g/dL) | GAVE pattern | Endoscopic therapy | Resolution of GAVE post-OLT |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 66 | F | 33.7 | NASH | 26 | 8 | Diffuse punctate GAVE | APC | Yes |
| 2 | 55 | F | 28 | cryptogenic | 15 | 8.7 | Pre-OLT: Striped GAVEPost-OLT: Nodular polypoid GAVE with exudate | None | No |
| 3 | 50 | M | 42.7 | NASH | 32 | 7 | Linear/striped GAVE | APC | Yes |
| 4 | 57 | F | 32.9 | cryptogenic | 13 | 7.2 | Striped and nodular GAVE | Banding | Yes |
| 5 | 59 | F | 35.7 | NASH | 14 | 6.7 | Pre and post-OLT: Nodular polypoid GAVE with exudate | Banding | No |
| 6 | 51 | M | 23 | Alcohol | 30 | 7.1 | Diffuse punctate GAVE | APC | Yes |
| 7 | 51 | M | 27 | NASH | 23 | 7.2 | Diffuse punctate GAVE | APC | Yes |
| 8 | 57 | F | 22 | Alcohol | 23 | 7.5 | Diffuse punctate GAVE | None | Yes |
| 9 | 59 | F | 32.6 | NASH | 31 | 7.7 | Diffuse punctate GAVE | Banding | Yes |
| 10 | 40 | M | 36.6 | Alcohol, HCV | Post-OLT diagnosis | 6.9 | Post-OLT: Linear striped; then polypoid GAVE | None | No |
| 11 | 55 | M | 30.8 | PSC | 13 | 8.8 | Nodular polypoid GAVE with exudate | None | Yes |
| 12 | 58 | M | 17.8 | Alcohol | 12 | 7.3 | Diffuse punctate GAVE | None | Yes |
| 13 | 62 | F | 47.6 | HCV | 30 | 7.6 | Linear/striped GAVE | None | Yes |
| 14 | 57 | F | 25.5 | Budd Chiari syndrome | Post-OLT diagnosis | | Post-OLT: Patchy mild punctate GAVE | None | No |
| 15 | 67 | F | 35.5 | NASH | 15 | 9.6 | Patchy mild punctate GAVE | None | Yes |
| 16 | 60 | F | 32.5 | NASH | 8 | 7.5 | Linear/striped GAVE | APC | Yes |

APC: Argon Plasma Coagulation; BMI: Body mass index; F: Female; GAVE: Gastric antral vascular ectasia; HCV: Hepatitis C virus; Hgb: Hemoglobin; M: Male; MELD-Na: Model for End-Stage Liver Disease- Sodium level; NASH: Non-alcoholic Steatohepatitis; OLT: Orthotopic liver transplantation; PSC: Primary sclerosing cholangitis.

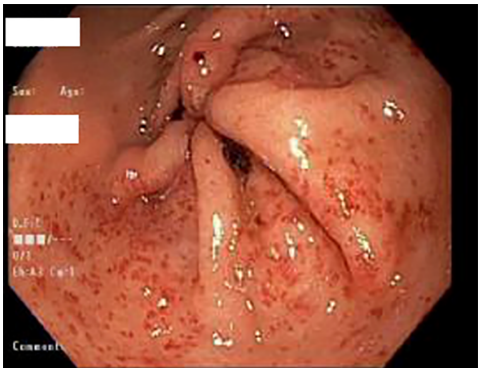

**Figure 1 Endoscopic image showing a diffuse punctate pattern of gastric antral vascular ectasia.**

common in non-cirrhotic patients[21]. In our cohort, 50% of patients were found to have diffuse punctate GAVE, 31% had a linear/striped pattern, and 19% with nodular type. The gender distribution in our study seems to be similar to other studies. In our study, the cirrhotic GAVE patients were predominately female (62.5%) with a mean age of 56.2 years. Other studies have shown a female predominance (71%) of GAVE in cirrhotic patients with a mean age of 73 years, and a male predominance (75%) in non-

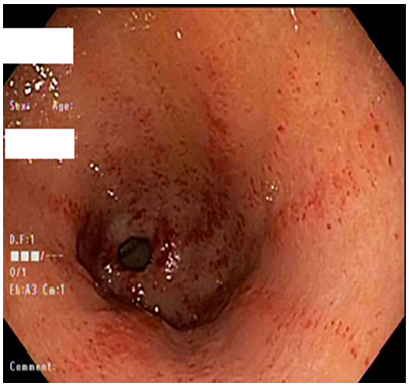

**Figure 2 Endoscopic image showing a linear and striped pattern of gastric antral vascular ectasia.**

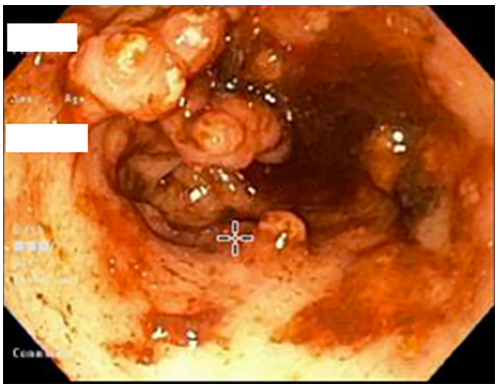

**Figure 3 Endoscopic image showing a nodular polypoid pattern of gastric antral vascular ectasia.**

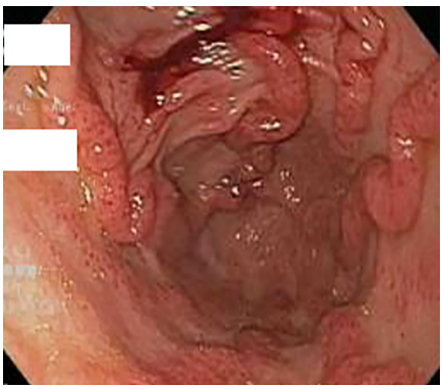

**Figure 4 Endoscopic image showing a nodular and striped pattern of gastric antral vascular ectasia.**

cirrhotic GAVE patients, with a mean age of 65 years[22,23].

The exact pathophysiological mechanism that leads to GAVE remains unclear. It is highly essential to differentiate GAVE from portal hypertensive gastropathy (PHG) because they are treated differently. PHG mainly causes mucosal changes in the fundus and corpus[20], whereas GAVE is limited to antrum[18]. It is possible that portal hypertension has no significant role in the development of GAVE for several reasons, such as absence of correlation between the severity of portal hypertension and degree of vascular ectasia[6], and non-resolution of GAVE after reduction in portal pressure[23,24]. Quintero *et al*[6] linked low levels of pepsinogen, high levels of gastrin, and achlorhydria, with GAVE, and other studies have reported improvement in GAVE and reduction in gastrin levels after cessation of proton pump inhibitor[25]. In our study, 13 patients (81%) were taking proton pump inhibitors before and after OLT. Prostaglandin E2 has a gastric vasodilator effect and has been considered to play a role in the development of GAVE[8]. Another study by Lowes *et al*[7] found a proliferation of

neuroendocrine cells that secretes 5-hydroxytryptamine and vasoactive peptide close to the ectatic blood vessels. Mechanical stress theory has been suggested by Charneua *et al*[9] to be playing a role in cirrhotic GAVE patients after they found abnormal motility patterns on antral motility studies. In non-cirrhotic GAVE patients, many other medical conditions have been described, such as autoimmune disease, acute myeloid leukemia, hypertension, chronic renal failure, ischemic heart disease, bone marrow transplantation, scleroderma, and familial Mediterranean fever[22,26-28]. The most frequent associated autoimmune conditions are connective tissue diseases followed by Raynaud's phenomenon and sclerodactyly[22].

Bleeding GAVE can be very problematic and challenging to manage. Several endoscopic interventions and medications are available[10-15]. In three previous small studies, GAVE and associated anemia resolved after liver transplantation[2,16,17]. Reduction in portal hypertension *via* medical use of beta-blockers or transjugular intrahepatic portosystemic shunt (TIPS) placement has failed to accomplish resolution of GAVE or associated anemia in cirrhotic patients with portal hypertension[23,24]. None of our patients had TIPS before the diagnosis of GAVE. One patient had TIPS a month just before the transplant, and TIPS was done eighteen months after diagnosis of GAVE for refractory ascites. Before the transplant, eight of our patients underwent endoscopic treatment with APC as the most commonly used method. None of our patients received medical treatment, such as Tranexamic acid.

Our study further supports that GAVE in cirrhotic patients may resolve after OLT. Six out of ten patients who underwent post-OLT EGD have complete resolution of GAVE. The potential explanations for persistent GAVE among the other four patients include an underlying rheumatologic condition which can also cause non-cirrhotic GAVE in the first patient, underlying chronic splenic vein thrombosis in the second patient, recurrent cirrhosis in the third patient, and delayed resolution of GAVE after transplant since GAVE was seen fairly recently post-transplant, noted at two and ten weeks post-transplant with the resolution of anemia in the fourth patient.

The associated anemia entirely resolved in half of our patients and improved in 87.5% of patients six to twelve months after OLT. Anemia is common in cirrhotic patients; however, it is usually complex, and may be influenced by several factors, including etiology of cirrhosis and degree of portal hypertension[29,30]. Portal hypertension and hypersplenism can be contributing factors to anemia in cirrhosis; however, the magnitude of the effect is unclear[31,32]. Furthermore, Vincent *et al*[17] reported resolution of GAVE and anemia in 2 patients post OLT even with persistent evidence of portal hypertension.

One would speculate that the associated resolution of liver dysfunction would lead to the resolution of GAVE after transplant. GAVE was frequently found in patients with more advanced liver disease and has been reported to resolve after liver transplantation[2,16,17]. In our cohort at the time of GAVE diagnosis, the mean MELD-NA score was 20.4.

The limitations of the current study include retrospective design with a small sample size. EGDs were also performed by several different endoscopists performed EGDs; however, we attempted to control for this by having one endoscopist/hepatologist review all endoscopic images. Also, only 10 patients had undergone EGD post-OLT. The patients were not treated with the same endoscopic modality, and there was no established endoscopic protocol to eradicate GAVE. Indeed, there is usually no standard treatment modality since the type of endoscopic treatment will depend on the pattern of GAVE and previous endoscopic therapies, with the preferable use of banding for most patients with nodular GAVE and APC in all other patterns of GAVE (linear and punctate GAVE). Not all patients in our cohort had histologic confirmation of GAVE, and this could raise the possibility of overlap between GAVE and PHG. Despite these limitations, this study offers the largest number of patients with GAVE who underwent liver transplantation, and our findings corroborate those from previous studies showing that GAVE and associated anemia resolve in the majority of patients who underwent liver transplantation. It also further explored the possible underlying mechanisms for persistent GAVE after OLT in four out of ten patients who had post-OLT EGD.

## CONCLUSION

GAVE is a significant cause of morbidity and mortality in patients with cirrhosis. Although GAVE and associated anemia completely resolved in the majority of our patients after liver transplantation, GAVE persisted in a few patients post-OLT. Large,

prospective studies using consistent diagnostic criteria for GAVE and planned follow-up EGDs after OLT are needed to better understand the factors may contribute to persistence of GAVE post-transplant.

## ARTICLE HIGHLIGHTS

### *Research background*

Gastric antral vascular ectasia (GAVE) is a well-recognized cause of gastrointestinal bleeding in cirrhotic patients. The etiology of GAVE remains unclear, although several humoral, autoimmune, mechanical stress, and pharmacological (proton pump inhibitor use) hypotheses have been described. Different treatment modalities have been proposed with varying results. Orthotopic liver transplantation (OLT), which is often recommended for patients with significant complications due to end-stage liver disease, has also been shown to be beneficial treatment for resolution of GAVE and associated anemia in a few small studies.

### *Research motivation*

To evaluate the relation between GAVE and associated anemia and OLT.

### *Research objectives*

To assess the impact of OLT on resolution of the natural course of GAVE and associated anemia.

### *Research methods*

A retrospective chart review was conducted of adult patient with GAVE who underwent liver transplant between September 2012 and September 2019. Demographics and other relevant findings, including hemoglobin levels, Model of End-stage Liver Disease-sodium score, GAVE presentation, and upper endoscopy findings before and after OLT were collected.

### *Research results*

Sixteen patients were identified, all Caucasians and predominantly female (62.5%) with a mean age of 56.5 years. The most common etiology for cirrhosis was NASH (44%). All patients presented with anemia, with 50% presenting with overt bleed and required transfusions prior to transplant. Mean pre-OLT Hgb was 7.7, and the mean 12 mo post-OLT Hgb was 11.9 ($P$ = 0.001). Anemia improved in 87.5% of patients ($n$ = 14) within 6 to 12 mo after OLT and resolved completely in half of the patients. Post-OLT esophagogastroduodenoscopy was performed in 10 patients, and GAVE resolved entirely in 6 of those patients (60%).

### *Research conclusions*

Although GAVE and associated anemia completely resolved in the majority of our patients after liver transplantation, GAVE persisted in a few patients after transplant.

### *Research perspectives*

Large prospective studies are needed to better understand what factors may contribute to persistent GAVE post-liver transplant.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1076-1088

DOI: 10.4254/wjh.v12.i11.1076

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Clinical Trials Study

# Hepatitis B surface antigen and hepatitis B core-related antigen kinetics after adding pegylated-interferon to nucleos(t)ids analogues in hepatitis B e antigen-negative patients

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**Author contributions:** Broquetas T completed statistical analysis and drafting of the manuscript; Broquetas T and Carrión JA analyzed and interpreted the data; Micó M and Hernandez JJ analyzed samples; Carrión JA completed concept, design and supervision of the study; all authors performed the acquisition of data, critical revision of the manuscript.

**Supported by** Instituto de Salud Carlos III, Ministerio de Economía y Competitividad No. PI14/00540; Fondo Europeo de Desarrollo Regional; Unión Europea; Una manera de hacer Europa.

**Institutional review board statement:** The study protocol was

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## Abstract

*BACKGROUND*

Hepatitis B e antigen-negative chronic hepatitis B patients under nucleos(t)ids analogues (NAs) rarely achieve hepatitis B surface antigen (HBsAg) loss.

*AIM*

To evaluate if the addition of pegylated interferon (Peg-IFN) could decrease HBsAg and hepatitis B core-related antigen (HBcrAg) levels and increase HBsAg loss rate in patients under NAs therapy.

*METHODS*

Prospective, non-randomized, open-label trial evaluating the combination of Peg-IFN 180 μg/week plus NAs during forty-eight weeks *vs* NAs in monotherapy. Hepatitis B e antigen-negative non-cirrhotic chronic hepatitis B patients of a tertiary hospital, under NAs therapy for at least 2 years and with undetectable viral load, were eligible. Patients with hepatitis C virus, hepatitis D virus or human immunodeficiency virus co-infection and liver transplanted patients were excluded. HBsAg and HBcrAg levels (log10 U/mL) were measured at baseline and during ninety-six weeks. HBsAg loss rate was evaluated in both groups.

Adverse events were recorded in both groups. The kinetic of HBsAg for each treatment group was evaluated from baseline to weeks 24 and 48 by the slope of the HBsAg decline (log10 IU/mL/week) using a linear regression model.

*RESULTS*

Sixty-five patients were enrolled, 61% receiving tenofovir and 33% entecavir. Thirty-six (55%) were included in Peg-IFN-NA group and 29 (44%) in NA group. After matching by age and treatment duration, baseline HBsAg levels were comparable between groups (3.1 *vs* 3.2) ($P$ = 0.25). HBsAg levels at weeks 24, 48 and 96 declined in Peg-IFN-NA group (-0.26, -0.40 and -0.44) and remained stable in NA group (-0.10, -0.10 and -0.10) ($P$ < 0.05). The slope of HBsAg decline in Peg-IFN-NA group (-0.02) was higher than in NA group (-0.00) ($P$ = 0.015). HBcrAg levels did not change. Eight (22%) patients discontinued Peg-IFN due to adverse events. The HBsAg loss was achieved in 3 (8.3%) patients of the Peg-IFN-NA group and 0 (0%) of the NA group.

*CONCLUSION*

The addition of Peg-IFN to NAs caused a greater and faster decrease of HBsAg levels compared to NA therapy. Side effects of Peg-IFN can limit its use in clinical practice.

**Key Words:** Chronic hepatitis B; Hepatitis B e antigen-negative; Hepatitis B surface antigen; Hepatitis B core-related antigen; Pegylated-interferon; Nucleos(t)ids analogues



**Core Tip:** The functional cure of chronic hepatitis B defined as the loss of the hepatitis B surface antigen is the optimal end-point with the currently available therapies. However, it is rarely achieved in hepatitis B e antigen-negative chronic hepatitis B patients under nucleos(t)ids analogues (NAs). In the present study, we report that the addition of pegylated interferon (Peg-IFN) to NAs during forty-eight weeks caused a greater and faster decrease of hepatitis B surface antigen levels compared to NA monotherapy. No changes in hepatitis B core-related antigen were observed. However, the low applicability and poor tolerance of Peg-IFN make difficult its use in clinical practice.

**Citation:** Broquetas T, Garcia-Retortillo M, Micó M, Canillas L, Puigvehí M, Cañete N, Coll S, Viu A, Hernandez JJ, Bessa X, Carrión JA. Hepatitis B surface antigen and hepatitis B core-related antigen kinetics after adding pegylated-interferon to nucleos(t)ids analogues in hepatitis B e antigen-negative patients. *World J Hepatol* 2020; 12(11): 1076-1088
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1076.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1076

## INTRODUCTION

Chronic hepatitis B (CHB) affects around 240 million people worldwide[1]. Hepatitis B virus (HBV) cannot be completely eradicated with the available therapies due to the presence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes[2]. Hepatitis B surface antigen (HBsAg) loss is the optimal treatment endpoint, representing a functional cure of CHB and improving long-term outcome[3].

Although liver biopsy for the quantification of intrahepatic cccDNA and intrahepatic HBV DNA remains the most accurate measurement for viral reservoir, it is limited by its invasive nature and the potential for sampling error. Therefore, noninvasive serological tests are necessary as surrogate markers of intrahepatic viral replicative activity. Serum HBsAg is the glycosylated envelope protein of the mature HBV, which is produced by transcription and translation of the surface genes[4]. On the other hand, the hepatitis B core-related antigen (HBcrAg) combines the antigenic reactivity resulting from denatured hepatitis B e antigen (HBeAg), HBV core antigen and a core-related protein (p22cr), all products of the precore/core gene share an

reviewed and approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica - Parc de Salut Mar", study reference 2014/5787/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

**Clinical trial registration statement:** The study was registered at http://clinicaltrials.gov with the number NCT02743182.

**Informed consent statement:** Study participants, or their legal guardian, provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** Authors declare no conflict-of-interest.

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

**CONSORT 2010 statement:** The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.



**Manuscript source:** Invited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Spain

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): B, B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** June 2, 2020
**Peer-review started:** June 2, 2020
**First decision:** June 15, 2020
**Revised:** June 23, 2020
**Accepted:** September 4, 2020
**Article in press:** September 4, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Barone M, Kasirga E, Tuna N
**S-Editor:** Gao CC
**L-Editor:** A
**P-Editor:** Li JH

identical 149 amino acid sequence[5].

Currently, there are two strategies to treat HBeAg-negative CHB patients, a finite course with pegylated interferon (Peg-IFN) or a long-term therapy with nucleos(t)ids analogues (NAs). Entecavir or tenofovir monotherapy have been shown to achieve the virological response in almost all adherent patients[6]. However, the reduction of HBsAg levels in HBeAg-negative CHB patients under NAs is very slow (-0.1 log IU/mL/yr)[7,8] with HBsAg loss rates < 1% after five years of NAs therapy[7,9] compared to 4% after 48 wk of Peg-IFN[10]. Moreover, it has been suggested that interleukin 28B (IL28B) rs12979860 polymorphism CC could confer a better probability of response to Peg-IFN in HBeAg-negative CHB patients infected by genotype D[11]. On the other hand, differences in Peg-IFN response rates have been demonstrated according to HBV genotype especially in HBeAg-positive patients[12]. Despite NAs are the most used therapy in HBeAg-negative CHB patients because of its safety, long term therapy is needed. In contrast, the addition of the immunomodulatory effect of Peg-IFN could improve HBsAg loss rates[10,13]. However, this strategy has been mostly evaluated in naïve treatment or HBeAg-positive patients being the information about pre-treatment predictors and the kinetics of serological markers (HBsAg and HBcrAg) scarce during the add-on strategy in HBeAg-negative patients.

In the present study, we have prospectively evaluated the levels of HBsAg and HBcrAg in HBeAg-negative non-cirrhotic CHB patients receiving NAs after the addition of Peg-IFN during forty-eight weeks. The primary aim was to compare the HBsAg and HBcrAg kinetics in both treatment strategies (NA group *vs* Peg-IFN-NA group). The secondary aim was to evaluate the proportion of HBsAg loss at week 96.

## MATERIALS AND METHODS

### *Patients and study design*

This is a single center, prospective, non-randomized, open-label trial including HBeAg-negative non-cirrhotic CHB patients, receiving NAs for at least 2 years. Recruitment period was from August 2014 to February 2016 in a tertiary center (Hospital del Mar, Barcelona, Spain). Patients were eligible if they received a stable NAs dose with virological response (undetectable HBV-DNA viral load during the last twelve months). Exclusion criteria were as follows: Patients with a previous Peg-IFN treatment, NA treatment for HBV reactivation prophylaxis, patients with human immunodeficiency virus, hepatitis D virus or hepatitis C virus co-infection, and liver transplanted patients. All patients provided written informed consent.

Patients with any malignancy in the last 5 years, those with psychiatric, thyroid or autoimmune disorders, and non-liver transplanted patients were only eligible for NAs monotherapy. Peg-IFN alpha-2a was offered to be added in all eligible patients. Those who accepted it, received 180 μg/week during forty-eight weeks (Peg-IFN-NA group) and all the other participants remained in NAs monotherapy (NA group). At week 48 all the patients continued with NAs in monotherapy and were followed up until week 96 or loss of follow-up. Protocol visits were at weeks 0, 12, 24, 48, 72 and 96. Figure 1 shows the flowchart of patients and study design.

The study protocol was approved by the Ethical Committee of our Institution "Comitè Ètic d'Investigació Clínica-Parc de Salut Mar", study reference 2014/5787/I, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

### *Clinical variables and definitions*

Demographic data, liver stiffness measurement (LSM) and polymorphism rs12979860 of IL28B were assessed at baseline. HBV-genotype was collected from electronic data as it had been performed prior to the initiation of NAs therapy. The levels of HBV-DNA, HBsAg and HBcrAg were analyzed at weeks 0, 24, 48, 96. Adverse events were recorded at each protocol visit, following the Common Terminology Criteria for Adverse Events. All the data were collected and tabulated in a database with an access code to ensure patient confidentiality.

LSM was performed at baseline by a single experienced operator (> 5000 examinations), using the FibroScan® 502 Touch (FibroScan® EchosensTM, Paris, France) following the manufacturer's recommendations as previously described[14]. Liver fibrosis was categorized according to previously published cut-offs for LSM considering significant fibrosis for LSM > 7.2 kPa. Patients with LSM > 12 kPa were considered as having cirrhosis and were excluded[15].

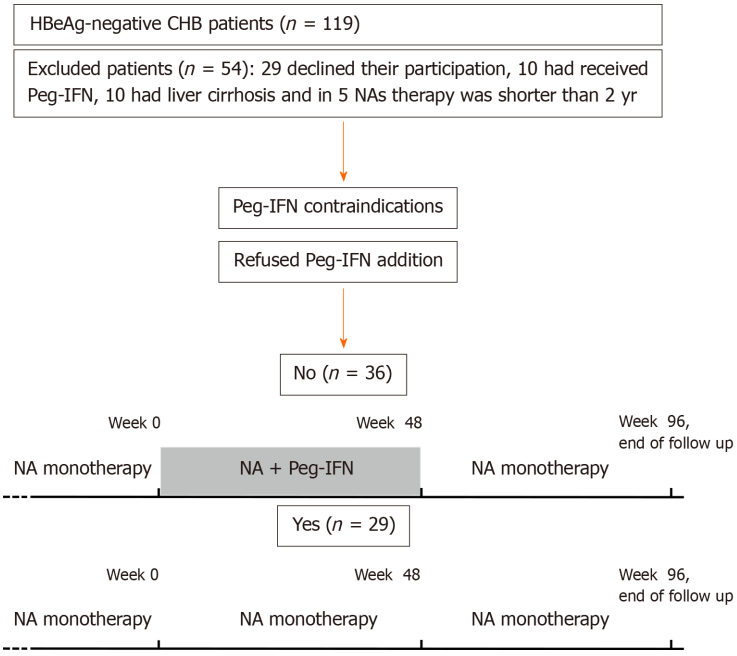


**Figure 1 Flowchart of patients and study design.** HBeAg: Hepatitis B e antigen; CHB: Chronic hepatitis B; Peg-IFN: Pegylated interferon; NAs: Nucleos(t)ids analogues.

### *Virological markers*

HBV DNA was measured by polymerase chain reaction with a limit of quantification of 10 IU/mL (ABBOTT RealTime HBV m2000®, Abbott Molecular Inc., IL, United States). Serum HBsAg was quantified by Electro-chemiluminescence immunoassay Elecsys® HBsAgII QuantII (Roche Diagnostic, Rotkreuz, Switzerland) according to the manufacturer's instructions. The assay ranged from 0.05 to 117000 IU/mL. In highly concentrated samples above the upper limit, the value of manual dilution was multiplied by the dilution factor. Serum HBcrAg was measured using a quantitative fully automated chemiluminiscent enzyme immunoassay (LUMIPULSE®, Fujirebio Europe, Belgium).

The monoclonal antibodies used in this two-step immunoassay measure simultaneously denatured HBeAg, HBV core antigen and the precore protein p22cr (aa-28 to aa-150). Samples were processed according to the manufacturer's instructions. The lower limit of detection was 2.0 log U/mL, and a linear range of 3.0 log U/mL-7.0 log U/mL (1 kU/mL was equal to 3 log U/mL).

### *Statistical analysis*

Quantitative variables were expressed as medians and ranges. Categorical variables were expressed as proportions. Continuous variables were compared by the Mann–Whitney *U* test or Kruskall-Wallis when appropriate and categorical by the Pearson chi-square test, Fisher test or the Mc Nemar test. Patients were categorized according to antiviral treatment (Peg-IFN-NA group *vs* NA group). Differences between NA and Peg-IFN-NA groups regarding age, sex, IL28B polymorphism, ethnicity, liver function, liver stiffness, treatment duration, viral genotype, HBsAg and HBcrAg levels and HBsAg loss rate were analyzed by univariate analysis. A two-sided $P$ value < 0.05 was considered to indicate statistical significance. The kinetic of HBsAg for each treatment group was evaluated from baseline to weeks 24 and 48 by the slope of the HBsAg decline (log10 IU/mL per week) using a linear regression model (LRM). Statistical analyses were performed with the SPSS® 25.0 (SPSS Inc., Chicago, IL, United States) and LRM with the Prism 7.0 (© 1994-2016 GraphPad Software, Inc.).

## RESULTS

### *Study population and baseline characteristics*

From August 2014 to February 2016, 119 HBeAg-negative CHB patients were evaluated. Twenty-nine (24%) patients declined their participation, 10 (8.4%) had

previously received Peg-IFN, 10 (8.4%) had liver cirrhosis and in 5 (4.2%) patients NAs therapy duration was shorter than 2 years. Among the 65 included patients, 5 were only eligible for the NA therapy due to Peg-IFN contraindications and 60 were eligible for both therapies: 36 accepted to receive Peg-IFN and 24 refused the addition of Peg-IFN. Therefore, 36 (55.4%) patients were included in the Peg-IFN-NA group and 29 (44.6%) in the NA group. Two patients in NA group were receiving low doses of corticosteroids (prednisone 2.5 to 5 mg/d) for rheumatoid arthritis and no kidney transplanted patients were included because none of them fulfilled the inclusion criteria.

Figure 1 shows the flowchart and Table 1 the main characteristics of the included patients. Patients in Peg-IFN-NA group compared to NA group were younger (age 45 *vs* 53, $P = 0.01$) and had a shorter previous NA treatment duration (259 *vs* 393 wk, $P = 0.01$), but were comparable in gender, IL28B polymorphism, ethnicity, liver function, liver stiffness, type of NA, HBV genotype and baseline HBcrAg and HBsAg levels. Due to the baseline differences, patients of both treatment groups were individually matched for age and treatment duration. Therefore, pre-treatment predictors and the kinetic of serological markers (HBsAg and HBcrAg) were performed in 48 patients. Table 2 shows the characteristics of matched patients.

### HBcrAg kinetics according to baseline variables and treatment group

The median (range) HBcrAg values (log 10 U/mL) was 2.7 (< 2-4.9) in NA group and 2.3 (< 2-3.7) in Peg-IFN-NA group ($P = 0.18$) at baseline. The rate of patients with HBcrAg values below the limit of detection (HBcrAg < 2 log10 U/mL) was 25% and 38%, respectively ($P = 0.39$). The HBcrAg kinetics was described as the delta (Δ) of its levels at weeks 24, 48 and 96. The HBcrAg levels remained stable at weeks 24, 48 and 96 (Table 2). We did not detect differences on HBcrAg levels between both treatment strategies according to the treatment group, the IL28B polymorphism or the HBV genotype. We did not find any correlation between HBcrAg and HBsAg levels nor HBsAg loss rate (data not shown).

### HBsAg kinetics according to baseline variables and treatment group

The baseline levels of HBsAg (log 10 IU/mL) were similar in NA and Peg-IFN-NA groups (3.1 *vs* 3.2) ($P = 0.25$). The HBsAg kinetics was described as the delta (Δ) of their levels at weeks 24, 48 and 96. The decline of the HBsAg level was greater in Peg-IFN-NA group (-0.26, -0.40, -0.44) compared to NA group (-0.11, -0.10, -0.12) ($P < 0.05$ in all determinations) (Figure 2).

The HBsAg kinetics was different between treatment arms according to IL28B polymorphism and HBV genotype. In patients with IL28B CC polymorphism ($n = 22$) the decline of HBsAg at weeks 24, 48 and 96 was greater in Peg-IFN-NA group (-0.27, -0.92 and -0.64) than in NA group (-0.11, -0.11 and -0.10) ($P < 0.05$ in all cases) (Figure 3A). In contrast, in patients with IL28B CT/TT ($n = 26$) we did not find differences on HBsAg kinetics at weeks 24, 48 and 96 between Peg-IFN-NA group (-0.09, -0.11 and -0.19) and NA group (-0.10, -0.07 and 0.13) (not significant in all determinations) (Figure 3B). Moreover, the decline of HBsAg were different between NA and Peg-IFN-NA group at weeks 48 and 96 in patients infected by HBV genotype A (-0.07 *vs* -1.05 and -0.08 *vs* -0.53) and genotype D (-0.08 *vs* -0.42 and -0.51 *vs* -0.80) ($P < 0.05$ in all cases) (data not shown).

### LRM to recognize different HBsAg kinetics

In order to demonstrate the existence of different HBsAg kinetics for each treatment strategy, we evaluated the slope of the HBsAg decline (log10 IU/mL per week) from baseline to weeks 24 and 48 using a LRM (Figure 4). In patients receiving NA monotherapy, HBsAg levels did not decrease during the forty-eight weeks. The slope of HBsAg kinetics in NA group (-0.00) was similar to zero ($P = 0.6$). On the contrary, in patients receiving Peg-IFN-NA, HBsAg levels significantly decreased during the forty-eight weeks and the slope of HBsAg kinetic (-0.02) was different to zero ($P < 0.001$) and greater than that found in NA group ($P = 0.015$).

### Rate of low HBsAg levels and HBsAg loss during follow-up

The proportion of patients reaching low levels of HBsAg (HBsAg < 100 IU/mL) at baseline and at weeks 24, 48 and 96 are depicted in Figure 5. In the NA group the rate of patients with low HBsAg levels was 21% at baseline, but did not change at weeks 24, 48 and 96 (not significant) (Figure 5A). On the contrary, rate of patients with low HBsAg levels in Peg-IFN-NA group was 4.2% at baseline and increased at weeks 24 (16.7%), 48 (29.6%) and 96 (16.7%) ($P = 0.001$) (Figure 5B). The proportion of patients

**Table 1 Main characteristics of the included patients**

| | NA group (*n* = 29) | Peg-IFN-NA group (*n* = 36) | *P* value |
|---|---|---|---|
| Age (yr) | 53 (36-70) | 45 (26-72) | 0.01 |
| Males, *n* (%) | 21 (72) | 29 (81) | 0.44 |
| IL28B polymorphism, *n* (%) | | | 0.16 |
| CC | 11 (37.9) | 20 (55.6) | |
| CT/TT | 14 (62.1) | 16 (44.4) | |
| Origin (ethnicity), *n* (%) | | | 0.70 |
| Europe | 20 (69) | 20 (56) | |
| Asia | 12 (33) | 12 (33) | |
| Africa | 3 (10) | 3 (8) | |
| AST (IU/mL) | 20 (15-59) | 22 (12-62) | 0.37 |
| ALT (IU/mL) | 19 (12-101) | 25 (12-91) | 0.20 |
| GGT (IU/mL) | 19 (9-197) | 22 (10-125) | 0.33 |
| LSM, *n* (%) | | | 0.91 |
| < 7.2 kPa | 28 (97) | 34 (97) | |
| 7.2-12 kPa | 1 (3) | 1 (3) | |
| NA treatment, *n* (%) | | | |
| Tenofovir | 20 (69) | 22 (61) | 0.46 |
| Entecavir | 7 (24) | 11 (31) | |
| Others | 2 (7) | 3 (8) | |
| NA treatment duration (wk) | 393 (113-763) | 259 (118-496) | 0.01 |
| HBV genotype, *n* (%) | | | 0.99 |
| Non-D | 7 (24.1) | 16 (44.4) | |
| D | 12 (41.4) | 13 (36.1) | |
| Not available | 10 (34.5) | 7 (19.4) | |
| Baseline HBcrAg (log 10 U/mL) | 2.65 (< 2-4.9) | 2.30 (< 2-3.7) | 0.18 |
| Baseline HBsAg (log 10 IU/mL) | 2.96 (1.3-4.2) | 3.22 (1.6-4.6) | 0.07 |

Quantitative variables are expressed as median (range); qualitative variables are expressed as *n* (%). NA: Nucleos(t)id analogue; Peg-IFN: Pegylated interferon; IL28B: Interleukin 28B; AST: Aspartate aminotransferase; ALT Alanine aminotransferase; GGT: Gamma-glutamyl transferase; LSM: Liver stiffness measurement; HBV: Hepatitis B virus; HBcrAg: Hepatitis B core-related antigen; HBsAg: Hepatitis B surface antigen.

achieving HBsAg loss in the Peg-IFN-NA group (*n* = 3, 12.5%) was higher compared to NA group (*n* = 0, 0%), but the difference did not reach the statistical significance (*P* = 0.07).

Patients with HBsAg loss were male, with low fibrosis stage (F0-F1), and infected by HBV-genotype A (*n* = 1) or B (*n* = 2). Two patients had an IL28B CC polymorphism and the other a CT polymorphism. All of them had been on NAs therapy for more than 5 years before the addition of Peg-IFN. The NAs treatment was entecavir (*n* = 1), tenofovir (*n* = 1) and telbivudine (*n* = 1). Baseline levels of HBsAg (log10 IU/mL) were 4.0, 2.1 and 1.6, and baseline levels of HBcrAg (log10 U/mL) were 2.7, < 2 and 3.4, respectively. All of them received Peg-IFN during forty-eight weeks. Two patients lost HBsAg during therapy (week 24 and 36) and one at week 24 after Peg-IFN discontinuation (week 72).

### *Safety*

No serious adverse events were observed during treatment and follow-up. However, 8 (22%) patients did not complete Peg-IFN treatment. The reasons for Peg-IFN discontinuation were flu-like symptoms and asthenia (*n* = 3), DNA flare (*n* = 3),

**Table 2 Characteristics of matched patients in each treatment group**

| | NA group (*n* = 24) | Peg-IFN-NA group (*n* = 24) | *P* value |
|---|---|---|---|
| Age (yr) | 54 (36-60) | 45 (26-63) | 0.07 |
| Male sex, *n* (%) | 18 (75) | 22 (91) | 0.12 |
| IL28B polymorphism, *n* (%) | | | 0.25 |
| CC | 9 (38) | 13 (54) | |
| CT/CT | 15 (62) | 11 (46) | |
| Origin (ethnicity), *n* (%) | | | 0.20 |
| European | 17 (70) | 12 (50) | |
| Asia | 3 (12) | 9 (38) | |
| Africa | 2 (8) | 3 (12) | |
| AST (IU/mL) | 20 (15-59) | 22 (15-38) | 0.69 |
| ALT (IU/mL) | 20 (12-101) | 23 (15-50) | 0.41 |
| GGT (IU/mL) | 23 (9-197) | 22 (11-125) | 0.44 |
| LSM, *n* (%) | | | 0.32 |
| < 7.2 kPa | 23 (96) | 24 (100) | |
| 7.2-12 kPa | 1 (4) | 0 (0) | |
| NA treatment, *n* (%) | | | 0.32 |
| Tenofovir | 16 (67) | 12 (50) | |
| Entecavir | 6 (25) | 9 (38) | |
| Others | 2 (8) | 3 (12) | |
| NA treatment duration (wk) | 378 (113-763) | 272 (139-495) | 0.06 |
| HBV genotype, *n* (%) | | | 0.43 |
| A | 5 (21) | 4 (17) | |
| B | 1 (4) | 3 (12) | |
| C | 0 (0) | 2 (8) | |
| D | 10 (42) | 8 (33) | |
| E | 1 (4) | 2 (8) | |
| F | 0 (0) | 1 (4) | |
| Not available | 7 (29) | 4 (18) | |
| Baseline HBcrAg (log 10 U/mL) | 2.7 (< 2-4.9) | 2.3 (< 2-3.7) | 0.18 |
| Baseline HBcrAg (log10 U/mL), *n* (%) | | | 0.39 |
| < 2 | 6 (25) | 9 (38) | |
| 2-2.5 | 4 (17) | 6 (25) | |
| 2.5-3 | 6 (25) | 3 (12) | |
| 3-3.5 | 2 (8) | 3 (12) | |
| 3.5-4 | 3 (13) | 3 (12) | |
| > 4 | 3 (13) | 0 (0) | |
| Baseline HBsAg (log10 IU/mL) | 3.1 (1.3-4.2) | 3.2 (1.6-4.4) | 0.25 |
| Baseline HBsAg (IU/mL), *n* (%) | | | 0.22 |
| > 1000 | 12 (50) | 14 (48) | |
| 100-1000 | 7 (29) | 9 (38) | |
| < 100 | 5 (21) | 1 (4) | |

| | | | |
|---|---|---|---|
| HBcrAg decline (log10 U/mL) | | | |
| Δ Week 24 | 0.00 (-1.10-1.21) | 0.00 (-0.71-0.30) | 0.96 |
| Δ Week 48 | 0.00 (-1.00-0.30) | 0.00 (-1.31-1.10) | 0.25 |
| Δ Week 96 | 0.00 (-1.00-0.10) | 0.00 (-0.71-0.71) | 0.12 |
| HBsAg decline (log10 IU/mL) | | | |
| Δ Week 24 | -0.11 (-0.04-0.00) | -0.26 (-3.8-0.1) | 0.01 |
| Δ Week 48 | -0.10 (-1.17-0.04) | -0.40 (-4-0.02) | 0.00 |
| Δ Week 96 | -0.12 (-1.39-0.96) | -0.44 (-4-0.01) | 0.00 |
| HBsAg Loss; *n* (%) | 0 (0) | 3 (12.5) | 0.07 |

Quantitative variables are expressed as median (range); qualitative variables are expressed as *n* (%). NA: Nucleos(t)id analogue; Peg-IFN: Pegylated interferon; IL28B: Interleukin28B; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; LSM: Liver stiffness measurement; HBV: Hepatitis B virus; HBcrAg: Hepatitis B core related antigen; HBsAg: Hepatitis B surface antigen.

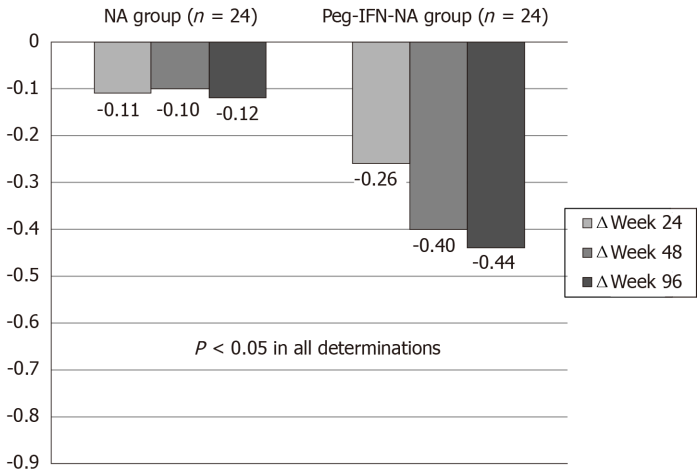


**Figure 2 Hepatitis B surface antigen delta (Δ) (log10 IU/mL) at wk 24, 48 and 96 according to treatment group.** Peg-IFN: Pegylated interferon; NA: Nucleos(t)ids analogue.

polyarthritis (*n* = 1) and Graves' thyroiditis (*n* = 1). No patients discontinued antiviral treatment in NA group.

## DISCUSSION

In this controlled trial of HBeAg-negative CHB non-cirrhotic patients under NAs treatment and with undetectable DNA, the addition of 48 wk of Peg-IFN alfa-2a reduced HBsAg levels further and faster than continuing with NAs monotherapy. However, the proportion of patients with HBsAg loss during the first ninety-six weeks did not reach the statistical significance with this add-on strategy.

HBsAg kinetics has been shown as one of the best predictors of treatment response[8,16,17]. However, patients of our Peg-IFN-NA group were younger and had a shorter previous NA treatment duration compared to NA group. According to previously published studies showing a decrease of HBsAg levels with NA therapy[18] and a higher probability to HBsAg clearance in aged populations[19] we decided to match the included patients for age and treatment duration.

The present study prospectively confirms our previously published results[7] regarding the slow decline of HBsAg levels in HBeAg-negative CHB patients receiving NAs therapy. The current study has demonstrated a very low decline (-0.12 log10 IU/mL at week 96) and very slow change (-0.00 log10 IU/mL per week) of HBsAg levels in patients receiving NAs. As a consequence, the rate of patients with low HBsAg levels (< 100 IU/mL) did not change at weeks 24, 48 and 96, and no patient achieved HBsAg loss. On the contrary, the addition of Peg-IFN clearly increased the

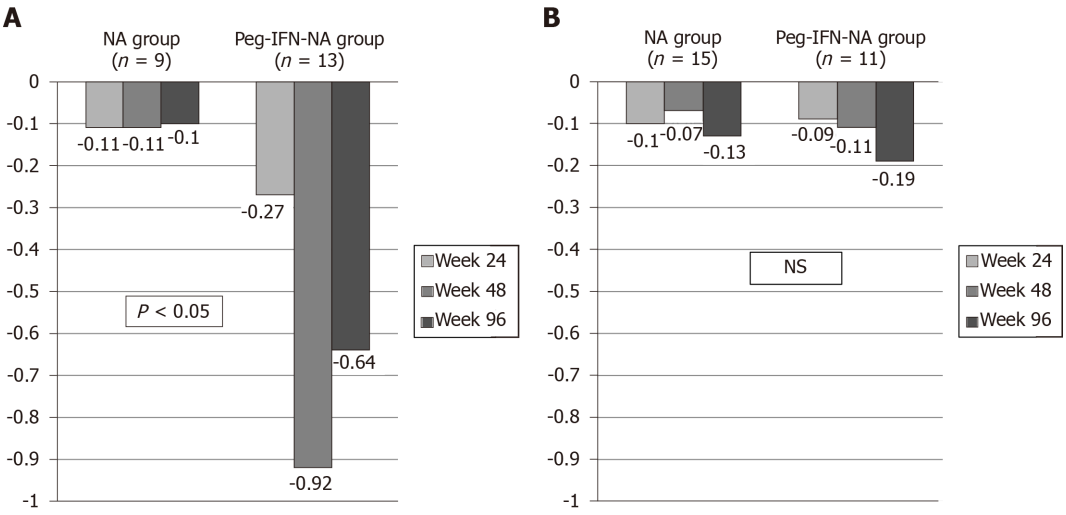


**Figure 3 Hepatitis B surface antigen delta (Δ) (log10 IU/mL) according to interleukin 28B polymorphism and treatment group.** A: Hepatitis B surface antigen delta (Δ) in interleukin 28B CC patients (*n* = 22); B: Hepatitis B surface antigen delta (Δ) in interleukin 28B CT/TT patients (*n* = 26). NS: Not significant; NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon.

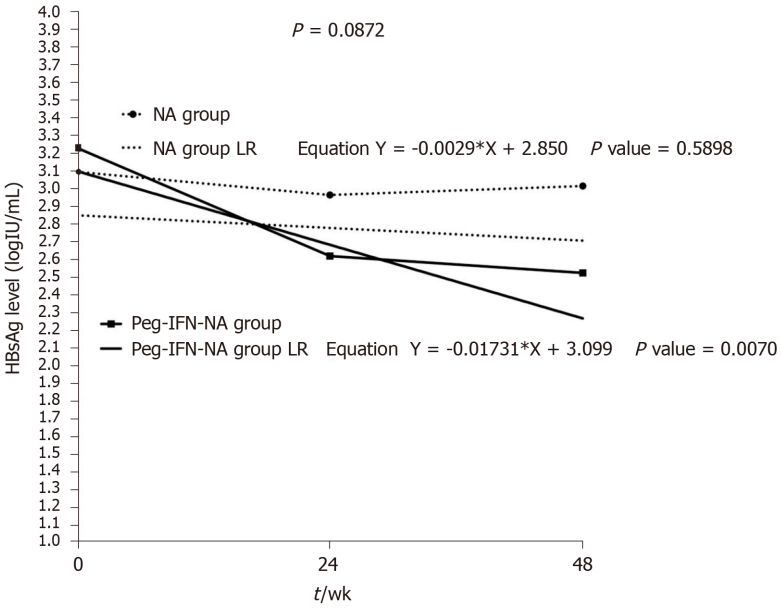


**Figure 4 Linear regression model of hepatitis B surface antigen levels according to treatment group.** NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon; LR: Linear regression.

decline (-0.44 log10 IU/mL at week 96) and accelerate the decrease (-0.02 log10 IU/mL per week) of HBsAg levels compared to NA group. Therefore, in the Peg-IFN-NA group the rate of patients with low HBsAg levels was higher at weeks 24 (16.7%) and 48 (29.6%) and the rate of HBsAg loss increased (*n* = 3, 12.5%) compared to NA group ( *n* = 0, 0%).

We also analyzed the HBcrAg levels during the study in both treatment strategies. However, levels of HBcrAg remained stable during the 96 wk without differences between both treatment strategies and without correlation with HBsAg levels or HBsAg loss rate. This could be explained by the fact that baseline levels of HBcrAg in our cohort of HBeAg negative patients, receiving NAs during a long time period before inclusion, were already low. As described before, the rate of patients with a baseline HBcrAg value below the limit of detection (< 2 log10 U/mL) was high in both treatment groups (25% and 38%). Recent studies have shown that HBcrAg can reflect cccDNA transcriptional activity in the different phases of HBV infection[20,21]. However as HBeAg is included in HBcrAg, this could explain the low baseline HBcrAg levels in our cohort of HBeAg-negative patients. Moreover, recent studies, have described that

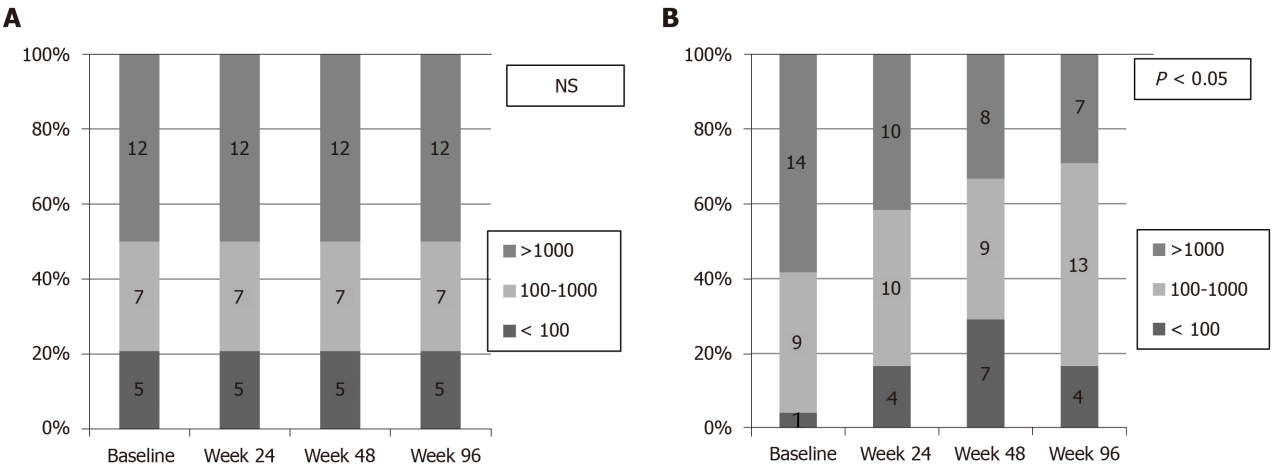


**Figure 5 Rate of patients with low hepatitis B surface antigen levels (Hepatitis B surface antigen < 100 IU/mL and 100-1000 IU/mL) according to treatment group.** A: NA group; B: Peg-IFN-NA group. NS: Not significant; NA: Nucleos(t)ids analogue; Peg-IFN: Pegylated interferon.

HBcrAg levels can decline over the time in patients undergoing NAs therapy, especially in HBeAg-negative patients[22,23]. Thus, according to our results, we have not found that HBcrAg determination could be a useful serum marker in clinical practice for monitoring treatment response in HBeAg-negative patients receiving NAs or Peg-IFN-NAs.

It has been suggested that low levels of HBsAg are related to higher rates of HBsAg loss after NA discontinuation, being advisable to achieve low levels of HBsAg before stopping NA therapy[24,25]. Our study showed that the rate of patients with HBsAg < 100 IU/mL increased in the Peg-IFN-NA group from 4.2% at baseline to 29.6% at 48 wk ($P$ = 0.001). The NAs have shown to restore partly adaptive immunity, whereas Peg-IFN boosts innate immunity and depletes the ccc-DNA, which leads to a major HBsAg loss[26-29]. The analysis performed in matched patients by age and treatment duration showed that the proportion of HBsAg loss during the first 96 wk was higher in the Peg-IFN-NA group compared to the NA group. However, this difference did not reach the statistical significance probably due to the limited number of included patients and the short follow-up time of our study. Nevertheless, our results are in accordance with smaller studies previously published[30,31] and in line with the results published by Bourlière *et al*[32] during the execution of the current study.

Previous studies have linked the presence of IL28B CC polymorphisms with the HBsAg loss in HBeAg-negative CHB patients receiving Peg-IFN. It has been shown that CC polymorphism could confer a better response profile to Peg-IFN therapy than CT/TT polymorphisms, especially in patients infected by HBV genotype D[11,33]. We analyzed the HBsAg kinetics according to IL28B polymorphism, and we found that patients with CC polymorphism showed a higher HBsAg decline in Peg-IFN-NA group compared to NA group. On the contrary, HBsAg kinetics was similar in both treatment strategies in CT/TT patients. Therefore, the add-on strategy should not be recommended in patients with IL28B CT or TT polymorphism.

Our study has several limitations. First, the treatment assignment was not randomized. However, patients on both treatment strategies were individually matched for age and treatment duration to make the cohort comparable. Second, the acceptance of the add-on strategy was low and only 40% of eligible patients with a previous (well-tolerated) NA therapy accepted the addition of Peg-IFN due to its potential toxicity. Third, the frequent adverse events of Peg-IFN (22% of discontinuations) caused a low number of patients completing 48 wk of therapy making this therapeutic strategy difficult to be introduced in clinical practice. However, this applicability and tolerability are in line with previous published data[32]. Fourth, the treatment duration of Peg-IFN was limited to 48 wk and the follow-up period to 96 wk. Therefore, patients with a rapid HBsAg decline could have taken advantage of a longer therapy or longer follow-up. Finally, the low rate of HBsAg loss did not allow to identify predictors associated with HBsAg loss. However, the LRM demonstrated different HBsAg kinetics after adding Peg-IFN.

## CONCLUSION

In conclusion, our prospective, non-randomized, open-label clinical trial has demonstrated that the addition of Peg-IFN to NAs decreased HBsAg levels further and faster compared to NA monotherapy. The HBcrAg levels remained stable. Despite the low applicability and poor tolerance of Peg-IFN making difficult its use in clinical practice, it could be considered in selected patients with favorable HBV genotype and IL28B polymorphism.

## ARTICLE HIGHLIGHTS

### *Research background*

Functional cure of chronic hepatitis B (CHB), defined as the loss of hepatitis B surface antigen (HBsAg), is very unusual with current antiviral treatments in hepatitis B e antigen (HBeAg)-negative patients. HBsAg levels decline very slow in patients receiving nucleos(t)ids analogues (NAs). Therefore, they need long-term antiviral treatment.

### *Research motivation*

The hypothesis that we wanted to answer with our study was that the addition of pegylated-interferon (Peg-IFN) could accelerate the decline of HBsAg levels in patients that were receiving NAs and that this therapeutic strategy could increase the HBsAg loss rate.

### *Research objectives*

In our study we wanted to evaluate in patients under NAs therapy if the addition of Peg-IFN could decrease HBsAg and hepatitis B core-related antigen (HBcrAg) levels, and increase HBsAg loss rate. If HBeAg-negative patients could achieve low levels of HBsAg it could be a good strategy to shorten the antiviral treatment.

### *Research methods*

We have performed a prospective, non-randomized, open-label trial evaluating the combination of Peg-IFN 180 μg/wk plus NAs during forty-eight weeks *vs* NAs in monotherapy, in HBeAg-negative non-cirrhotic CHB patients after a minimum of two years of NA therapy and with virological response.

### *Research results*

We have shown that the addition of Peg-IFN 180 μg/wk during forty-eight weeks to NAs caused a greater and faster decrease of HBsAg levels compared to NA therapy alone, especially in those patients with interleukin 28B polymorphism CC. However, the HBcrAg levels remained stable after adding Peg-IFN to NAs. We have also shown that, the low acceptance by the patients of this therapeutic strategy and the side effects of Peg-IFN can limit its use in clinical practice.

### *Research conclusions*

This study shows that the addition of Peg-IFN to NA therapy accelerates the decline of HBsAg, especially in patients with interleukin 28B polymorphism CC. Therefore, even Peg-IFN has several side effects, this treatment strategy could be offered to some selected patients in order to achieve the functional cure of CHB. On the other hand, our study shows that HBcrAg levels do not seem useful to monitor this kind of treatment, neither as a predictor of HBsAg loss.

### *Research perspectives*

It is well known that patients with HBeAg-negative CHB usually need a long-term therapy with NAs, even lifelong, to achieve HBsAg loss. However, it has been suggested that low levels of HBsAg are related to higher rates of HBsAg loss after NA discontinuation, being advisable to achieve low levels of HBsAg before stopping NA therapy.

## ACKNOWLEDGEMENTS

The authors thank Duran X in Hospital del Mar Medical Research Institute for assistance with the statistical analysis.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1089-1097

DOI: 10.4254/wjh.v12.i11.1089

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

**Observational Study**

# Occurrence of seeding metastases in resectable perihilar cholangiocarcinoma and the role of low-dose radiotherapy to prevent this

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**Author contributions:** Franken L, Saris J, van Gulik TM designed the research study; Franken LC, Saris J performed the research. van Hooft JE, Roos E, van Delden OM, Verheij J, Besselink MG, Erdmann JI, Busch OR, van Gulik TM contributed essential reagents or tools; van Tienhoven G contributed essential reagents or tools, interpretation of the data, revised work for important intellectual content; Franken L, Saris J analyzed the data; Roos E, van Gulik TM, van Delden OM, Erdmann JI, Busch OR, van Gulik TM interpretation of data; Franken L, van Gulik TM, Roos E and van Gulik TM wrote the paper; Van Hooft J, Roos E, van Delden OM, Verheij J, Besselink MG, Erdmann

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## Abstract

### *BACKGROUND*

Preoperative biliary drainage in patients with presumed resectable perihilar cholangiocarcinoma (PHC) is hypothesized to promote the occurrence of seeding metastases. Seeding metastases can occur at the surgical scars or at the site of postoperative drains, and in case of percutaneous biliary drainage, at the catheter port-site. To prevent seeding metastases after resection, we routinely treated PHC patients with preoperative radiotherapy (RT) for over 25 years until January 2018.

### *AIM*

To investigate the incidence of seeding metastases following resection of PHC.

### *METHODS*

All patients who underwent resection for pathology proven PHC between January 2000 and March 2019 were included in this retrospective study. Between

JI, Busch OR and van Gulik TM revised work for important intellectual content.

**Institutional review board statement:** The need for ethical approval was waived by the Medical Ethics Review Committee of the Amsterdam UMC, location AMC (W19_155).

**Informed consent statement:** The need for individual informed consent was waived by the Medical Ethics Review Committee of the Amsterdam UMC, location AMC (W19_155).

**Conflict-of-interest statement:** The authors declare no conflict of interest.

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

**STROBE statement:** The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.



**Manuscript source:** Unsolicited manuscript

**Specialty type:** Medicine, research and experimental

**Country/Territory of origin:** Netherlands

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): 0

2000-January 2018, patients received preoperative RT (3 × 3.5 Gray). RT was omitted in patients treated after January 2018.

*RESULTS*

A total of 171 patients underwent resection for PHC between January 2000 and March 2019. Of 171 patients undergoing resection, 111 patients (65%) were treated with preoperative RT. Intraoperative bile cytology showed no difference in the presence of viable tumor cells in bile of patients undergoing preoperative RT or not. Overall, two patients (1.2%) with seeding metastases were identified, both in the laparotomy scar and both after preoperative RT (one patient with endoscopic and the other with percutaneous and endoscopic biliary drainage).

*CONCLUSION*

The incidence of seeding metastases in patients with resected PHC in our series was low (1.2%). This low incidence and the inability of providing evidence that preoperative low-dose RT prevents seeding metastases, has led us to discontinue preoperative RT in patients with resectable PHC in our center.

**Key Words:** Perihilar cholangiocarcinoma; Seeding metastases; Preventive radiotherapy



**Core Tip:** Routine preoperative radiotherapy (3 times 3.5 Gray) to prevent the occurrence of seeding metastases was used in our tertiary center for 28 years in patients undergoing resection for perihilar cholangiocarcinoma. Seeding metastases occurred in 2 out of 171 patients (1.2%) undergoing resection between 2000 and 2019. Intraoperative bile cytology showed no significant difference in the presence of tumor cells in the bile of patients undergoing preoperative radiotherapy or not. Due to the current low incidence of seeding metastases, preoperative radiotherapy to prevent seeding metastases is now abandoned in our institution.

**Citation:** Franken LC, Roos E, Saris J, van Hooft JE, van Delden OM, Verheij J, Erdmann JI, Besselink MG, Busch OR, van Tienhoven G, van Gulik TM. Occurrence of seeding metastases in resectable perihilar cholangiocarcinoma and the role of low-dose radiotherapy to prevent this. *World J Hepatol* 2020; 12(11): 1089-1097

**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1089.htm

**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1089

## INTRODUCTION

Patients with perihilar cholangiocarcinoma (PHC) have been treated with low-dose radiotherapy (RT) preoperatively at our institution since the early 90s with the aim to prevent seeding metastases. Preoperative radiotherapy was introduced in our practice based on a study published by our institution in 1999, showing that 8 of 41 patients (20%) undergoing resection for PHC who were drained preoperatively, developed implantation metastases within 1 year after resection. In this study, endoscopic biliary drainage was performed in all 41 patients, combined with percutaneous biliary drainage in 4 of these patients. In addition, in 11 patients without biliary drainage, no seeding metastases were found[1]. It was hypothesized that biliary drainage, with perturbation of the tumor, enhances bile contamination with tumor cells, thereby increasing the risk of seeding metastases with bile spill incurred during resection.

In an effort to prevent this complication, all patients with resectable PHC at our tertiary center, received three times 3.5 Gray (Gy) external radiation therapy to the primary tumor on three consecutive days before surgery to kill free-floating tumor cells in the bile and destabilize the tumor cells that might be spilled during the operation. This concept of preventive, preoperative radiation therapy and dose regimen was based on a suggestion of a Mayo clinic study[2] and a study[3] in bladder carcinoma in which 3 times 3.5 Gy external radiation therapy had shown to be effective to reduce scar implementation metastases following surgery. Initial results of

Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** April 13, 2020
**Peer-review started:** April 13, 2020
**First decision:** June 18, 2020
**Revised:** August 11, 2020
**Accepted:** September 3, 2020
**Article in press:** September 3, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Coco D, Sperti C
**S-Editor:** Zhang L
**L-Editor:** A
**P-Editor:** Liu JH

twenty-one patients with resected PHC who had undergone preoperative radiation, were promising with none of the patients developing seeding metastases[4].

Seeding metastases resulting from spill of bile during operation may become manifest in the tract of previous abdominal drains or laparotomy scar after resection. In addition, most patients with resectable PHC undergo preoperative biliary drainage which in itself, was thought to promote seeding metastases. Biliary drainage can be performed by two different approaches: Percutaneously or by the endoscopic route. Several studies have suggested that percutaneous biliary drainage carries an additional risk of developing seeding metastases along the catheter tract compared to endoscopic approaches, in which no catheter tract is created. Based on a described incidence of seeding metastases in the catheter tract after percutaneous biliary drainage of 2.6%-6.3%, several Asian authors[5-10] suggested that endoscopic biliary drainage should be the preferred approach.

The aim of this study was to update the occurrence of seeding metastases in our institution and evaluate the role of preventive pre-operative low-dose radiotherapy in patients with resectable PHC.

## MATERIALS AND METHODS

All consecutive patients who underwent resection for pathology proven PHC at the Amsterdam UMC (location AMC) between January 2000 and March 2019 were included in this retrospective study. The need for ethical approval was waived by the Medical Ethics Review Committee of the Amsterdam UMC, location AMC (W19_155).

Whereas patients with intraductal papillary neoplasm of the bile duct (IPNB) were included in this analysis, patients with other final pathology diagnosis were excluded. Patient and tumor characteristics were obtained from a prospectively maintained database including all patients undergoing resection for suspicion of PHC. Data regarding preoperative work-up, biliary drainage, radiotherapy, type of surgery, postoperative course, follow-up and recurrence was collected. Jaundiced patients were treated by endoscopic biliary drainage or percutaneous biliary drainage, or a combination of both, with a preference at our institution for initial endoscopic biliary drainage.

Patients were stratified according to type of biliary drainage, while patients undergoing both percutaneous and endoscopic biliary drainage were analyzed as a separate group. Surgical procedures generally consisted of resection of the extrahepatic bile duct in combination with (extended) hemihepatectomy. After 2009, intra-operative bile sampling for cytology was routinely performed in the majority of the patients. Results of bile cytology were compared between patients undergoing preoperative radiotherapy or not. Standard follow up after resection was 5 years. Imaging was not standard incorporated in oncological follow-up, but performed when patients showed symptoms of recurrent disease.

Between 2000 and January 2018, patients undergoing resection for PHC were irradiated to 3 times 3.5 Gy to the tumor in the liver hilum, administered on three consecutive days prior to resection. Up to 2004, the hilar area was identified using a conventional simulator, based on the position of the biliary stents. From 2004 onwards, computerized tomography (CT) based planning was used. Throughout, a 3D conformal technique was applied. Patients treated between 2000 and 2012, were also included in the study by Wiggers *et al*[11]. Primary outcome of this study was the occurrence of seeding metastases, defined as metastases occurring in the laparotomy scar or in tracts of previous abdominal drains. Recurrence was defined as radiologically suspected or pathologically proven recurrence and time of recurrence was documented. Peritoneal recurrence, unlike in other studies, was not considered as seeding metastases. Only the initial location of recurrence was registered.

### *Statistical analyses*

Descriptive statistics were used to describe the data using statistical product and service solutions version 25 (International Business Machines Corporation, Armonk, NY, United States). The Chi-square test was used to compare the incidence of seeding metastases and the occurrence of tumor cells in bile cytology between groups. Duration of drainage was compared using Mann-Witney *U* test.

## RESULTS

A total of 171 patients who underwent resection for pathology proven PHC between 2000 and March 2019 were identified (Table 1). The median follow-up of survivors was 36 mo [interquartile range (IQR): 16-81]. Biliary drainage prior to resection was performed in 145 patients (85%). In 81 (56%) patients endoscopic biliary drainage took place, 14 (10%) patients underwent percutaneous biliary drainage and 50 (34%) patients underwent both drainage routes, due to the absence of therapeutic success of one route. Median duration between (first) biliary drainage and surgery was 76 d (IQR: 53-101 d) for endoscopic drainage, 53 d (IQR: 36-80 d) for percutaneous biliary drainage and 76 d (range 61-99 d) for both endoscopic and percutaneous biliary drainage, respectively ($P$ = 0.026). Of these 171 patients, there were 161 patients treated between January 2000-January 2018 and ten patients treated between February 2018 and March 2019.

### Radiotherapy

A total of 111 patients (65%) underwent preoperative radiotherapy (3 times 3.5 Gy) to reduce the risk of seeding metastases (Figure 1). As part of another study[12], 13 patients were additionally treated with postoperative radiotherapy (as well as preoperative radiotherapy) between 2000 and 2001 (20 × 2.2 Gy).

### Intraoperative bile cytology

After 2009, intraoperative bile cytology was obtained in 76 of 125 patients (61%). Bile cytology showed (suspicion of) tumor cells in 24 of 76 patients (32%) and was negative in 33 of 76 patients (45%). In the remaining patients, bile cytology was inconclusive in 9 (12%) and not assessable in 10 of 76 patients (14%). Malignant cells were found in the conclusive bile samples of 18 of 41 patients (44%) treated with radiotherapy and in 6 of 16 (37%) patients not treated with radiotherapy. No difference in the distribution of positive or negative bile cytology was observed between patients who were treated with preoperative radiotherapy or not ($P$ = 0.660).

### Seeding metastases

Two patients with metastases in the laparotomy scar (1.2%) were identified (Figure 1). Both patients had been treated with preoperative radiotherapy and one patient was additionally treated with postoperative radiotherapy. Histopathology showed moderately differentiated adenocarcinoma in both cases and both resections were R1 resections. The seeding metastases occurred 21 and 17 mo after resection, respectively. Overall, the incidence of seeding metastases was 1.4% (1 of 81 patients) after endoscopic biliary drainage, 2% (1 of 50 patients) after combined endoscopic and percutaneous biliary drainage, and 0% after both percutaneous biliary drainage (14 patients) and no biliary drainage (26 patients) ($P$ = 0.852).

## DISCUSSION

The hypothesis underlying this study was that low-dose preoperative radiotherapy eradicates free-floating vital tumor cells in bile, thereby reducing the risk of seeding metastases incurred during bile spill at the time of resection. The outcome of this study including 171 patients who had undergone resection for PHC, was that only two patients (1.2%) developed seeding metastases (both at the laparotomy scar). Intraoperative bile cytology showed no significant difference in the presence of tumor cells in bile of patients who underwent preoperative radiotherapy or not.

To our knowledge, literature on application of preoperative radiotherapy in patients with resectable PHC from other centers is lacking. As mentioned above, preoperative radiotherapy was introduced at our institution based on a 20% incidence of seeding metastases reported back in 1999 that has shown a striking decrease to currently 1.2%, suggesting a positive effect of preoperative radiotherapy. However, Wiggers *et al*[11] published a combined analysis of 234 patients from our institution (Academic Medical Center, Amsterdam and Memorial Sloan Kettering Cancer Center, New York)[11]. This study included 106 patients from the Academic Medical Center (Amsterdam) who were subjected to preoperative radiotherapy and 128 patients from the Memorial Sloan Kettering Cancer Center (New York) were no preoperative radiotherapy is given. The difference in the percentage of seeding metastases between both centers (2 of 106; 1.9% *vs* 5 of 128; 3.9% $P$ = 0.46) was not significant and the overall incidence was low (3.0%)[11]. Therefore, evidence of a potential benefit of low dose preoperative radiotherapy

**Table 1 Characteristics of patients undergoing resection for perihilar cholangiocarcinoma**

| Characteristics | Patients with PHC (*n* = 171) |
|---|---|
| Age, mean ± SD | 64 (10) |
| Male, *n* (%) | 104 (61) |
| ASA, *n* (%) | |
| 1 | 30 (18) |
| 2 | 112 (65) |
| 3 | 27 (16) |
| Missing | 2 (1) |
| Drainage, *n* (%) | |
| None | 26 (15) |
| Endoscopic biliary drainage | 81 (48) |
| Percunatenous biliary drainage | 14 (8) |
| Both | 50 (29) |
| Bismuth-Corlette type, *n* (%) | |
| I | 4 (2) |
| II | 12 (7) |
| IIIa | 85 (50) |
| IIIb | 42 (25) |
| IV | 24 (14) |
| Missing | 4 (2) |
| Portal vein embolization, *n* (%) | 20 (12) |
| Radiotherapy, *n* (%) | 111 (65) |
| Resection type, *n* (%) | |
| Left hemihepatectomy | 61 (36) |
| Extended left hemihepatectomy | 6 (4) |
| Right hemihepatectomy | 40 (23) |
| Extended right hemihepatectomy | 47 (27) |
| Minor liver resection | 4 (2) |
| External bile duct resection only | 13 (8) |
| + Pancreatoduodenectomy | 4 (2) |
| + Portal vein reconstruction | 43 (26) |
| 90-d mortality, *n* (%) | 22 (13) |
| 90-d morbidity, *n* (%) | 126 (74) |
| Severe morbidity (Clavien-Dindo ≥ 3), *n* (%) | 96 (56) |
| Recurrence, *n* (%) | 61 (36) |
| Local recurrence | 37 (22) |
| Liver metastases | 10 (6) |
| Lung metastases | 2 (1) |
| Peritoneal metastases | 7 (4) |
| Other | 5 (3) |

SD: Standard deviation; ASA: American society of anesthesiologists.

cannot be delivered, since this would require a randomized study with approximately 1200 patients per arm, which is not feasible for such a rare disease. In view of the incidence of local and distant recurrences and the overall prognosis of PHC, the overall incidence of seeding metastases is clinically less relevant. Hence, in January 2018 we decided to discontinue low dose preoperative radiotherapy. Interestingly, the potential of treatment with stereotactic body radiation therapy for patients with unresectable perihilar cholangiocarcinoma is currently being investigated by multiple group[13,14].

The concept of 3 times 3.5 Gy preoperative radiation therapy to reduce the risk of seeding metastases was based on a study in bladder carcinoma first published in 1969[15]. Follow-up studies on the effect of preoperative low-dose radiotherapy in bladder carcinoma however, are lacking while low-dose radiotherapy seems to have been abandoned in these patients. In current literature, indeed, a trend of a lower seeding metastases rate is observed (Table 2)[5-10,16]. For example, incidence reported by investigators from Hokkaido University Hospital decreased from 6.3% (3 of 48)[5] to 4.5% (3 of 67)[16] in their publications from 2011 and 2014, respectively. Likewise in Nagoya University Hospital, catheter tract recurrence was reported to be 5.6% (19 of 339) in a study performed by Kim *et al*[10] in 2015[10], whilst this decreased to 3.6% (6 of 168) in a study published in 2017[6]. Surgical techniques for resection of PHC have been refined reducing bile spill, which may have contributed to this decrease.

Our reported incidence of 1.2%, based one of the largest series on this topic, remains lower than the incidence reported in other studies (up to 6.3%)[5-10,16]. Several factors may contribute to the development of seeding metastases. As mentioned above, preoperative biliary drainage is thought to increase the risk of seeding metastases by enhancing bile contamination with tumor cells. There are some drainage related factors that potentially influence the development of seeding metastases, one of them being the duration of biliary drainage. A recently published study by Komaya *et al*[6], including 341 resected PHC patients, did not describe the duration of biliary drainage in their patients, although in another study originating from the same group, duration of percutaneous biliary drainage of 60 d or more was identified as an independent risk factor for the development of catheter tract recurrence in distal and perihilar cholangiocarcinoma[7]. In other studies reporting on seeding metastases, duration of biliary drainage was often not reported. In our series, the median duration of biliary drainage of 73 d in case of endoscopic biliary drainage and 77 d after percutaneous biliary drainage. Although this would suggest an increased risk in the majority of our patients, the overall incidence was low (1.2%). Therefore, we cannot fully exclude the possibility that there was an effect of preoperative radiotherapy based on this series alone.

Related to biliary drainage, the type (endoscopic or percutaneous), placement (proximal or transtumoral) may also play a role. Komaya *et al*[6] showed that the incidence of seeding metastases was higher after percutaneous biliary drainage (44 of 165, 26.7%) compared to endoscopic biliary drainage (25 of 150, 16.7%). Their definition of seeding metastases included right-sided pleural dissemination as well as peritoneal dissemination and catheter tract recurrences, due to the use of the transpleural approach of percutaneous biliary drainage in this cohort, resulting in relatively high seeding metastases rates. Separate analysis of catheter tract recurrence, showed occurrence of catheter tract recurrence in 6 of 164 patients (3.7%) undergoing percutaneous drainage, while recurrence at the laparotomy scar was not described[6]. In contrary, the previously mentioned study by Wiggers including 234 patients showed no significant difference in the occurrence of seeding metastasis between different drainage approaches: 3.4% after percutaneous and 2.7% after endoscopic biliary drainage ($P = 0.71$)[11].

This study has several limitations, the main limitation being the retrospective design of this single-center study, introducing a significant risk of bias. A direct comparison between patients undergoing preoperative radiation to those who did not is not possible within this study. Before January 2018, patients who did not undergo preoperative radiotherapy (35%) were not randomly assigned and most likely there was a reason not to treat them with preoperative radiotherapy (for example because they did not undergo biliary drainage or for logistic reasons). For some patients treated after stopping preoperative radiotherapy in January 2018, follow-up might be too short for seeding metastases to develop, as seeding metastases occurred after a median of 17 mo in the study by Wiggers *et al*[11]. In addition, due to the absence of standardized follow-up imaging after resection, not all seeding metastases or recurrences may have been detected. When comparing cytology results of patients with and without radiotherapy, we could not account for viability of the cells. Although cells may stain positively, this does not mean that these cells are still viable

**Table 2 Overview of studies reporting on incidence of seeding metastases in patients with perihilar cholangiocarcinoma**

| | Incidence | Patients |
|---|---|---|
| Sakata *et al*[8], 2005 | 3/67 (4.4%), cumulative 6% | Niigata University Graduate School of Medical and Dental Sciences; Percutaneous biliary drainage, 1998-2002 |
| Kang *et al*[9], 2013 | 6/232 (2.6%) + 8 abdominal wall/wound implant metastases (3.4%) | Seoul National University Hospital; Percutaneous biliary drainage, 1991-2011 |
| Kim *et al*[10], 2015 | 2/52 (3.8%) | Samsung Medical Center Seoul; Percutaneous biliary drainage, 2000-2012 |
| Kawakami *et al*[5], 2011 | 3/48 (6.3%) | Hokkaido University Hospital; Percutaneous biliary drainage, 1999-2009 |
| Hirano *et al*[16], 2014 | 3/67 (4.5%) | Hokkaido University hospital; Percutaneous biliary drainage, 2000-2008 |
| Takahashi *et al*[7], 2010 | 19/339 (5.6%) | Nagoya University hospital, 1977-2007 |
| Komaya *et al*[6], 2017 | 6/168 (3.6%) | Nagoya University Hospital Percutaneous biliary drainage, 2003-2012 |
| Ten Hoopen *et al*[1], 1999 | 8/41 (20%) | AMC Amsterdam; Biliary drainage, 1983-1990 |
| Wiggers *et al*[11], 2013 | 2/106 (1.9%) | AMC Amsterdam; Percutaenous + endoscopic biliary drainage, 1991-2012 |

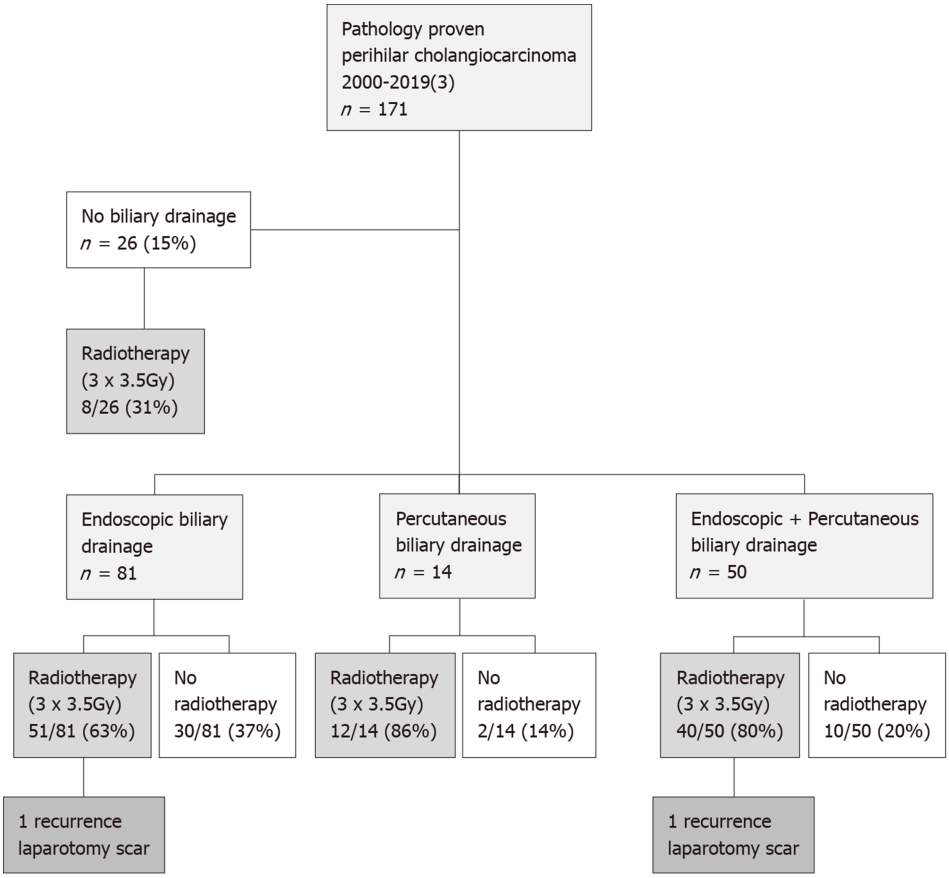


**Figure 1 Flowchart of patients with perihilar cholangiocarcinoma.**

and therefore, we could have overlooked an effect of radiotherapy. Due to the overall low number of events (seeding metastases), proper statistical analysis was not feasible in this series and confidence limits are huge.

## CONCLUSION

The incidence of seeding metastases in patients with resected PHC has decreased and is currently low (1.2% in our institution). As the only center in the world reporting on preventive low-dose radiotherapy, we were unable to deliver evidence of a potential benefit of preoperative radiotherapy and therefore preoperative radiotherapy in patients with resectable PHC has been discontinued in our institution.

## ARTICLE HIGHLIGHTS

### *Research background*

Routine preoperative radiotherapy (3 times 3.5 Gray) to prevent the occurrence of seeding metastases was used in our tertiary center for 28 years in patients undergoing resection for perihilar cholangiocarcinoma.

### *Research motivation*

Previous research from our department showed that seeding metastases occurred in up to 20% of the patients undergoing resection of perihilar cholangiocarcinoma.

### *Research objectives*

To investigate the occurrence of seeding metastases among patients with resectable perihilar cholangiocarcinoma.

### *Research methods*

A retrospective study was conducted, including all patients undergoing resection of perihilar cholangiocarcinoma in a larger tertiary center between 2000 and March 2019.

### *Research results*

Seeding metastases occurred in 2 out of 171 patients (1.2%) undergoing resection for perihilar cholangiocarcinoma. These seeding metastases occurred at the laparotomy scar in both patients, after 17 and 21 mo, respectively. Intraoperative bile cytology showed no significant difference in the presence of tumor cells in the bile of patients undergoing preoperative radiotherapy or not.

### *Research conclusions*

The incidence of seeding metastases in patients with resected perihilar cholangiocarcinoma has decreased. Evidence of a potential benefit of preoperative radiotherapy could not be delivered and therefore preoperative radiotherapy in patients with resectable perihilar cholangiocarcinoma has been discontinued in our institution.

### *Research perspectives*

As we are the only center reporting on the use of low-dose radiotherapy to prevent seeding metastases in patients with perihilar cholangiocarcinoma, it is unlikely that other reports on this topic will appear and future research may focus more on the potential of stereotactic body radiation therapy to treat patients with unresectable perihilar cholangiocarcinoma.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1098-1114

DOI: 10.4254/wjh.v12.i11.1098

ISSN 1948-5182 (online)

ORIGINAL ARTICLE

**Randomized Controlled Trial**

# Metalloproteinase expression after desflurane preconditioning in hepatectomies: A randomized clinical trial

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**Author contributions:** Koraki E, Tsalis K, Kesisoglou I and Mantzoros I designed the research; Chatzakis C, Gkiouliava A, Cheva A, Lavrentieva A, Sifaki F and Argiriadou H analyzed and interpreted the data; Koraki E, Mantzoros I, Chatzakis C, Gkiouliava A, Galanos-Demiris K and Bitsianis S wrote the paper.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board of the General Hospital of Thessaloniki, Georgios Papanikolaou, No. 763/26.06.2016.

**Clinical trial registration statement:**

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## Abstract

*BACKGROUND*

Hepatectomy with inflow occlusion results in ischemia-reperfusion injury; however, pharmacological preconditioning can prevent such injury and optimize the postoperative recovery of hepatectomized patients. The normal inflammatory response after a hepatectomy involves increased expression of metalloproteinases, which may signal pathologic hepatic tissue reformation.

*AIM*

To investigate the effect of desflurane preconditioning on these inflammatory indices in patients with inflow occlusion undergoing hepatectomy.

*METHODS*

The study is registered at clinicaltrials.gov. The registration identification number is NCT03848780.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** The authors declare no conflicts of interest.

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

**CONSORT 2010 statement:** The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.



**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Greece

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

**Received:** July 21, 2020
**Peer-review started:** July 21, 2020
**First decision:** September 14, 2020
**Revised:** September 26, 2020

This is a single-center, prospective, randomized controlled trial conducted at the 4th Department of Surgery of the Medical School of Aristotle University of Thessaloniki, between August 2016 and December 2017. Forty-six patients were randomized to either the desflurane treatment group for pharmacological preconditioning (by replacement of propofol with desflurane, administered 30 min before induction of ischemia) or the control group for standard intravenous propofol. The primary endpoint of expression levels of matrix metalloproteinases and their inhibitors was determined preoperatively and at 30 min posthepatic reperfusion. The secondary endpoints of neutrophil infiltration, coagulation profile, activity of antithrombin III (AT III), protein C (PC), protein S and biochemical markers of liver function were determined for 5 d postoperatively and compared between the groups.

*RESULTS*

The desflurane treatment group showed significantly increased levels of tissue inhibitor of metalloproteinases 1 and 2, significantly decreased levels of matrix metalloproteinases 2 and 9, decreased neutrophil infiltration, and less profound changes in the coagulation profile. During the 5-d postoperative period, all patients showed significantly decreased activity of AT III, PC and protein S (*vs* baseline values, $P < 0.05$). The activity of AT III and PC differed significantly between the two groups from postoperative day 1 to postoperative day 5 ($P < 0.05$), showing a moderate drop in activity of AT III and PC in the desflurane treatment group and a dramatic drop in the control group. Compared to the control group, the desflurane treatment group also had significantly lower international normalized ratio values on all postoperative days ($P < 0.005$) and lower serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase values on postoperative days 2 and 3 ($P < 0.05$). Total length of stay was significantly less in the desflurane group ($P = 0.009$).

*CONCLUSION*

Desflurane preconditioning can lessen the inflammatory response related to ischemia-reperfusion injury and may shorten length of hospitalization.

**Key Words:** Desflurane; Preconditioning; Hepatectomy; Inflammation; Metalloproteinases; Reperfusion injury



**Core Tip:** Ischemia-reperfusion injury remains a leading cause of morbidity and mortality in hepatectomies. In our study, 46 patients were randomly and equally allocated to receive pharmacological preconditioning with desflurane (intervention group) or not (control group) to compare inflammatory indices between the two groups. We found significantly reduced levels of matrix metalloproteinases 2 and 9, increased levels of tissue inhibitor matrix metalloproteinases 1 and 2, and decreased neutrophil infiltration in the intervention group. Thus, hepatoprotective strategies may ameliorate the pathophysiologic effects of ischemia-reperfusion during liver surgery, with our study suggesting a novel promising strategy that may benefit patients postoperatively.

**Citation:** Koraki E, Mantzoros I, Chatzakis C, Gkiouliava A, Cheva A, Lavrentieva A, Sifaki F, Argiriadou H, Kesisoglou I, Galanos-Demiris K, Bitsianis S, Tsalis K. Metalloproteinase expression after desflurane preconditioning in hepatectomies: A randomized clinical trial. *World J Hepatol* 2020; 12(11): 1098-1114
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1098.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1098

**Accepted:** October 12, 2020
**Article in press:** October 12, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Li YY, Tan X, Luo H
**S-Editor:** Gao CC
**L-Editor:** Webster JR
**P-Editor:** Wang LL

## INTRODUCTION

Hepatectomy is one of the most frequently performed strategies to treat benign and malignant liver diseases, and the number of patients undergoing hepatectomy is increasing[1]. Unfortunately, the ischemia-reperfusion (IR) technique in the hepatectomy procedure can cause liver damage and represents one of the most important reasons for postoperative liver failure[2]. When prolonged ischemia is applied to a tissue, the cellular metabolism inevitably becomes anaerobic, leading to loss of cellular function and ultimately cell death[3,4]. The extent of the resection along with IR injury (IRI) results in tissue trauma and triggers the acute phase response. It consequently impacts the physiology of the liver and affects the mechanisms underlying production of clotting factors, including that of inflammatory cytokines, chemokines and complement products, and the recruitment of neutrophils to the site of injury[5,6]. Indeed, previous studies have demonstrated increased neutrophil infiltration in the livers of animals which have suffered from IRI and that exposure to pharmacological agents attenuating neutrophil activities leads to milder hepatic IRI[7-9].

The inflammatory response complicating IRI leads to induction of matrix metalloproteinases (MMPs), which are produced by hepatic stellate cells, Kupffer cells and hepatocytes[10]. While the MMPs in general play a major role in tissue remodeling and molecular signaling, and correlate with the inflammation process, MMP2 and MMP9 are expressed under pathological conditions and are responsible for the extracellular matrix disruption linked to IRI[11,12]. Parallel to this, the balance between MMPs and their endogenous inhibitors (known as the tissue inhibitors of metalloproteinases, or TIMPs) regulate their activity in pathologic conditions[13].

Pharmacological preconditioning may limit the anticipated rise in the concentration of MMPs following hepatic IRI. Although this procedure has been studied extensively in cardiac surgery[14-16], its efficacy in hepatic surgery remains unknown. Desflurane, a volatile anesthetic in routine clinical use, protects hepatic blood flow. It is also known to cause less toxicity to the liver than other volatile anesthetics, to have minor biological degradation, with a 0.02% calculated metabolism ratio, and to be less soluble in plasma and tissues[17]. Moreover, it was found to be superior to total intravenous anesthesia, regarding patient outcomes following liver surgery[18,19].

Taking the role of MMPs and pharmacological preconditioning in IRI into consideration, we aimed to investigate the effect of preconditioning with desflurane on MMP induction.

## MATERIALS AND METHODS

This single-center, prospective, randomized controlled trial was conducted in patients undergoing liver resection at the 4$^{th}$ Department of Surgery of the Medical School of Aristotle University of Thessaloniki, between August 2016 and December 2017. Patients were randomized, in a 1:1 ratio, into the hepatectomy with pharmacological preconditioning with desflurane group (intervention group) or the hepatectomy without preconditioning group (control group). The study was carried out following review and approval by the Institutional Review Board of the General Hospital "Georgios Papanikolaou" where the 4$^{th}$ Surgical Department of Aristotle University in Thessaloniki is located. Written informed consent was obtained from all participants. The study was also conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and is registered at clinicaltrials.gov (NCT03848780).

Patients eligible for study inclusion were older than 18 years of age and undergoing an elective extensive hepatic resection that included more than two segments of the liver. Exclusion criteria were infection with hepatitis B or C or human immunodeficiency virus, liver cirrhosis, autoimmune disease, inflammatory bowel disease, or pregnancy. Patients who received additional ablation therapies (cryosurgery or radiofrequency) or liver resections without inflow occlusion were also not eligible for study inclusion. During the study, there were no changes in the eligibility criteria.

Ultimately, 46 consecutive patients undergoing elective major hepatectomy were included in this prospective study. The patients were randomized equally into either the intervention group (receiving preconditioning with desflurane) or the control group. The randomization sequence was generated by a computer and sealed in envelopes. Each patient was operated by the same experienced hepatobiliary surgical team, who were all blinded to the intervention assignment.

### Anesthetic technique

The preoperative physical status of the patients was categorized into classes, according to the criteria of the American Society of Anesthesiologists (commonly known as ASA). Standard monitoring, along with invasive blood pressure, cardiac output/stroke volume variation, a central venous line and depth of anesthesia (measured with the bispectral index), were conducted routinely. General anesthesia was accomplished with 3 μg/kg fentanyl, 2-2.5 mg/kg propofol, 1 mg/kg lidocaine, and 0.2 mg/kg cis-atracurium. Anesthesia was maintained by the continuous infusion of 0.05-0.1 mg/kg/min propofol, 5 μg/kg fentanyl, 2-4 mg boluses cis-atracurium (according to clinical need) and 0.3-0.6 μg/kg/min remifentanil. Both the control and desflurane groups underwent the same protocol for administration of fentanyl and remifentanil.

In patients with preconditioning, propofol anesthesia was replaced by desflurane according to previous practice for pharmacological preconditioning in IR[20]. Specifically, at 30 min before the induction of ischemia, propofol administration was stopped and replaced by desflurane, set to achieve a minimal alveolar concentration of 1 (induction time of 5 min). The pharmacological preconditioning was performed for 20 min, after which the following 5 min were used to cease desflurane administration and reinitiate propofol (with washout of 5 min). Hepatic ischemia was then begun. The hemodynamic tolerance to clamping was the same in both groups.

### Surgical technique

Surgical procedures were performed in a standardized manner, by the same experienced hepatobiliary surgeon. At 30 min before clamping of the portal triad, the anesthesiologist was informed as to whether a pharmacological preconditioning with desflurane was to be performed or not, according to the randomized assignment. The surgeon remained blinded to the assignment for the entire operation. During resection, we aimed for a low central venous pressure (0-5 mmHg). Liver transection was performed by parenchyma crushing, using a 3-mm tip Kelly clamp. Vessels ≤ 2 mm were coagulated at 120W with the irrigated bipolar forceps. Clipping or ligation was applied for all other elements. A stapler device was used only for the transection of hepatic veins. The Pringle maneuver was applied intermittently and the cumulative duration was at least 30 min. Specifically, every interval of 10 min of inflow occlusion was followed by 5 min reperfusion time[21]. Postoperatively, all patients in both groups received thromboprophylaxis with tinzaparin.

### Study endpoints

**Primary endpoints:** Primary outcomes of the study were the serum levels of MMP2, MMP9, TIMP1 and TIMP2 in both groups. Blood samples were taken preoperatively and at 30 min after hepatic reperfusion had been permanently established. The samples were tested to determine the relative gene expression using real-time polymerase chain reaction (PCR). The comparative log fold-change method (also referred to as the $2^{-\Delta\Delta CT}$ method) was used to calculate the fold-change and then convert it to a percentage.

**Secondary endpoints:** A sample of hepatic tissue was taken immediately before ischemia induction and at 30 min after liver reperfusion, for histological analysis. Hematoxylin-eosin staining was used to assess the degree of steatosis, while Gomori and Masson staining was used to determine the level of fibrosis. Steatosis was characterized (100 × magnification) as mild (10%-30%), moderate (30%-60%) or severe (> 60%), according to the presence of fat droplets in the hepatic cells. Fibrosis was graded based on the METAVIR score, with absence graded as F0, portal fibrosis without septa as F1, with rare septa as F2 or numerous septa as F3, and cirrhosis as F4.

Coagulation markers and the activity of anticoagulation factors were monitored perioperatively and followed for the first 5 d after liver resection. Blood samples were collected preoperatively and on postoperative days (PODs) 1-5. Standard coagulation tests, international normalized ratio (INR), prothrombin time (PT), activated partial thromboplastin time (aPTT), the fibrin degradation product D-dimer (D-d), platelet count, and natural anticoagulant activity levels [antithrombin III (AT III), protein C (PC), and protein S (PS)] were measured and compared between the two groups using chromogenic assays for plasma AT III and PC and electro-immunodiffusion assay for free PS (all from Dade-Behring Inc., Deerfield, IL, United States). The AT III assay had normal values at 80%-120%, an intra-assay coefficient of 7.7% and inter-assay coefficient of 8.2%. The PC assay had normal values at 70%-130%, an intra-assay coefficient of 8.1% and inter-assay coefficient of 8.6%. The PS assay had normal values of 70%-130%, an intra-assay coefficient of 8.2% and inter-assay coefficient of 8.7%. To

assess activation of the fibrinolytic system, fibrinogen and D-d were measured, with the latter measured by latex semiquantitative assay (Diagnostica Stago, Asnières-sur-Seine, France) wherein a negative result was indicated by concentrations of < 0.5 μg/mL.

The lengths of intensive care unit and hospital stays were also recorded. In addition, biochemical markers of liver function, including aspartate aminotransferase, alanine transaminase, total bilirubin, gamma-glutamyl transferase and alkaline phosphatase, were evaluated in all patients preoperatively and up to POD 5. No changes were made to the trial outcomes after the trial commenced.

### Statistical analysis

In order to determine the appropriate sample size of the study, power analysis was carried out in G*Power (version 3.1.7; Universität Kiel, Germany). Data from a previous study[20] were used as a proxy of the anticipated findings in the present study, with 80% power and 5% significance level. Power calculation resulted in 20 patients per group.

The independent samples *t*-test and independent samples Mann-Whitney test were implemented for group (intervention *vs* control) comparisons. Furthermore, Fisher's exact test and the $\chi^2$ test were used for comparisons of groups relative to qualitative variables. Data on biological markers were analyzed within the frame of general linear models with the ANOVA method, according to the model which involves one factor between patients (factor "group" with two levels) and one factor within patients (factor "time" with five levels, with repeated measures). Comparisons of means were carried out with the least significant difference criterion. The significance level was preset at $P \leq 0.05$ for all hypotheses testing procedures. The SPSS v.22.0 software (IBM Corp., Armonk, NY, United States) was utilized for all statistical analyses. All data were subjected to the Kolmogorov-Smirnov test and found to be normally distributed; as such, they are presented as mean ± standard deviation in the tables. The statistical methods used in this study were reviewed by Anna Bettina Haidich, Assistant Professor in Hygiene-Medical Statistics Department of the Aristotle University of Thessaloniki.

## RESULTS

### Patients' characteristics

The selection process is displayed in Figure 1, as a flow diagram. Patients' characteristics are presented in Table 1. The leading reason for liver resection among the study population was hepatic metastasis ($n$ = 20). There was no significant difference between the two groups in terms of sex ($P$ = 0.719), age ($P$ = 0.612), body mass index (BMI) or ASA class ($P$ = 0.963).

The mean duration of ischemia during the hepatectomies was 61 ± 30 min in the intervention group and 55 ± 26 min in the control group ($P$ = 0.654). Median duration of operation was 300 min (range: 241-380 min) in the intervention group and 270 min (range: 210-340 min) in the control group ($P$ = 0.364). There was no statistically significant difference between the two groups in the units of blood administered intraoperatively (Table 1). The median length of intensive care unit stay after liver resection was also similar between the two groups [1 (1-2) *vs* 1 (1-2) d; $P$ = 0.373]; however, a shorter hospital stay was observed for patients undergoing liver resection with desflurane preconditioning [9.5 (8-11) d *vs* 12 (11-20.3) d; $P$ = 0.009]. Postoperative complications are presented in Table 2.

### Primary endpoints

**MMPs:** After hepatic IR, the control group showed significantly higher levels of both MMP2 and MMP9 (*vs* the intervention group; Table 3).

**TIMPs:** After hepatic IR, the intervention group showed significantly higher levels of both TIMP1 and TIMP2 (*vs* the control group; Table 3).

### Secondary endpoints

**Neutrophil infiltration:** After hepatic IR, the control group showed a significantly higher infiltration of neutrophils (*vs* the intervention group; Table 4).

**Hepatic fibrosis and steatosis:** After hepatic IR, the rates and grades of fibrosis and steatosis were not significantly different between the two groups (Table 4). Also, after

**Table 1 Patients' characteristics**

| Characteristics | Desflurane group, *n* = 23 | Control group, *n* = 23 | *P* value |
|---|---|---|---|
| Age, yr | 64.5 ± 10.6 | 61.5 ± 11,4 | 0.612 |
| Males | 12 (52.1) | 14 (60.8) | 0.719 |
| BMI, kg/m$^2$ | 25.6 ± 5.1 | 26.2 ± 4.8 | 0.953 |
| Cause of liver resection | | | 0.914 |
| Hepatic cancer | 10 (44) | 9 (39) | |
| Liver metastasis | 9 (39) | 11 (48) | |
| Hemangioma | 1 (4.3) | 1 (4.3) | |
| Klatskin tumor | 2 (8.6) | 1 (4.3) | |
| Focal nodular hyperplasia | 1 (4.4) | 1 (4.3) | |
| No of tumors | | | 0.772 |
| Solitary | 14 (60.9) | 12 (52.2) | |
| 2 | 5 (21.7) | 7 (30.4) | |
| 3 | 3 (13) | 4 (17.4) | |
| 4 | 1 (4.3) | 0 | |
| Size of largest tumor, cm | 12.5 ± 8.7 | 11.9 ± 7.5 | 0.711 |
| No of segments | | | 1 |
| 1 | 10 (43.5) | 11 (47.8) | |
| 2-3 | 8 (34.6) | 7 (30.4) | |
| > 3 | 5 (21.7) | 5 (21.7) | |
| Type of hepatectomy | | | 0.853 |
| Right extended | 3 (13) | 2 (8.6) | |
| Right | 8 (34.7) | 10 (43.5) | |
| Left | 10 (43.5) | 8 (34.7) | |
| Left extended | 2 (8.6) | 3 (13) | |
| Previous treatment status | | | 0.552 |
| No preoperative treatment | 14 (60.9) | 12 (52.2) | |
| Neo-adjuvant chemotherapy | 9 (39.1) | 11 (47.8) | |
| Intraoperative blood loss, mL | 630 ± 550 | 690 ± 580 | 0.847 |
| Intraoperative blood transfusion, units of red blood cells | | | 0.875 |
| 0 | 5 (21.7) | 5 (21.7) | |
| 1 | 6 (26) | 7 (30.4) | |
| 2 | 8 (34.7) | 7 (30.4) | |
| 3 | 3 (13) | 2 (8.6) | |
| 4 | 1 (4.3) | 2 (8.6) | |

Quantitative variables are expressed as mean and standard deviation, while qualitative variables are expressed as absolute (*n*) and relative values (%). The *t*-test was used for comparison of patient's age between the two groups. The $\chi^2$ test was used for sex (male). Fisher's exact test was used for etiologies of hepatectomy, type of hepatectomy, and intraoperative transfusion. BMI: Body mass index.

reperfusion, the findings were identical to baseline among the participants.

**Coagulation markers:** Upon intensive care unit admission, all patients had significant acquired reduction in the activity of AT III, PC and PS. However, the postoperative activity of AT III (Figure 2) and PC (Figure 3) differed significantly between the two

**Table 2 Postoperative complications, *n* (%)**

| Postoperative complications | Desflurane group, *n* = 23 | Control group, *n* = 23 | *P* value |
|---|---|---|---|
| Postoperative blood transfusion (red blood cells) | | | 0.555 |
| 0 U | 13 (56.5) | 11 (47.8) | |
| 1 U | 10 (43.5) | 10 (43.5) | |
| 2 U | 0 | 2 (8.7) | |
| Bile leak | 2 (8.7) | 2 (8.7) | 1.00 |
| Deep vein thrombosis | 2 (8.7) | 1 (4.3) | 0.555 |
| Pneumonia | 4 (17.4) | 5 (21.7) | 0.71 |

Variables are expressed as absolute and relative values (%). Fisher's exact test was used for comparisons between the two groups.

**Table 3 Matrix metalloproteinases and tissue inhibitors of metalloproteinases in the different groups**

| | Groups | | *P* (95%CI) |
|---|---|---|---|
| MMP2 | Desflurane | 80.6 ± 42.3 | [a](-46.4 to 3.1) |
| | Control | 123.5 ± 51.7 | |
| MMP9 | Desflurane | 178.5 ± 80.3 | [b](-473.9 to 203.1) |
| | Control | 449.9 ± 298.8 | |
| TIMP1 | Desflurane | 173.3 ± 87.6 | [b](25.9 to 104.3) |
| | Control | 104.4 ± 39.7 | |
| TIMP2 | Desflurane | 154.4 ± 65.4 | [b](35.7 to 93.6) |
| | Control | 90.6 ± 29.3 | |

[a]$P < 0.05$ statistical significance *vs* intervention group.
[b]$P < 0.01$ statistical significance *vs* intervention group. Results are expressed as means and standard deviation. The *t*-test was used for between-groups comparisons, resulting in *P* values and 95% confidence intervals (CIs, in brackets). MMP: Matrix metalloproteinase; TIMP: Tissue inhibitors of metalloproteinase; CI: Confidence interval.

groups from POD 1 to POD 5. The intervention group showed a postoperative moderate drop in the activities of both AT III and PC, while the control group showed a substantial drop (Table 5).

**INR and aPTT:** Both groups showed significantly elevated INR postoperatively. At baseline, there was no difference in INR between the two groups; however, from POD 1 to POD 5, INR in the intervention group was significantly lower than that in the control group (Figure 4, Table 5). The aPTT did not differ significantly between the two groups (Table 6).

**Platelets:** From POD 1, all patients showed a reduction in platelets in comparison to preoperative levels ($P < 0.05$), although no differences were observed in platelet levels between the two groups (Table 6).

**Fibrinogen and D-d:** Although fibrinogen and D-d levels increased from POD 1 to POD 5 in all patients ($P < 0.05$), no statistically significant differences were observed between the two groups (Table 6).

**White blood cell count:** White blood cell count was significantly lower in the intervention group from POD 1 to POD 5, in comparison to that in the control group ( $P < 0.02$) (Table 6).

**Serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase:** Both serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase increased in the intervention and control groups in POD 2 and remained elevated until POD 3. However, the intervention group showed a less extensive

**Table 4 Histological findings between the different groups, *n* (%)**

| Score | Desflurane group, *n* = 23 | Control group, *n* = 23 | *P* value |
|---|---|---|---|
| Neutrophil infiltration | | | |
| 0 | 9 (39) | NA | [b] |
| 1 | 9 (39) | 5 (23) | |
| 2 | 5 (23) | 9 (39) | |
| 3 | NA | 9 (39) | |
| Fibrosis | | | |
| 0 | 11 (48) | 7 (30) | 0.767 |
| 1 | 7 (30) | 9 (39) | |
| 2 | 3 (13) | 4 (18) | |
| 3 | 2 (9) | 3 (13) | |
| Steatosis | | | |
| 0 | 11 (48) | 9 (39) | 0.780 |
| 1 | 10 (43) | 10 (43) | |
| 2 | 2 (9) | 4 (18) | |
| 3 | | | |

[a]$P < 0.05$ statistical significance *vs* intervention group.
[b]$P < 0.01$ statistical significance *vs* intervention group. Results are expressed as absolute and relative values. The $\chi^2$ test or Fisher's exact test was used for comparisons between the groups. NA: Not available.

increase on those days ($P < 0.05$). There were no significant differences in gamma-glutamyl transferase or total bilirubin concentrations between the two groups (Table 6).

## DISCUSSION

Pharmacological preconditioning as a strategy for IRI prevention in humans has been the focus of researchers in many fields of medicine[14,15], especially hepatic IRI. Pharmacological interventions that transiently subject the liver to ischemic conditions may lessen the systemic inflammatory response after surgery and enhance liver function[22,23]. The inflammatory response complicating IR leads to induction of MMPs[10], which have a key role in the inflammation process and their levels reflect the severity of that process[24-26]. Desflurane preconditioning has been promisingly associated with the upregulation of antiapoptotic molecules in an IRI model[27]. We found that levels of MMP2 and MMP9 were significantly increased in the control group, in comparison to the desflurane preconditioning intervention group. These findings indicate, for the first time in a clinical trial, that preconditioning with desflurane limits the anticipated rise in the concentration of MMPs following hepatic IRI.

In a laboratory model, MMP2 activity was found to be reduced after preconditioning in isolated rat hearts[28], while MMP2 inhibition halted IRI in a rat myocardium IR model[29]. In an inducible nitric oxide synthase knockout mouse study, researchers observed inhibition of MMP9 activity and mitigated leukocyte infiltration and liver trauma[30]; Romanic *et al*[31] reported similar results in MMP9 knockout mice. Taking into consideration the known physiologic and pathogenic actions of MMPs and enhanced liver function after their inhibition in research models, MMPs represent a significant topic of interest for investigation as they are directly involved in IR injury[32,33].

In the present study, we showed that the post-hepatectomy characteristic infiltration of neutrophils in the hepatic parenchyma was greater in the control group than in the desflurane preconditioning group of patients. During the inflammatory process,

**Table 5 Relative changes between baseline and postoperative values in the two groups**

| Change, % | AT III | *P* (95%CI) | PC | *P* (95%CI) | INR | *P* (95%CI) |
|---|---|---|---|---|---|---|
| Day 1 | | [a](4.6 to 15.4) | | [a](2.2 to 13.5) | | [a](-22.6 to -7.5) |
| Desflurane | -17.9 ± 16.7 | | -11.9 ± 13.2 | | 12.5 ± 17.1 | |
| Control | -27.9 ± 14.9 | | -20.9 ± 16.1 | | 27.5 ± 13.6 | |
| Day 2 | | [a](5.3 to 18.5) | | [b](9.7 to 18.1) | | [b](-39.5 to -11.7) |
| Desflurane | -15.4 ± 16.6 | | -8.2 ± 10.1 | | 14.5 ± 20.1 | |
| Control | -27.3 ± 18.9 | | -22.1 ± 16.5 | | 40.1 ± 31.1 | |
| Day 3 | | [b](14.3 to 23.7) | | [b](10.2 to 18.9) | | [a](-27.6 to -4.2) |
| Desflurane | -12.1 ± 16.6 | | -8.3 ± 11.9 | | 17.9 ± 15.6 | |
| Control | -31.1 ± 28.2 | | -22.9 ± 15.7 | | 33.6 ± 28.2 | |
| Day 4 | | [a](10.9 to 18.2) | | [a](5.6 to 15.5) | | [a](-26.8 to -4.1) |
| Desflurane | -12.3 ± 19.8 | | -1.4 ± 14.8 | | 14.4 ± 20.1 | |
| Control | -23.7 ± 16.2 | | -11.9 ± 15.4 | | 29.8 ±23.6 | |
| Day 5 | | [b](9.8 to 22.7) | | [a](2.5 to 14.3) | | [a](-23.7 to -2.2) |
| Desflurane | -3.8 ± 17.7 | | 3.3 ± 13.6 | | 7.7 ± 18.7 | |
| Control | -12.4 ± 17.4 | | -5.5 ±11.5 | | 22.2 ± 17.8 | |

[a]*P* < 0.05 statistical significance *vs* intervention group.
[b]*P* < 0.01 statistical significance *vs* intervention group. AT III: Antithrombin III; CI: Confidence interval; INR: International normalized ratio; PC: Protein C.

recruitment of neutrophils is facilitated by nuclear factor-κappa beta (NF-κβ) regulation of cytokine release [*e.g.*, tumor necrosis factor alpha (TNFα), interleukin (IL) 1, and IL8] and the production of adhesion molecules [*e.g.*, intercellular cell adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1][34]. In parallel, TNFα promotes NF-κB action as well as ICAM1 and MMP9 expression[35]. Desflurane can halt these pathways through its direct inhibition of expression of adhesion molecules[36], NF-κB and TNFα[37] all of which lead to decreased penetration of neutrophils in the hepatic tissue. Therefore, our results indicate that preconditioning with desflurane decreases the inflammatory response following hepatic IR, which in turn protects the hepatic tissue. Regarding the pathology of hepatic parenchyma, fibrosis and steatosis may be risk factors for intraoperative hemorrhage, transfusion and postoperative complications[38,39]. However, histological results in the two groups were similar.

Although, pharmacological anesthetic preconditioning, such as the administration of volatile and intravenous anesthetics has been extensively studied, no conclusive data recommend the use of a specific technique. In liver transplantation, especially in living liver donors, optimization of liver function is of outmost importance. Desflurane was questioned in this population and was found to be superior to sevoflurane and isoflurane in two studies comparing postoperative hepatic function[40,41]. Beck-Schimmer *et al*[20], in a randomized controlled trial of patients undergoing liver surgery, showed that ischemic preconditioning with sevoflurane before inflow occlusion dampened postoperative liver injury, even in patients with steatosis. Nguyen *et al*[42] also pointed that hepatectomy patients receiving sevoflurane, presented better liver function postoperatively. However, the protective effect of pharmacological preconditioning with volatile anesthetics on the remnant liver has been disputed by findings from other studies[43].

With regard to coagulation parameters, our study showed that in the desflurane preconditioning group, the activity of AT III and PC showed a moderate drop postoperatively, while the control group experienced a substantial drop. In addition, INR in the desflurane preconditioning intervention group was significantly lower in comparison to that in the control group, in the postoperative period. Coagulation system homeostasis is frequently impaired in patients undergoing major surgery, especially in those indicated for hepatectomy[44-46]. The coagulopathy present in surgical patients is associated with the marked depletion and decreased activity of the endogenous regulators of blood coagulation[47]. Numerous studies have provided

**Table 6 Postoperative course of coagulation and inflammation laboratory indices**

| Day | Group | WBCs ($10^3$/μL) | SGOT (U/L) | SGPT (U/L) | γ-GT (U/L) | Tbil (mg/dL) | Fib (g/L) | Plts ($10^9$/L) | D-dimer (μg/mL) | Protein S (%) | aPTT (s) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | Control | 6.1 ± 1.6 | 30 ± 13 | 44 ± 30 | 47 ± 26 | 0.78 ± 0.45 | 2.4 ± 0.5 | 229.2 ± 78 | 0.57 ± 0.3 | 83.5 ± 12 | 26 ± 1.4 |
| | Desflurane | 5.8 ± 1.8 | 29 ± 10 | 38 ± 33 | 39 ± 24 | 0.72 ± 0.36 | 2.4 ± 0.4 | 202.5 ± 92 | 0.55 ± 0.3 | 84.1 ± 14 | 26.2 ± 1.6 |
| *P* | | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |
| 1 | Control | 8 ± 2 | 32 ± 16 | 44 ± 32 | 50 ± 24 | 0.88 ± 0.48 | 3.6 ± 1 | 126.1 ± 32 | 3.8 ± 1.4 | 60.8 ± 11 | 28 ± 2 |
| | Desflurane | 6 ± 1.8 | 26 ± 10 | 39 ± 23 | 37 ± 22 | 0.69 ± 0.38 | 2.7 ± 0.6 | 168.3 ± 86 | 2.6 ± 1.3 | 64.8 ± 16 | 27.8 ± 3 |
| *P* | | [b] | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |
| 2 | Control | 11.6 ± 6.4 | 395 ± 256 | 373 ± 263 | 113 ± 96 | 1.91 ± 0.94 | 3.2 ± 0.8 | 130.5 ± 29 | 4.1 ± 0.9 | 60 ± 12 | 31 ± 4.8 |
| | Desflurane | 9.2 ± 4.7 | 197 ± 83 | 227 ± 170 | 140 ± 109 | 1.65 ± 0.83 | 2.9 ± 0.5 | 160.2 ± 81 | 3.7 ± 1.1 | 65.4 ± 14 | 28 ± 3.9 |
| *P* | | [a] | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |
| 3 | Control | 12.5 ± 3.8 | 543 ± 395 | 395 ± 326 | 129 ± 106 | 2.29 ± 1.22 | 2.9 ± 0.7 | 137.8 ± 89 | 4.2 ± 2.3 | 59.9 ± 13 | 29.5 ± 3.7 |
| | Desflurane | 10.1 ± 2 | 256 ± 140 | 197 ± 83 | 131 ± 70 | 2.4 ± 1.72 | 3.06 ± 0.6 | 146.2 ± 54 | 4.9 ± 3.1 | 66.6 ± 14 | 27.2 ± 2.5 |
| *P* | | [b] | [a] | [a] | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |
| 4 | Control | 11.7 ± 2.9 | 187 ± 109 | 304 ± 236 | 124 ± 107 | 1.31 ± 0.72 | 3 ± 0.4 | 166.4 ± 51 | 3.9 ± 1.4 | 67 ± 12 | 28 ± 2.6 |
| | Desflurane | 9.9 ± 2.1 | 137 ± 102 | 177 ± 106 | 101 ± 93 | 1.6 ± 1.1 | 3.5 ± 0.9 | 157.2 ± 41 | 4.1 ± 1.1 | 77 ± 14 | 26 ± 2.7 |
| *P* | | [b] | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |
| 5 | Control | 9.5 ± 2.8 | 105 ± 89 | 135 ± 81 | 120 ± 73 | 0.88 ± 0.48 | 3.2 ± 0.5 | 186.3 ± 31 | 3.2 ± 1.6 | 85 ± 11 | 26.7 ± 3 |
| | Desflurane | 8.2 ± 2 | 84 ± 71 | 162 ± 91 | 92 ± 66 | 0.69 ± 0.38 | 4.1 ± 0.6 | 176.5 ± 76 | 3.6 ± 1.6 | 87 ± 16 | 25.9 ± 3 |
| *P* | | [a] | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 | > 0.05 |

[a]$P < 0.05$ statistical significance *vs* intervention group.

[b]$P < 0.01$ statistical significance *vs* intervention group. WBCs: White blood cells; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; γ-Gt: Gamma-glutamyl transferase; Tbil: Total bilirubin; Fib: Fibrinogen; Plts: Platelets; aPTT: Activated partial thromboplastin time.

evidence of the platelet count being reduced while INR, PT and aPTT are increased in the first PODs after hepatectomy[47-49], which coincides with the results of our study. Cerruti *et al*[48] studied the perioperative coagulation profile of living liver donors with the use of routine tests, including those for platelet count, PT-INR and aPTT, as well as testing by thromboelastogram. They reported that in the postoperative period, despite the presence of decreased platelet counts, increased PT-INR and normal aPTT values, thromboelastogram demonstrated the progressive development of hypercoagulability. The complex interaction between coagulation and inflammation may provide insight into derangements of both pathways[50]. Our findings indicate that preconditioning with desflurane may prevent coagulopathies following hepatectomy. The attenuated indices in either pathway may be at least partially attributed to the effect of desflurane on inflammatory mediators. In terms of intraoperative transfusion, the rate is relatively high in both arms compared to the literature despite the use of low central venous pressure[51]. The Pringle maneuver prevents bleeding only from portal inflow but cannot control backflow bleeding from hepatic veins. Thus, blood loss occurs during both transection and reperfusion of the liver. This may also be attributed to the characteristics of the population which includes mostly hepatic tumors and extensive resections[52].

Except for the molecular and biochemical findings, total length of hospital stay was significantly shorter in the intervention group. This may have socioeconomic implications including reduced cost of hospitalization and greater patient satisfaction. Further prospective cohorts should be designed to identify superiority of any pharmacological regimen in relation to outcomes of survival and morbidity[53].

Lastly, this is the first study to investigate the role of desflurane as a volatile preconditioning factor in liver resection. The study is a well-designed and performed randomized controlled trial with excellent allocation concealment, as the surgeon was blinded to the preconditioning intervention. Furthermore, all the hepatectomies were

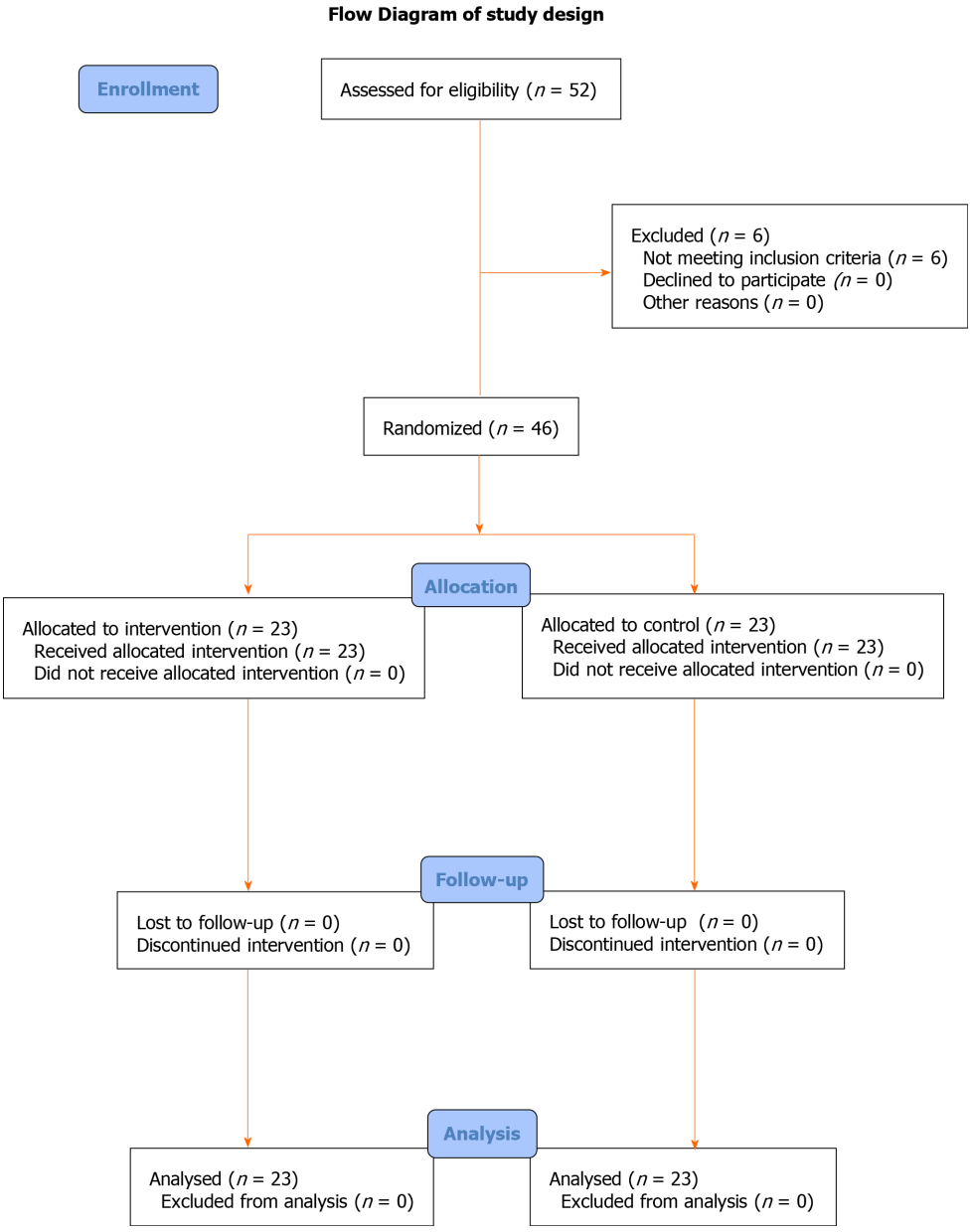


**Figure 1 Flow diagram of study design.**

performed by the same expert hepatobiliary surgeon, in order to avoid bias due to inter-surgeon differences in operation techniques. In this study, hepatic IRI was assessed from different aspects, including levels of MMPs, neutrophil infiltration of hepatic parenchyma, and coagulation status. However, as with all studies, a limitation exists; that being, our inability to assess the coagulation status of the patients by thromboelastography. In addition, a larger scale study focusing on more pathways of inflammation and coagulation may be needed in order to elucidate the potential mechanisms of IRI that can be inhibited and introduce desflurane preconditioning in clinical practice.

## CONCLUSION

To summarize, desflurane preconditioning was shown to decrease the inflammatory response and ameliorate the coagulation status following hepatic IRI, thereby protecting hepatic tissue in patients undergoing hepatectomy.

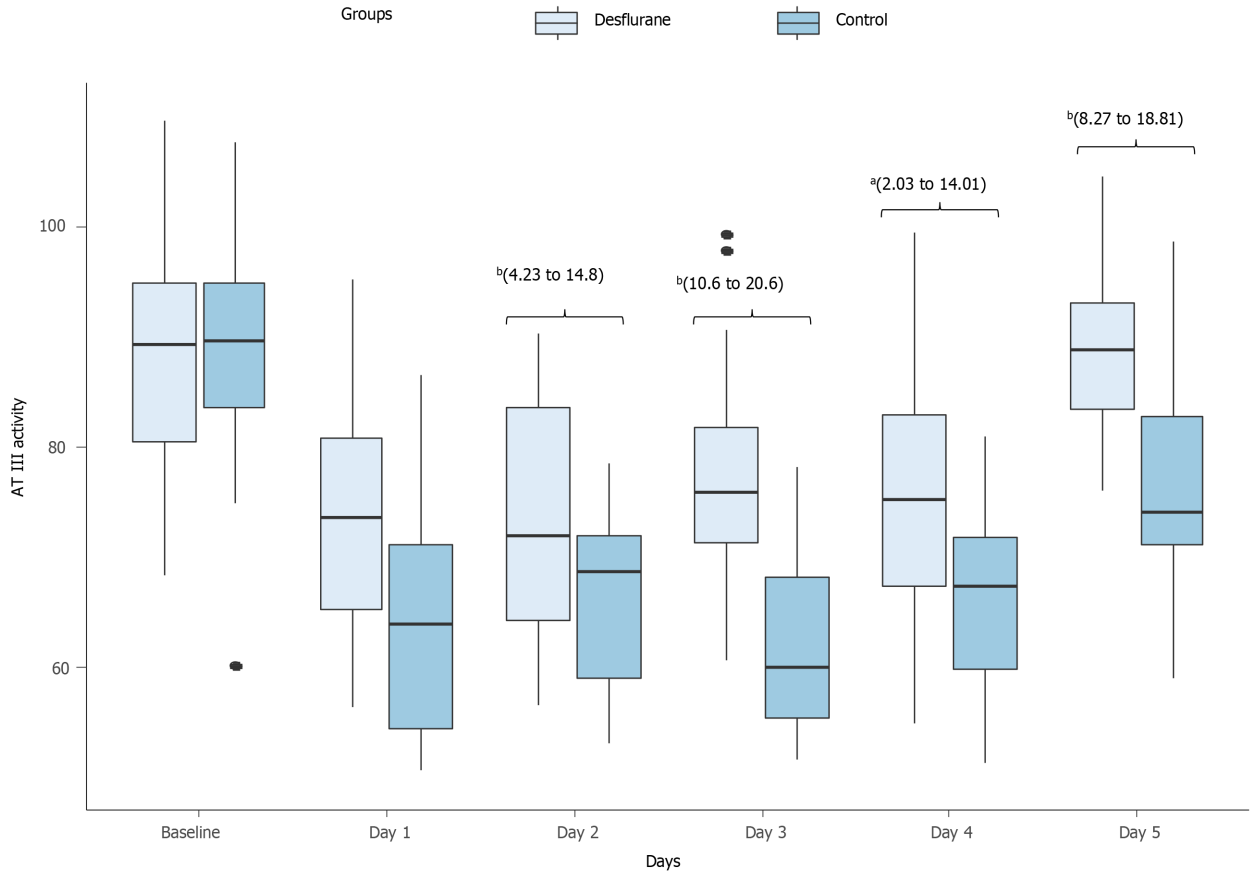


**Figure 2 Antithrombin activity compared between groups.** The *t*-test was used for between-groups comparisons, resulting in *P* values and 95% confidence intervals (CIs, provided in brackets). [a]*P* < 0.05 statistical significance between desflurane *vs* control group, [b]*P* < 0.01 statistical significance between desflurane *vs* control group. ATIII: Antithrombin III.

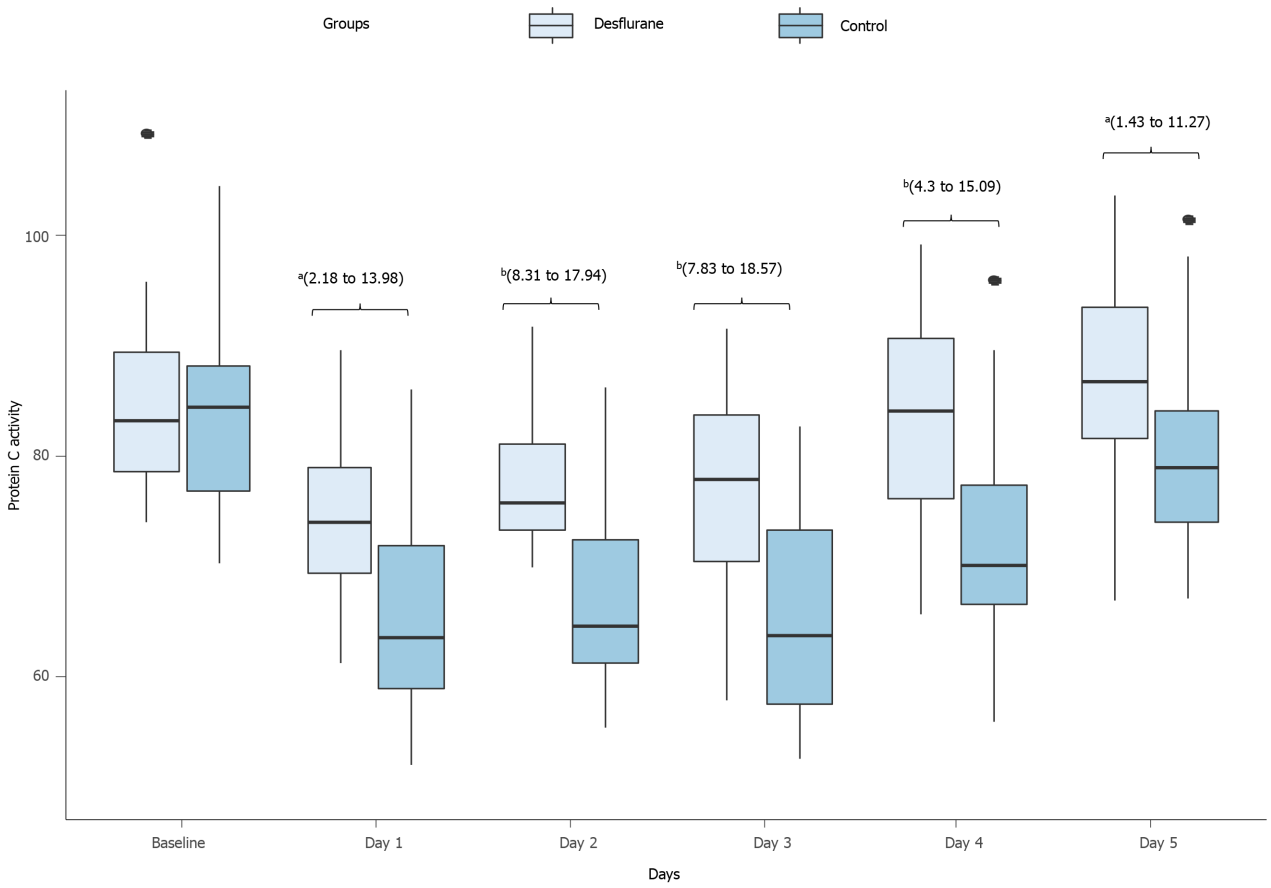


**Figure 3 Protein C activity compared between groups.** The *t*-test was used for between-groups comparisons, resulting in *P* values and 95% confidence intervals (CIs, provided in brackets). [a]*P* < 0.05 statistical significance between desflurane *vs* control group, [b]*P* < 0.01 statistical significance between desflurane *vs* control group.

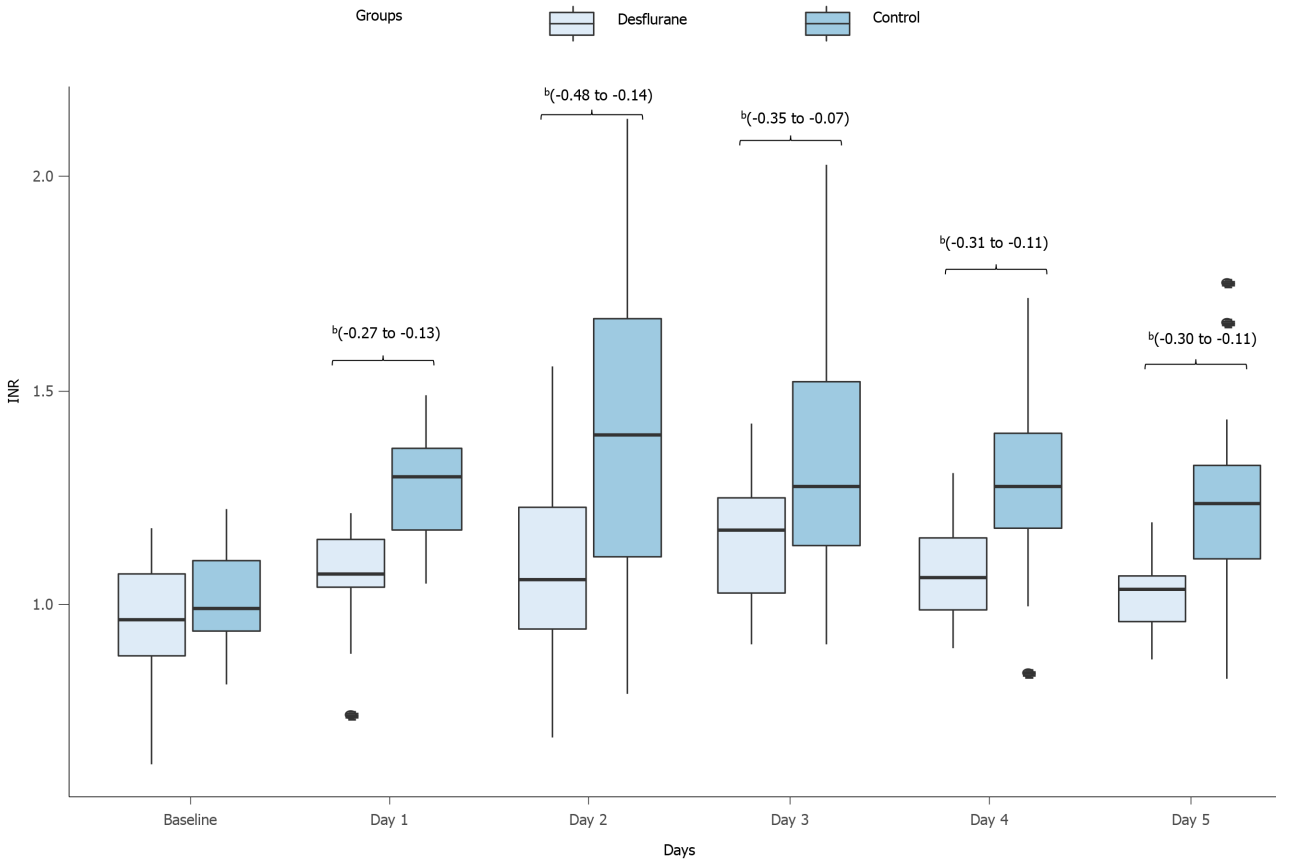


**Figure 4 International normalized ratio between groups.** The *t*-test was used for between-groups comparisons, resulting in *P* values and 95% confidence intervals (CIs, in brackets). [a]*P* < 0.05 statistical significance between desflurane *vs* control group, [b]*P* < 0.01 statistical significance between desflurane *vs* control group. INR: International normalized ratio.

## ARTICLE HIGHLIGHTS

### *Research background*

The primary cause of morbidity and mortality in hepatectomies is ischemia-reperfusion injury (IRI).

### *Research motivation*

Understanding the pathophysiology accompanying IRI can offer novel therapeutic targets. Metalloproteinases have been identified as regulators of IRI, and ischemic preconditioning has shown promising results in attenuating IRI.

### *Research objectives*

Our aim was to investigate the effect of ischemic preconditioning with desflurane, primarily on metalloproteinases and their inhibitors, and on the indices of liver and coagulation function.

### *Research methods*

Patients undergoing liver resection were randomized to receive pharmacologic preconditioning with desflurane or not. Blood samples and liver tissue specimens were collected for laboratory analysis.

### *Research results*

Desflurane preconditioning resulted in an attenuated inflammatory response compared to the control group.

### *Research conclusions*

Desflurane preconditioning may be effective in ameliorating IRI in hepatectomies, as indicated by the reduction in the expression of matrix metalloproteinases observed in the intervention group. Large scale studies are needed to verify our findings, with data

on long-term clinical outcomes.

### *Research perspectives*

Metalloproteinases may represent a useful target for managing IRI after hepatectomy.

## ACKNOWLEDGEMENTS

The assistance provided by Haidich AB, the responsible statistician, was greatly appreciated.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1115-1127

DOI: 10.4254/wjh.v12.i11.1115

ISSN 1948-5182 (online)

SYSTEMATIC REVIEWS

# Clinical utility of viscoelastic testing in chronic liver disease: A systematic review

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**Author contributions:** Wei H and Child L designed the research, analysed the data and wrote the paper; all authors read and approved the final manuscript.

**Conflict-of-interest statement:** All authors declare that they have no competing interests.

**PRISMA 2009 Checklist statement:** The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised in accordance with this checklist.



**Manuscript source:** Unsolicited manuscript

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## Abstract

### *BACKGROUND*

Conventional coagulation tests are widely used in chronic liver disease to assess haemostasis and to guide blood product transfusion. This is despite the fact that conventional tests do not reliably separate those with a clinically significant coagulopathy from those who do not. Viscoelastic testing such as thromboelastography (TEG) correlate with bleeding risk and are more accurate in identifying those who will benefit from blood product transfusion. Despite this, viscoelastic tests have not been widely used in patients with chronic liver disease outside the transplant setting.

### *AIM*

To assess the utility of Viscoelastic Testing guided transfusion in chronic liver disease patients presenting with bleeding or who require an invasive procedure.

### *METHODS*

PubMed and Google Scholar searches were performed using the key words "thromboelastography", "TEG" or "viscoelastic" and "liver transplantation", "cirrhosis" or "liver disease" and "transfusion", "haemostasis", "blood management" or "haemorrhage". A full text review was undertaken and data was extracted from randomised control trials that evaluated the outcomes of viscoelastic test guided transfusion in those with liver disease. The study subjects, inclusion and exclusion criteria, methods, outcomes and length of follow up were examined. Data was extracted by two independent individuals using a standardized collection form. The risk of bias was assessed in the included studies.

### *RESULTS*

A total of five randomised control trials included in the analysis examined the use

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** New Zealand

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** May 4, 2020
**Peer-review started:** May 4, 2020
**First decision:** May 24, 2020
**Revised:** June 1, 2020
**Accepted:** September 1, 2020
**Article in press:** September 1, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Adam EH, Hilmi I, Ruiz-Margáin A
**S-Editor:** Gong ZM
**L-Editor:** A
**P-Editor:** Li JH

of TEG guided blood product transfusion in cirrhosis prior to invasive procedures ($n$ = 118), non-variceal haemorrhage ($n$ = 96), variceal haemorrhage ($n$ = 60) and liver transplantation ($n$ = 28). TEG guided transfusion was effective in all five studies with a statistically significant reduction in overall blood product transfusion compared to standard of care. Four of the five studies reported a significant reduction in transfusion of fresh frozen plasma and platelets. Two studies showed a significant reduction in cryoprecipitate transfusion. No increased risk of bleeding was reported in the three trials where TEG was used perioperatively or prior to an invasive procedure. Two trials in the setting of cirrhotic variceal and non-variceal bleeding showed no difference in control of initial bleeding. In those with variceal bleeding, there was a statistically significant reduction in rate of re-bleeding at 42 d in the TEG arm 10% (*vs* 26.7% in the standard of care arm $P$ = 0.012). Mortality data reported at various time points for all five trials from 6 wk up to 3 years was not statistically different between each arm. One trial in the setting of non-variceal bleeding demonstrated a significant reduction in adverse transfusion events in the TEG arm 30.6% (*vs* 74.5% in the control arm $P$ < 0.01). In this study there was no significant difference in total hospital stay although length of stay in intensive care unit was reduced by an average of 2 d in the TEG arm ($P$ = 0.012).

*CONCLUSION*
Viscoelastic testing has been shown to reduce blood product usage in chronic liver disease without compromising safety and may enable guidelines to be developed to ensure patients with liver disease are optimally managed.

**Key Words:** Viscoelastic testing; Thromboelastography; Rotational thrombelastometry; Coagulation; Chronic liver disease; End stage liver disease; Cirrhosis; Haemostasis



**Core Tip:** Conventional coagulation tests do not predict bleeding or thrombosis risk in liver cirrhosis. Viscoelastic testing such as thromboelastography is a point of care test which can better predict clinically significant coagulopathy and the need for blood product transfusion compared to conventional coagulation tests. Randomized control trials have shown the clinical benefits of viscoelastic testing in liver cirrhosis in the perioperative setting and in those presenting acutely with bleeding. The primary aim of this systematic review is to verify the utility of viscoelastic testing guided transfusion in chronic liver disease patients presenting with bleeding or who require an invasive procedure.

**Citation:** Wei H, Child LJ. Clinical utility of viscoelastic testing in chronic liver disease: A systematic review. *World J Hepatol* 2020; 12(11): 1115-1127
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1115.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1115

## INTRODUCTION

The liver plays a fundamental role in maintaining normal haemostasis. It synthesises the majority of clotting factors and anticoagulants and is involved in the regulation of platelet production through the synthesis of thrombopoietin (TPO). Chronic liver disease is associated with complex changes that result in a state of rebalanced homeostasis, where abnormalities in procoagulant factors are balanced by changes to anticoagulant factors[1,2]. The most significant changes to coagulation are summarised in Table 1[2-5].

Conventional coagulation tests such as prothrombin time (PT), international normalised ratio (INR) and activated partial thromboplastin time (APTT) are commonly used to assess haemostasis in patients with chronic liver disease. This is despite the fact these tests were never developed to provide information on complex

**Table 1 Rebalanced haemostasis in chronic liver disease**

| | Procoagulant factors | Anticoagulant factors |
|---|---|---|
| **Primary haemostasis** | Increased vWF | Thrombocytopenia |
| | Reduced ADAMTS13 | +/- platelet dysfunction |
| **Secondary haemostasis/coagulation** | High FVIII | Reduced synthesis of FII, FV, FVII, FIX and FXI |
| | Reduced protein C, protein S and antithrombin | Dysfibrinogenaemia |
| | | Low fibrinogen (in end stage disease) |
| **Fibrinolysis** | Low plasminogen | Low antiplasmin |
| | | Low TAFI |
| | High PAI-1 | High tPA |

ADAMTS13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; PAI-1: Plasminogen activator inhibitor-1; TAFI: Thrombin-activatable fibrinolysis inhibitor; tPA: Tissue plasminogen activator; vWF: von Willebrand factor.

haemostatic abnormalities and are not validated for predicting bleeding or thrombosis risk in cirrhosis[5]. Conventional coagulation tests only measure the first 5%-10% of fibrin formation and provide no information on clot strength and stability, in vivo activity of natural anticoagulants or the complex interaction between clotting factors, platelets and the endothelium[1]. While increases in PT and INR are associated with mortality in liver disease, there is no correlation between a raised PT, INR or APTT and risk of bleeding[3]. A systematic review published in 2005 found no correlation between a prolonged PT and bleeding risk in patients undergoing liver biopsy[6]. Pre-operative PT/INR is also not predictive of bleeding risk in those undergoing liver transplantation[5,7].

Fresh frozen plasma (FFP) is often transfused in an attempt to normalise PT/INR in patients with liver disease[3,5]. Major societal guidelines differ in their recommendations on the management of cirrhotic patients with gastrointestinal bleeding and abnormal coagulation profiles. The American Association for the Study of Liver Disease does not recommend correcting an abnormal INR in cirrhotic patients with portal hypertensive bleeding[8]. In contrast, the British Society of Gastroenterology and the American Society for Gastrointestinal Endoscopy recommended correction of an abnormal INR in patients with acute variceal bleeding[9,10]. Despite these recommendations, there is a lack of data to support the use of FFP in this setting. Multiple studies have demonstrated that transfusion of FFP has minimal in vivo effect on a mildly prolonged PT/INR in patients with liver disease[3,5]. Other studies have demonstrated that thrombin generation, a dynamic and global measure of clot formation, remains normal in patients with cirrhosis despite a prolonged PT/INR and APTT[11-13]. The use of FFP in patients with cirrhosis is not without harm and epidemiological studies have shown an increased risk of transfusion associated acute lung injury (TRALI)[14]. FFP administration also results in volume expansion which can exacerbate portal hypertension, paradoxically increasing the risk of variceal bleeding[15].

The concept of rebalanced haemostasis in liver disease and limitations of the conventional tests of coagulation has led to renewed interest in the use of global haemostatic assays including viscoelastic tests of coagulation (VETs) in patients with liver disease[2]. Compared to conventional tests, VETs such as thrombelastography (TEG) or rotational thrombelastometry (ROTEM) provide real time global assessment of clot formation in whole blood and information on the interaction between platelets and coagulation factors[3]. The general concept of TEG and an example of a normal trace are provided in Figures 1 and 2. Table 2 provides a comparison of TEG and ROTEM parameters.

### *Relevance of this review*

The use of VETs to guide perioperative transfusion is well established and widely used in liver transplantation[2]. Despite this, VETs are not commonly used in patients with chronic liver disease outside the transplant setting. Observational and cohort studies in patients with liver disease have shown that alterations in TEG parameters correlate with bleeding risk. Pre-operative TEG MA is highly predictive of massive transfusion during liver transplantation[16]. Unlike conventional tests of coagulation,

**Table 2 Comparison of thromboelastography and rotational thrombelastometry parameters**

| | Measurement | TEG | ROTEM |
|---|---|---|---|
| Period of initial fibrin formation | Time (min) to reach an amplitude of 2 mm | Reaction time (R) | Clotting time |
| Clot kinetics | Time (min) for clot amplitude to increase from 2 mm to 20 mm | Kinetics time (K) | Clot formation time |
| Clot kinetics | Angle of tangent line from clot initiation to the slope of the developing curve | Alpha angle (α) | Alpha angle (α) |
| Maximum clot strength | Peak amplitude (mm) | Maximum amplitude | Maximum clot firmness |
| Clot stability/fibrinolysis | Percent reduction in curve at 30 and 60 minutes | Lysis 30 (LY30) and lysis 60 (LY60) | Lysis index 30 (LI 30) |

TEG: Thromboelastography; ROTEM: Rotational thrombelastometry.

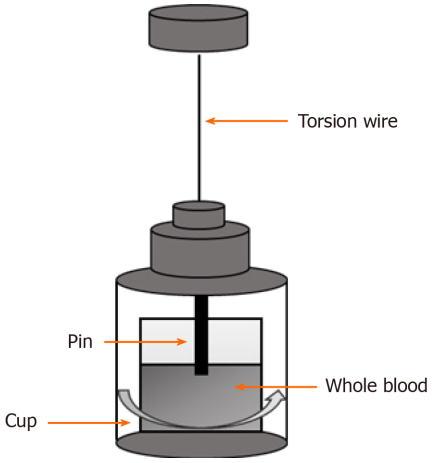


**Figure 1 Technique of thromboelastography.** Whole blood is pipetted into a cup which then rotates around a pin. As clot forms, the resistance to rotation increases. This resistance is measured *via* an electromechanical transducer and displayed graphically with additional calculations performed by an integrated computer. Thromboelastography (TEG) was originally performed on non-citrated whole blood and required up to 60 min to complete. Citrated blood, re-calcified at the time of testing, is now commonly used and reduces the turnaround time to 30 min. Initiators of clotting such as Kaolin can also be added which further reduces test time. TEG has a faster turnaround time than conventional coagulation tests with initial results available within 10 min.

TEG has also been shown to predict re-bleeding in acute variceal haemorrhage[17]. In cirrhotic patients with an acute infection, TEG parameters become hypocoagulable suggesting that cirrhotic patients have little haemostatic reserve. These observations explain the established link between infection and variceal bleeding in chronic liver disease[17,18].

As abnormalities on conventional tests of coagulation do not correlate with bleeding risk they cannot be used to distinguish between surgical or anatomic causes of bleeding such as portal hypertension and bleeding due to an underlying coagulopathy. Recent randomised control trials suggest that VETs have the potential to more accurately identify those who will benefit from blood product transfusion thereby avoiding unnecessary transfusions which has financial, resource and safety implications[19-23]. VETs may enable consistent and evidence-based guidelines to be developed to ensure that patients with liver disease, are optimally managed.

The aim of this systematic review is to assess the benefits and harms of using viscoelastic tests to guide blood product transfusion in patients with chronic liver disease who present with bleeding or require invasive procedures. To ensure that implementation of TEG and ROTEM is both safe and efficacious, this review will compare VETs with the conventional tests of coagulation and evaluate the implications of using TEG and ROTEM in patients with chronic liver disease.

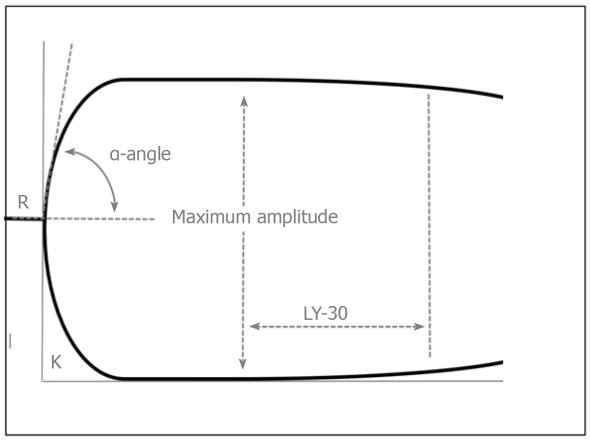


**Figure 2 Example of a thromboelastography tracing.** Five parameters are routinely measured on thromboelastography (TEG). The reaction (R) time is recorded in minutes and measures the time taken from the start of blood clotting until initial fibrin formation, defined as when the TEG trace amplitude reaches 2 mm. R is dependent on coagulation factors and generally corresponds to INR/PT. The kinetic (K) time assesses the rate of clot formation. It is measured from R to the point where the trace amplification reaches 20 mm which corresponds to standard clot firmness. The K value is dependent on the clotting factors of the intrinsic pathway, fibrinogen and platelets. The α-angle also corresponds to the kinetics of clot formation and is measured from a line drawn from the base-line to the tangent of the curve at R. The maximum amplitude (MA) of the trace reflects clot strength and is largely dependent on platelet count/ function and to a lesser extent, fibrinogen concentration. Clot lysis is measured at 30 min (Ly-30) and reflects the degree of fibrinolysis.

## MATERIALS AND METHODS

This article adheres to the Preferred Reporting Items for Systemic Reviews and Meta-Analysis (PRISMA) guideline[24].

### *Criteria for considering studies for this review*

**Types of studies:** We included original, randomised control trials that have been published in a medical journal in the English language irrespective of the blinding status. We excluded unpublished or observational trials from the analysis. Studies published in a language other than English were also excluded.

**Types of participants:** We included trials that examined the use of VETs in adult patients with chronic liver disease who presented with bleeding or required an invasive procedure. Trials involving children were excluded. There were no other subgroups of patient population that were excluded.

**Types of interventions:** We included trials comparing VET guided transfusion strategy with conventional tests of coagulation. Given the lack of consensus regarding transfusion strategies with conventional tests of coagulation, transfusion in the conventional care arm could be defined by the standard of care or guidelines that were in place at the centre performing the randomised control trial.

### *Types of outcome measures*

**Primary outcome:** Amount of blood products transfused. The amount of fresh frozen plasma, platelets and fibrinogen transfused were assessed. This includes the proportion of patients requiring transfusion of each blood product and the average amount of blood products transfused per patient.

**Secondary outcomes:** (1) Rates of bleeding in those undergoing an invasive procedure; (2) Rates of rebleeding in those presenting with bleeding; (3) Rates of adverse events related to blood transfusion; (4) Overall mortality using the longest follow-up data from each trial; (5) Length of stay in the intensive care unit; and (6) Number of days in hospital.

### *Search methods for identification of studies*

**Electronic searches:** A literature search was performed on MEDLINE (PubMed) and Google Scholar to identify original, English language articles assessing the use of VETs in patients with chronic liver disease. MEDLINE and Google Scholar were chosen primarily because of the large number of individual articles and range of journals that are included on these databases. In addition, MEDLINE targets healthcare professionals and researchers ensuring that the articles are relevant. It is authoritative

and peer-reviewed so that the research included is well-designed and statistics are accurately represented. The search included articles published up until the 17th of November 2019.

**Selection of studies and data extraction:** The following search string was used on PubMed: [(thromboelastography OR TEG OR viscoelastic) AND ("liver transplantation" OR cirrhosis OR liver disease) AND (transfusion OR haemostasis OR "blood management" OR haemorrhage)].

The following search string was used on Google Scholar: All of the words (thromboelastography, TEG, viscoelastic, liver transplantation, cirrhosis, liver disease, transfusion, haemostasis, blood management, haemorrhage).

The titles and abstracts of the articles were screened using the predefined inclusion and exclusion criteria detailed above. A full text review was then undertaken on the articles that met the inclusion criteria. Data was extracted from randomised control trials that evaluated the outcomes of VET guided transfusion in those with liver disease. The study subjects, inclusion and exclusion criteria, methods, outcomes and length of follow up were examined.

Data was extracted by two independent individuals using a data extraction form developed for this purpose. The risk of bias was assessed in the included studies by use of the Cochrane Collaboration tool for assessment of risk of bias[25].

## RESULTS

### *Results of the search*

The MEDLINE search generated 348 results and the Google scholar search generated 483 results. There were 71 duplicates across the two databases which left a total of 760 results. Five articles met the eligibility criteria based on title and abstract review and were included in the analysis following full text review (Figure 3)[19-23].

### *Analysis of the randomised control trials*

**Included studies:** The five randomised control trials included in the analysis examined the use of TEG in guiding blood product transfusion[19-23]. None of the studies utilised ROTEM. A range of different TEG methods were used. One trial used the TEG5000 analyser with native blood[21] whereas another two trials used the TEG5000 analyser with a kaolin activator[20,23]. Two trials used the MonoTEM-A analyser on native whole blood[19,22]. As a consequence of the differing TEG analysers and methodology used, the thresholds for TEG guided transfusion differed significantly between the clinical trials (Table 3). The thresholds for transfusion were consistent across the studies utilising the same TEG analyser and method.

All clinical trials utilised conventional coagulation tests as the control. The thresholds for transfusion were based on major societal guidelines and the thresholds for transfusion of FFP and platelets were consistent across all five studies. FFP was administered when the INR was ≥ 1.8 (PT used in one study[20]) and platelets transfusion when the platelet count was < $50 \times 10^9$[19-23].

The included trials examined the utility of TEG in guiding blood product transfusion in a variety of settings. One randomised control trial examined the use of TEG in orthotopic liver transplantation[20]. Two trials examined the use of TEG prior to invasive procedures[19,21]. One trial examined the use of TEG in patients presenting with a variceal bleeding[22] and the final study examined the use of TEG in cirrhotic patients with non-variceal bleeding[23].

The study by Wang *et al*[20] in the setting of orthotopic liver transplantation was relevant to investigate the consistency of TEG to safely guide transfusion therapies in the setting of major surgery. This study included advanced liver disease patients with an overall model for end-stage liver disease score of 11.3 and deranged coagulation parameters considered to be at high risk of bleeding. These were patients who had similar baseline factors and definitions of coagulopathy compared to the two randomized trials examining the use of TEG prior to invasive procedures outside of the transplant setting[19,21].

**Risk of bias in the included studies:** The overall methodologic quality of the studies was moderate to high (Figure 4) with an overall low risk of bias seen in 2 of the 5 studies (40%)[22,23] and no studies demonstrating an overall high risk of bias. The randomisation process was satisfactorily performed in 40% of the studies[22,23] and data regarding deviations from the pre-set protocol was satisfactorily reported in

**Table 3 Randomised control trials assessing the use of thromboelastography in liver disease**

| Ref. | Year | No. of patients | Method of TEG | TEG thresholds for transfusion | SOC thresholds for transfusion | Outcomes: Blood product usage | Outcomes: Other |
|---|---|---|---|---|---|---|---|
| Wang *et al*[20] | 2010 | 28<br>14 TEG<br>14 SOC | TEG 5000<br>Kaolin activated | FFP titrated to maintain R time < 10 min | FFP titrated to maintain PT and APTT at less than one and a half times control | Statistically significant reduction in FFP use in TEG group (12.8 units in TEG group *vs* 21.5 units in control group, $P < 0.05$) | Trend towards reduction in blood loss in the TEG arm (not statistically significant) |
| | | | | SDAP when MA < 55 mm* | | | |
| | | | | 5 pooled units of cryoprecipitate when alpha angle < 45 degrees** | Platelets to maintain a platelet count ≥ $50 \times 10^9$<br>Cryoprecipitate to maintain fibrinogen > 1 g/L | No reduction in RBC, Platelet or cryoprecipitate use | No statistically significant difference in mortality at 3 yr |
| De Pietri *et al*[21] | 2016 | 60<br>30 TEG<br>30 SOC | TEG 5000<br>Native blood (no activators) | FFP, 10 mL/kg*** when R time > 40 min[1] | FFP, 10 mL/kg*** when INR > 1.8 | Statistically significant reduction in FFP use in TEG group. (Total amount of FFP transfused in those undergoing a low risk procedure: 4000 mL in TEG group *vs* 11050 mL in SOC group, $P = 0.002$) (Total amount of FFP transfused in those undergoing a high-risk procedure: 0 mL in TEG group *vs* 6500 mL in SOC group) | No statistically significant difference in periprocedural bleeding complications. |
| | | | | SDAP when MA < 30 mm* | SDAP when platelets < $50 \times 10^9$* | Statistically significant reduction in platelets transfused. (6.7% required a platelet transfusion in the TEG arm *vs* 33.3% in the SOC arm, $P = 0.021$) | Periprocedural bleeding events were rare with only one patient experiencing post procedure bleeding. |
| Rout *et al*[22] | 2019 | 60<br>30 TEG<br>30 SOC | MonoTEM-A®<br>Native (no activators) | FFP, 5 mL/kg*** when R time > 15 min | FFP, 5 mL/kg*** when INR > 1.8 | Statistically significant reduction in FFP use. (13.3% receiving FFP in the TEG group *vs* 46.7% in the SOC group $P = 0.010$. 1345 ml LFFP transfused in the TEG group *vs* 4605 mL in the SOC) | No difference in initial control of bleeding |
| | | | | 3 pooled units of platelets when MA < 30 mm* | 3 pooled units of platelets when platelet count < $50 \times 10^9$* | | No difference in rates of re-bleeding at 5 d |
| | | | | | | Statistically significant reduction in platelets transfused (10% in TEG group *vs* 70% SOC group $P < 0.001$. Total vol. of platelets transfused: 450 mL platelets in the TEG group *vs* 3450 mL in the SOC) | Statistically significant reduction in rebleeding at 42 d (10% in the TEG group *vs* 36.7% in SOC, $P = 0.012$) |
| | | | | | | No difference in RBC transfusion | No difference in mortality at 6 wk (13.3% in TEG group *vs* 26.7% in SOC, $P = 0.176$) |
| Kumar *et al*[23] | 2019 | 96<br>49 TEG<br>47 SOC | TEG 5000<br>Kaolin activated | FFP, 10 mL/kg*** when R time > 10 min | FFP, 10 mL/kg*** if INR > 1.8 | Statistically significant reduction in FFP use (Total FFP transfused 440 mL in TEG *vs* 880 mL in SOC, $P < 0.01$) | Statistically significant reduction in transfusion related adverse events (30.6% in TEG group *vs* 74.5% in SOC $P < 0.01$)[2] |
| | | | | SDAP when MA < 55 mm* | SDAP when platelets < $50 \times 10^9$* | Statistically significant reduction in platelets transfused (Average of 1 SDAP unit per patient in TEG group *vs* 2 SDAP units in SOC, $P < 0.01$) | |
| | | | | 5 pooled units of cryoprecipitate when alpha angle < 45 degrees** | 5 pooled units of cryoprecipitate if fibrinogen < 80 mg/dL** | | Statistically significant reduction in ICU length of stay (median of 2 d in TEG arm *vs* 3 d in SOC. $P = 0.012$) |
| | | | | | | Statistically significant reduction in amount of cryoprecipitate used. (4 units in TEG group *vs* 16 in SOC group. $P < 0.01$) | No difference in failure to control bleeding at day 5 or rebleeding at day 42. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | No difference in 5-d and 42-d mortality |
| Vuyyuru *et al*[19] | 2019 | 58 | MonoTEM-A® | FFP 5 mL/kg when R time > 14 min*** | FFP 5 mL/kg*** if INR ≥ 1.8 | No statistically significant difference in the amount of FFP transfused (24.1% requiring FFP in the TEG group *vs* 27.6% in the SOC, $P = 0.764$) | No difference in post procedure bleeding complications (0% in both groups) |
| | | 29 TEG | Native (no activators) | | | | |
| | | 29SOC | | 3 pooled units of platelets when MA < 32 mm* | 3 pooled units of platelets when platelet count < 50 × $10^9$* | Statistically significant reduction in platelets transfused (10.3% requiring platelet transfusion in the TEG group *vs* 75.9% in the SOC group, $P < 0.001$) | No difference in pre and post procedure haemoglobin levels (TEG group: 11.3 ± 2.1 g/dL *vs* 11.2 ± 2.0 g/dL, $P = 0.979$; SOC group: 10.4 ± 2.1 g/dL *vs* 10.2 ± 2.0 g/dL, $P = 0.205$) |

*In the above clinical trials, 1 SDAP unit corresponds to 6-8 pooled platelet units from whole blood donation. **When whole blood is used as the source for cryoprecipitate, 5 pooled units of is equivalent to 700 mg of fibrinogen. ***Ideal body weight used.

[1]Natural whole blood used without added activators. Normal R time using this TEG method is 12-26 min.

[2]Higher than expected adverse reaction rate, including TRALI rates, not discussed in the paper. No information given about type of blood components used *i.e.*, leucodepleted *vs* non-leucodepleted. APTT: Activated partial thromboplastin time; FFP: Fresh frozen plasma; INR: International normalised ratio; MA: Maximum amplitude; PT: Prothrombin time; R: Reaction time; RBC: Red blood cells; SDAP: Single donor apheresis platelets; SOC: Standard of care; TEG: Thromboelastography.

60%[19,22,23]. None of the studies included had missing outcome data or selective outcome reporting.

### *Effects of Interventions on the primary outcomes*

All five studies reported a statistically significant reduction in overall blood product use with TEG guided transfusion[19-23]. The trials reported different outcomes with regards to the transfusion of specific blood products such as FFP, platelets, cryoprecipitate and red blood cells.

Four of the five studies reported a statistically significant reduction in FFP use[20-23]. In those presenting with variceal bleeding, 13.3% required FFP in the TEG arm compared to 46.7% in the conventional arm ($P = 0.010$)[22]. The absolute volume of FFP transfused was also markedly reduced in the TEG arm where 1345 mL of FFP was transfused compared with 4605 mL in the control arm[22]. The two studies examining the use of TEG prior to invasive procedures yielded different results with one trial showing a significant reduction in FFP use and the other showing no difference[19,21]. The two trials used different TEG analysers and methodology. As such, the transfusion thresholds in the TEG arm cannot be compared although the transfusion thresholds in the control group were identical. While not statistically significant, the trial which did not show a difference in FFP use had a higher number of patients with Childs Pugh B and C in the TEG arm than in the control arm (55.2% *vs* 31%)[19]. As the haemostatic rebalance is often lost in those with very advanced liver failure, this may have impacted on the results[26].

A statistically significant reduction in platelet transfusion was also reported in four of the five clinical trials[19,21-23] with no difference seen in the liver transplant trial[20]. Both trials that examined the use of TEG in cirrhotic patients requiring invasive

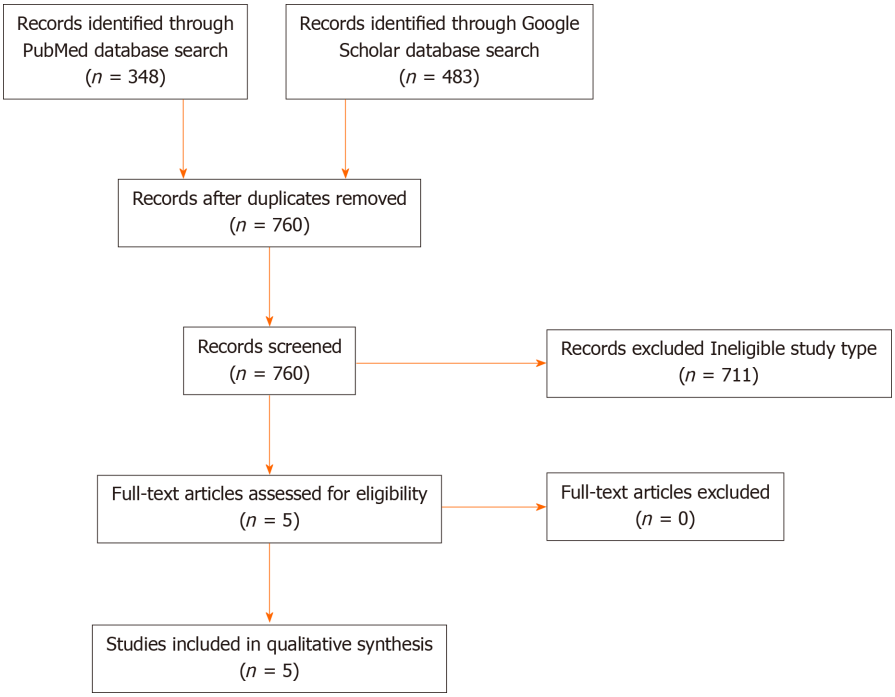


**Figure 3 PRISMA flow diagram.**

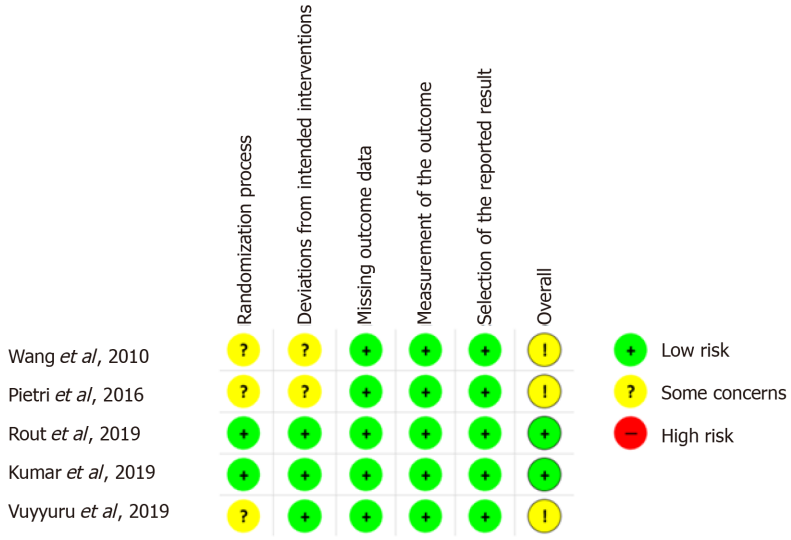


**Figure 4 Author-judged risk of bias for each included study.**

procedures showed significantly lower rates of platelet use[19,21]. In one study, 13.3% required platelet transfusion in the TEG group versus 46.7% in the control group ($P < 0.001$)[22]. Again, the total volume transfused was markedly lower with 450mL transfused in the TEG group *vs* 3405 mL in the standard of care arm[22].

The amount of cryoprecipitate transfused was only measured in two trials[20,23]. In the liver transplant trial, there was no statistically significant difference in cryoprecipitate transfusion between the two groups[20]. In the study examining the use of TEG in those with non-variceal bleeding a statistically significant reduction was seen where 4 units of cryoprecipitate were used in the TEG group compared with 16 in the standard of care group (where each unit consisted of 5 pooled units of cryoprecipitate)[23].

### *Effects of intervention on the secondary outcome*

**Rates of bleeding in those undergoing an invasive procedure:** There was no statistically significant difference in blood loss and/or bleeding events in the three

trials which examined the use of TEG perioperatively or prior to an invasive procedure[19-21]. One trial measured the haemoglobin levels prior to and following each invasive procedure and found no significant difference in the levels between the TEG group and control group[19]. In all three studies, periprocedural bleeding rates were low in both [19-21]. Despite several patients having Childs-Pugh B and C disease, one study reported no bleeding complications in either arm following high-risk procedures including percutaneous liver biopsies[19].

**Rates of rebleeding in those presenting with bleeding:** Two trials examined the use of TEG in cirrhotic patients presenting with bleeding complications[22,23]. There was no difference in the ability to control initial bleeding between the TEG and conventional care groups. In those presenting with variceal bleeding, there was a statistically and clinically significant difference in the rate of re-bleeding at 42 d. 10% of those in the TEG group re-bled compared to 26.7% in the standard of care arm ($P$ = 0.012)[22]. In the study of patients with non-variceal bleeding, no significant difference in rebleeding was seen at up to 42 d follow up[23].

**Rates of adverse events related to blood transfusion:** The rates of transfusion reactions and/or adverse events were reported in four out of the five studies[19,21-23] with no data available from the liver transplantation study[20]. Only one trial demonstrated a statistically significant reduction in adverse events related to transfusion where 30.6% had an adverse event in the TEG group versus 74.5% in the control arm[23]. These reaction rates are much higher than expected even in this high-risk population. The authors report a TRALI rate of 12.2% in the TEG arm versus 48.9% in the conventional arm[23]. The transfusion reactions were independently assessed by a panel of 3 experts to ensure appropriate classification. There is no mention of whether non-leucodepleted products were used in this trial which could potentially explain these unexpected results. In all other trials, transfusion reactions occurred infrequently with only two patients in all of the control groups and zero patients in the TEG groups experiencing an adverse transfusion event[19-22].

**Overall mortality using the longest follow-up data from each trial:** Mortality data was reported at various time points for all five trials with no statistically significant difference reported between the TEG group and control arm in any study[19-23]. In the liver transplant trial, there was no difference in overall survival at 3 years[20]. In the variceal bleeding trial, the mortality rate was high in both arms as one might expect in this high-risk population. The mortality rate was 13.3% at 6 wk in the TEG arm versus 26.7% in the control arm with a $P$ value of 0.76[22]. The small number of participants included in each individual trial means that not all trials were adequately powered to assess a statistically significant difference in mortality.

**Length of stay in the intensive care unit:** Only one trial reported on length of stay in the intensive care unit. Following a presentation with non-variceal bleeding, there was a statistically significant reduction in the length of ICU stay. This was reported to be an average of 2 d in the TEG group versus 3 days in the control arm ($P$ = 0.012)[23].

**Number of days in hospital:** Length of hospital stay was only reported in the study examining the use of TEG in non-variceal bleeding[23]. Length of hospital stay did not differ significantly between the two groups.

## DISCUSSION

It is now widely accepted that chronic liver disease results in a state of rebalanced haemostasis where a reduction in procoagulant factors is balanced by a reduction in anticoagulant factors[3]. While conventional tests of coagulation are commonly used in patients with chronic liver disease, there is no correlation between a prolonged PT or INR and risk of bleeding in this patient group. While a minority of patients with liver disease are at an increased risk of bleeding, the conventional tests of coagulation do not reliably separate those who have a clinically significant coagulopathy from those who do not[3]. The haemostatic management of cirrhotic patients with a baseline coagulopathy on conventional testing remains difficult with a significant variation in clinical practice. The use of FFP to correct an abnormal PT or INR remains common practice despite a lack of evidence demonstrating clinical benefit[3,5]. The potential harms of transfusion in this patient group are well documented[5].

VETs have significant potential to inform and improve the haemostatic management of patients with chronic liver disease. Alteration in TEG parameters have

been shown to correlate with bleeding risk in this patient group. TEG guided transfusion has been shown to reduce allogeneic blood product use in cirrhotic patients who require invasive procedures, including liver transplantation and in those presenting with variceal and non-variceal gastrointestinal bleeding[19-23]. The reduction in blood product use in five randomised control trials was not associated with an increased risk of bleeding, difference in the ability to control bleeding, morbidity or mortality when compared to standard care. In acute variceal haemorrhage, the rate of re-bleeding at 42 d was significantly lower with TEG guided transfusion[22]. Although the numbers included in each individual randomised control trial are small and there are differences in methodology and TEG cut-offs, the outcomes suggest a clinical benefit from TEG monitoring in chronic liver disease. The randomised control data available suggests that TEG provides a more accurate assessment of haemostasis, including bleeding risk and provides a more meaningful guide for blood product administration than conventional tests of coagulation in patients with chronic liver disease.

## CONCLUSION

In conclusion, the poor predictive value of conventional coagulation tests in chronic liver disease has led to renewed interest in the use of global measures of haemostasis. Randomised control trials have confirmed earlier observations that VETs are more accurate in assessing bleeding risk and reduce blood product usage in chronic liver disease without compromising safety. While additional prospective randomised trials are needed to establish appropriate transfusion thresholds, VETs may enable consistent and evidence-based guidelines to be developed to ensure that patients with liver disease, are optimally managed.

## ARTICLE HIGHLIGHTS

### *Research background*

Conventional coagulation tests do not predict bleeding or thrombosis risk in liver cirrhosis. Viscoelastic tests of coagulation (VETs) such as thrombelastography (TEG) is a point of care test that can predict clinically significant coagulopathy and the need for blood product transfusion. Despite this, VETs have not been widely used in patients with chronic liver disease outside the transplant setting.

### *Research motivation*

The systematic review provides a summary and evaluation of existing clinical evidence for VET guided transfusion in chronic liver disease. This data will be important to improve the haemostatic management in these patients.

### *Research objectives*

To verify the utility of VET guided transfusion in chronic liver disease patients presenting with bleeding or who require an invasive procedure.

### *Research methods*

A comprehensive systematic literature search was performed according to the methodology of evidenced-based medicine. We included randomized controlled trials that compared the use of VET guided transfusion to conventional coagulation tests in the setting of chronic liver disease who presented with bleeding or required an invasive procedure.

### *Research results*

Five studies were included in the analysis examining the use of TEG guided blood product transfusion in cirrhosis prior to invasive procedures, non-variceal haemorrhage, variceal haemorrhage and liver transplantation. TEG guided transfusion reduced overall blood product utilization compared to standard of care in all five studies. No increase in length of stay, mortality or risk of bleeding was observed. In those presenting with variceal bleeding, there was a statistically significant reduction in rate of re-bleeding at 42 d in the TEG arm versus standard of care.

### Research conclusions

This systematic review highlights the role of VET in reducing blood product utilization in chronic liver disease without compromising safety and may enable guidelines to be developed to ensure patients with liver disease are optimally managed.

### Research perspectives

There is an urgent need to develop protocols utilizing VET to guide transfusion in liver cirrhosis outside of the transplant setting in order to optimize haemostatic management of these patients.

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World Journal of Hepatology

Submit a Manuscript: https://www.f6publishing.com

*World J Hepatol* 2020 November 27; 12(11): 1128-1135

DOI: 10.4254/wjh.v12.i11.1128

ISSN 1948-5182 (online)

CASE REPORT

# Hepatocellular carcinoma with tumor thrombus extends to the right atrium and portal vein: A case report

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**Author contributions:** Gomez-Puerto D collected all of the data, wrote the first article draft, and reviewed the final version; Mirallas O drafted and critically reviewed the article, summarized each section, and edited the images of the article; Vidal-González J drafted some sections of the manuscript, obtained informed consent, and critically reviewed the entire article; Vargas V provided the final approval, expertise, mentorship and final revision.

**Informed consent statement:** Consent was obtained from the patient, and the signed Informed Consent Form was provided to the publisher.

**Conflict-of-interest statement:** The authors declare no conflicts of interest.

**CARE Checklist (2016) statement:** The authors have read the CARE Checklist (2016) and the manuscript was prepared and revised according to the CARE Checklist (2016).



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## Abstract

### *BACKGROUND*

Hepatocellular carcinoma (HCC) is the most important primary malignant liver disease. A large proportion of patients with advanced HCC have macrovascular invasion. HCC tends to infiltrate vascular structures, particularly the portal vein and its branches, and more rarely, the hepatic veins. The intravascular tumor thrombus can affect the inferior vena cava (IVC) or even the right atrium (RA), the latter having a poor prognosis.

### *CASE SUMMARY*

HCC is one of the most aggressive malignant tumors. Tumor thrombus (TT) formation in advanced HCC stages is common and usually involves the hepatic or portal veins. Herein, we report a 69-year-old woman who presented with dyspnea to the emergency department. A ventilation/perfusion lung scan was performed, ruling out pulmonary embolism. Hepatopulmonary syndrome and portopulmonary hypertension were discarded with contrasted echocardiography, but a mass in the RA was detected and confirmed by cardiac magnetic resonance imaging. Abdominal computed tomography showed a liver mass with a dynamic enhancement pattern compatible with HCC and an intraluminal IVC mass extending from the hepatic vein into the RA. HCC with TT expansion to IVC and RA is rare and indicates poor prognosis.

### *CONCLUSION*

HCC with TT expansion to IVC and RA is rare and indicates poor prognosis. There is no consensus about anticoagulation or other interventions in these patients.

**Key Words:** Hepatocellular carcinoma; Alpha-fetoprotein; Tumor thrombus; Right atrium;

**Manuscript source:** Unsolicited manuscript

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Spain

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

**Received:** July 12, 2020
**Peer-review started:** July 12, 2020
**First decision:** August 9, 2020
**Revised:** August 23, 2020
**Accepted:** September 15, 2020
**Article in press:** September 15, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Qi XS
**S-Editor:** Gong ZM
**L-Editor:** Filipodia
**P-Editor:** Li JH

Sorafenib; Case report



**Core Tip:** Hepatocellular carcinoma (HCC) with tumor thrombus (TT) in the right atrium (RA) is an unusual but critical condition. There is no standard treatment strategy or consensus. Alpha-fetoprotein is reportedly a new alternative biomarker to RECIST in order to detect tyrosine kinase inhibitors response in HCC, and our case supports this hypothesis. We report this HCC case due to the exceptionality of a TT extending into the RA in a patient with stable cirrhosis. We believe this case will warn professionals when facing similar cases, so systemic treatment can be started in a timely fashion and the treatment response can be evaluated with a serial blood test in patients suffering from advanced HCC.

**Citation:** Gomez-Puerto D, Mirallas O, Vidal-González J, Vargas V. Hepatocellular carcinoma with tumor thrombus extends to the right atrium and portal vein: A case report. *World J Hepatol* 2020; 12(11): 1128-1135
**URL:** https://www.wjgnet.com/1948-5182/full/v12/i11/1128.htm
**DOI:** https://dx.doi.org/10.4254/wjh.v12.i11.1128

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant liver disease and the fourth most common cause of cancer-related deaths, resulting in a global incidence of approximately 800000 cases per year[1]. Currently, liver cancer is the second most lethal tumor, after pancreatic cancer, with a 5-year survival rate of 18%[2]. The most common cause of HCC worldwide is hepatitis B virus chronic infection, but in Western countries, hepatitis C virus infection, chronic alcohol consumption and non-alcoholic steatohepatitis predominate.

HCC primarily develops in cirrhotic livers, which is the major cause of death in this population[3]. HCC also tends to infiltrate vascular structures, particularly the portal vein and its branches, and more rarely, the hepatic veins. Intravascular tumor thrombus (TT) can progress and extend to the inferior vena cava (IVC) or even to the right atrium (RA), the latter having a worse prognosis compared with patients with TT in the portal or hepatic vein[4,5]. IVC/RA tumor thrombus is an infrequent event of patients with HCC, with a reported incidence of 3% to 4%. Its diagnosis is challenging due to the lack of specific clinical signs[6-8].

## CASE PRESENTATION

### *Chief complaints*

A 69-year-old woman complained about experiencing asthenia and dyspnea with dizziness and diaphoresis.

### *History of present illness*

A 69-year-old woman arrived at the emergency department with a 15-d history of progressive asthenia, dyspnea on moderate exertion and progressive worsening shortness of breath associated with dizziness and diaphoresis.

### *History of past illness*

The patient was diagnosed in 2005 with alcoholic cirrhosis, without any decompensation since 2011. She followed an adequate follow-up in the hepatology department of the hospital and she had been abstinent since the last decompensation. Her last abdominal Doppler-ultrasonography from May 2019 showed liver cirrhosis with signs of portal hypertension, mild ascites and cholelithiasis. Furthermore her last upper gastrointestinal endoscopy from June 2019 showed esophageal varices and portal gastropathy. She also received treatment with beta-blockers and diuretics with

good tolerance and adherence. Thereafter, she had a biological aortic valve replacement for severe aortic stenosis in December 2018, and since then, she remained asymptomatic.

### *Physical examination*

The vital signs were within range and the physical examination was fairly normal. She had no chest discomfort or palpitations, orthopnea or an increase in the perimeter of lower limbs. She also denied having fever, chills, cough or expectoration, abdominal distention or any weight loss. In addition, she had a regular cardiac rhythm without cardiac murmur or rub noted, and the jugular venous pressure was not elevated. Her lungs were clear on auscultation and the oxygen saturation was correct without oxygen therapy. Her abdomen was also soft, non-tender and had no hepatosplenomegaly or clear signs of ascites. Moreover, she had no leg swelling, bruising or petechiae. There was no clubbing or cyanosis of her feet or hands. Signs of liver cirrhosis were found on examination, such as spider angioma and mild palmar erythema.

### *Laboratory examinations*

The blood test at admission revealed an impaired renal function with a creatinine of 1.82 mg/dL and a glomerular filtration rate of 28 mL/min/1.73 $m^2$, mild hyponatremia with sodium of 132.3 mmol/L, elevated pro-brain natriuretic peptide (923 pg/mL) and high D-dimer (917 ng/mL).

### *Imaging examinations*

Her chest X-ray showed pathological signs. Due to the high suspicion of pulmonary embolism (PE) in a patient allergic to iodine, a ventilation/perfusion lung scan was performed, which did not exhibit any signs of PE. Contrasted echocardiography was requested to rule out portopulmonary hypertension or hepatopulmonary syndrome as the cause of the dyspnea of the patient. The test did not reveal signs of pulmonary hypertension nor shunts and showed a preserved systolic function (left ventricular ejection fraction [LVEF]: 58%) and a normally functioning prosthetic aortic valve. Unexpectedly, a mass at the posterior wall of the RA of 32 by 20 mm suggestive of an intracavitary thrombus was detected.

### *Further diagnostic work-up*

Magnetic resonance imaging was performed to confirm this finding due to its higher sensitivity and specificity. The mass inside the RA coming from the liver through the IVC was noted. This image was suggestive of an infiltrating HCC tumor thrombus (TT) (Figure 1). With the previous premedication, an abdominal multiphase CT was finally performed, thereby confirming the presence of a lesion in segment VIII with extension to segments V and I, suggestive of hepatocarcinoma (Figure 2). Left suprahepatic vein thrombosis with extension to the IVC until the RA and thrombosis in the left portal vein was noted, suggestive of tumor infiltration (Figure 3). On further work-up, her alpha-fetoprotein level was 6856.1 ng/mL.

## MULTIDISCIPLINARY EXPERT CONSULTATION

A multidisciplinary committee was held to decide treatment and further work-up.

## FINAL DIAGNOSIS

The final diagnosis of the presented case is HCC with TT extended to the RA and portal vein.

## TREATMENT

A multidisciplinary committee evaluated the patient and determined that she was not a candidate for surgical or ablative therapies. Thus, systemic treatment with sorafenib was started at 400 mg every 12 h and required dose reduction for asthenia. Given that the patient presented an extended TT, anticoagulation was introduced with low

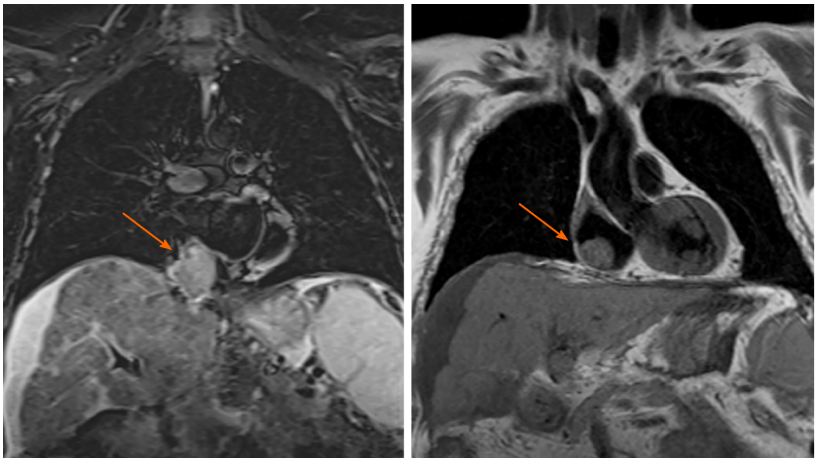

**Figure 1 Magnetic resonance imaging coronal view of a polylobulated mass of 59 mm × 51 mm × 52 mm inside the right atrium (right arrow), which extends from the liver through the inferior vena cava (left arrow) to the inside of the right atrium occupying its lower-posterior wall.** These findings suggest as the first diagnostic possibility: A tumor thrombus of an advanced infiltrating hepatocellular carcinoma. Volumes and biventricular function within physiological parameters.

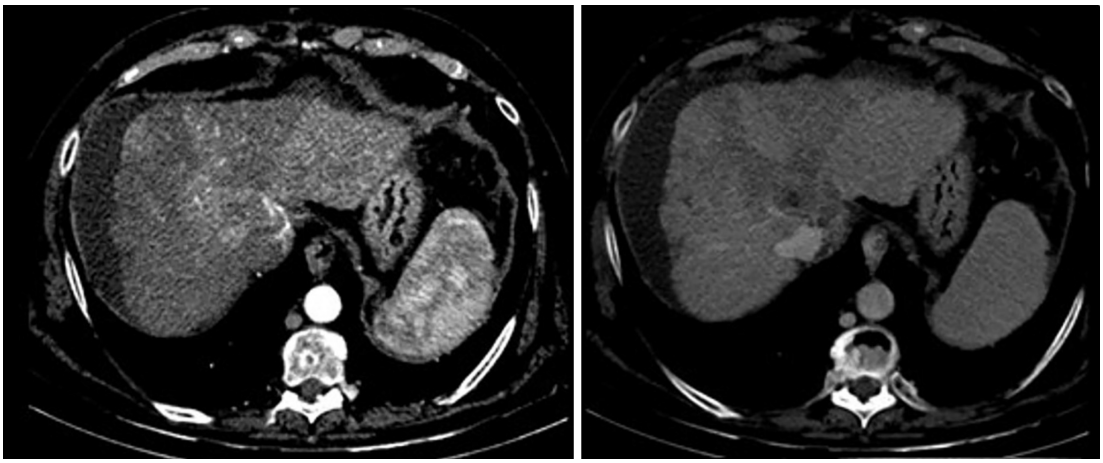

**Figure 2 Computed tomography scan axial view shows a poorly defined liver injury with margins of 55 mm × 90 mm located in segment VIII and with extension to segment V and segment I, enhanced in arterial phase with fast venous phase washing.**

molecular weight heparin at a dose of 1 mg/kg every 12 h.

## OUTCOME AND FOLLOW-UP

The patient was seen after 1 mo of being discharged, and she remained stable. She also showed an improved capacity for physical activities without any signs of embolism and diminished hepatomegaly in physical exploration. The dose of sorafenib was increased to a standard dose with positive tolerance. A non-contrast CT scan was completed 3 mo later (due to impaired kidney function). The limited evaluation presumed apparent stability of the tumoral mass (Figure 4). The alpha-fetoprotein level was 4156.1 ng/mL, thereby indicating a response to sorafenib. After 5 mo after receiving systemic therapy, the patient sought evaluation in the emergency department for being in a critical condition and was diagnosed with septic shock related to spontaneous bacterial peritonitis that ended fatally.

## DISCUSSION

HCC is the most common primary liver neoplasm. It is the fifth most frequently diagnosed cancer in men, and it is the ninth most commonly diagnosed cancer in

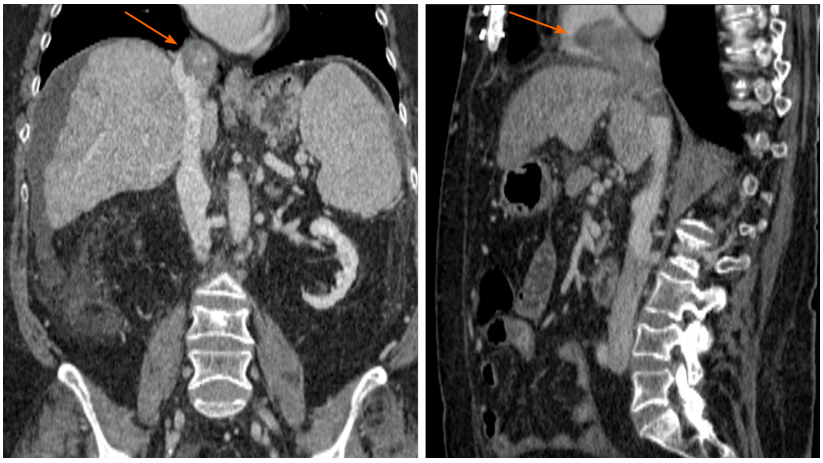

**Figure 3 Computed tomography scan coronal and sagittal views show a 55 mm × 90 mm mass in segment VIII with a tumor thrombus extending to the inferior vena cava (left arrow) and reaching up to right atrium (right arrow).** Left intrahepatic portal thrombosis. Portal vein thrombosis with hypercaptation of the thrombus, suggestive of infiltrative tumor.

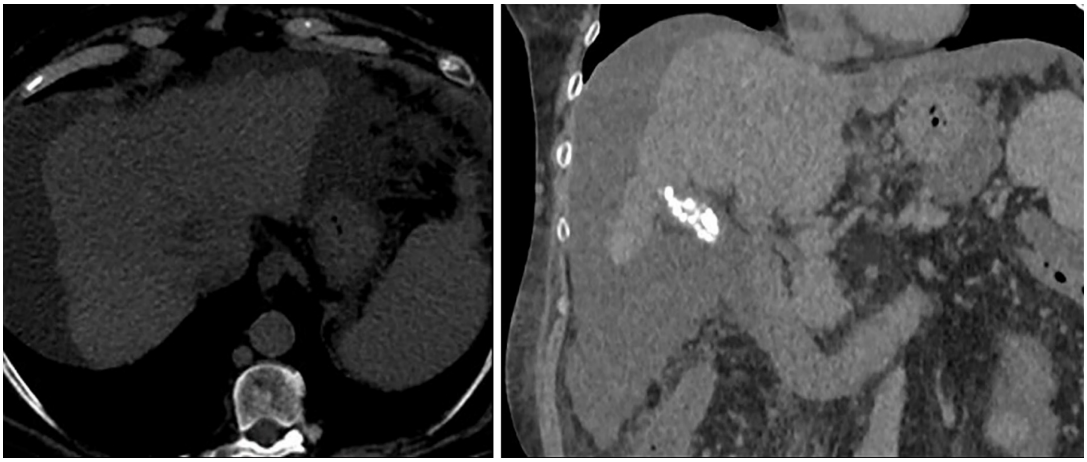

**Figure 4 Non-contrast computed tomography scan axial view showing persistence of the heterogeneous lesion in the segment VIII seen previously, not delimitable in the current study.** Hypodensity persists in the inferior vena cava and right atrium suggestive of tumor extension, with apparent small reduction, although the intravascular tumor thrombus cannot be properly determined in a non-contrast computed tomography.

women worldwide[9]. The development of HCC is intently related to the presence of chronic liver diseases.

The diagnosis of HCC can be difficult and often requires the use of one or more imaging approaches. Ideally, tumors should be detected when they are ≤ 2 cm so that all treatment options can be offered. Vascular invasion and tumor thrombosis formation are commonly seen in cases of advanced HCC due to the activation of hemostasis[10]. The incidence of vascular invasion increases with a larger tumor size, and it has been reported to be present in 82% of patients with serum AFP levels > 1000 μg/L and a tumor diameter of > 5 cm[11]. IVC thrombosis is often asymptomatic, but symptoms related to portal hypertension can develop, for example, upper gastrointestinal bleeding, abdominal pain and ascites. Contrarily, the intra-atrial growth of HCC may not cause any symptoms per se but may lead to pulmonary thrombosis and pulmonary metastasis[12]. Advanced HCC with the invasion of the IVC and RA is exceptional, but it has been associated with poor prognosis and limited treatment options. In a retrospective study of 50 patients with advanced HCC with RA involvement, the median survival was only 2 mo with supportive care and only marginally improved to 4 mo with aggressive therapies[13].

The European Association for the Study of the Liver (EASL) recommends patients with preserved liver function and minimally affected performance status (PS 0-2), stage C according to the Barcelona Clinic Liver Cancer Group (BCLC) and intermediate-stage (BCLC B) not eligible for locoregional therapies to undergo treatment with systemic therapy. Sorafenib, a multi-kinase inhibitor (TKI) was

introduced in the EASL guidelines in 2008. Sorafenib showed a survival benefit, documenting prolonged median survival and a nearly 3-mo extension of time to radiologic progression, which represented a breakthrough in the management of HCC[14]. Alpha-fetoprotein has been reported as a new alternative biomarker to RECIST to detect a sorafenib response in HCC, with the optimum cut-off points varying in published literature but suggesting that higher pre-treatment (AFP > 200 ng/mL) is associated with earlier recurrence and poorer overall survival[15,16]. Other experimental treatments were dismissed due to the age and medical history of the patient. Although sorafenib is the standard of care in these patients for more than a decade, further treatments and approaches have been undertaken in several reports as the resection of TT, the simultaneous resection of liver tumor and TT, external beam radiation therapy (EBRT), thalidomide treatment and transarterial chemoembolization (TACE). The postoperative survival period of patients with HCC and tumor thrombus in the RA varies from 18 d to 56 mo, with a mean survival of 20 mo[17]. In a retrospective cohort study of patients with HCC extending into the IVC/RA, treated with hepatectomy and thrombectomy, TACE and symptomatic treatment, the median survival was 19, 4.5 and 5 mo, respectively[17]. All these data indicate that removing the thrombus surgically combined with hepatectomy, or only TT extraction, might result in an improvement of survival in selected patients compared with other non-surgical procedures.

Radiofrequency ablation (commonly referred to as RFA) can result in long-term remission in small HCC. Nonetheless, performing RFA near the major vessels or diaphragm is difficult and challenging[18]. Several studies have validated that EBRT alone or combined with non-surgical treatment might achieve an excellent intrahepatic tumor control and a potential survival benefit of advanced HCC EBRT, presenting objective response rates ranging from 39% to 62% in patients with an associated macrovascular invasion[19]. The combination of TACE and RT has been beneficial compared with TACE alone for unresectable HCC in two meta-analyses[20,21]. Moreover, for major vessel invasion cases, combined TACE and RT had better OS and PFS than sorafenib, with a liver function not significantly worsened after treatment[22]. Further studies are crucial to accurately establish the incidence of adverse events and confirm these findings.

In our case, the patient was not a candidate for an invasive procedure; hence, systemic therapy with sorafenib was initiated. No predictive biomarkers of response to sorafenib or other TKI have been identified, and objective responses have been uncommon with the RECIST and modified RECIST criteria[23]. At a 3-mo follow-up, the patient improved symptoms with less dyspnea and asthenia, diminished hepatomegaly at physical exploration, a lower AFP levels and reached stable disease by CT scan.

## CONCLUSION

HCC is one of the most common and aggressive malignant tumors, being the TT formation in advanced stages a relatively common complication usually involving the portal vein, and less frequently, the hepatic veins. Hepatopulmonary syndrome and portopulmonary hypertension are the two main causes of dyspnea that must be discarded in a cirrhotic patient.

Non-invasive imaging is an essential part of HCC diagnosis and contributes to primary liver tumor staging. When dynamic explorations demonstrate a typical diagnostic pattern, no further diagnostic invasive procedures are necessary. HCC TT expansion to IVC and RA is rare and indicates poor prognosis. There is no consensus on anticoagulation or other interventions in these patients. Treatment response has formerly been evaluated using the RECIST criteria by CT scan, but alpha-fetoprotein may be employed to consider sorafenib or any other TKI's activity in HCC. Other treatment approaches could have been feasible as EBRT alone or combined with non-surgical treatment, but more data are essential to further establish the status of EBRT for the management of HCC.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge the important contribution by Dr. Alberto Escudero Rodriguez in the diagnostic, investigation and follow-up of the case reported.

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