

World Journal of *Hepatology*

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Spontaneous bacterial peritonitis due to carbapenemase-producing *Enterobacteriaceae*: Etiology and antibiotic treatment

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Abstract

Carbapenem antibiotics were first introduced in the 1980s and have long been considered the most active agents for the treatment of multidrug-resistant gram-negative bacteria. Over the last decade, carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as organisms causing spontaneous bacterial peritonitis. Infections caused by CRE have shown a higher mortality rate than those caused by bacteria sensitive to carbapenem antibiotics. Current antibiotic guidelines for the treatment of spontaneous bacterial peritonitis are insufficient, and rapid de-escalation of empiric antibiotic treatment is not widely recognized. This review summarizes the molecular characteristics, epidemiology and possible treatment of spontaneous bacterial peritonitis caused by CRE.

Key Words: Spontaneous bacterial peritonitis; Carbapenem-resistant *Enterobacteriaceae*; Carbapenem-resistant *Klebsiella pneumoniae*; Cirrhosis

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Core Tip: Carbapenem antibiotics were first introduced in the 1980s and have long been considered the most active agents for the treatment of multidrug-resistant gram-negative bacteria. Over the last decade carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as organisms causing spontaneous bacterial peritonitis (SBP). Infections caused by CRE have shown a higher mortality rate than those caused by bacteria sensitive to carbapenem antibiotics. Current antibiotic guidelines for the treatment of SBP are insufficient, and rapid de-escalation of empiric antibiotic treatment is not widely recognized. This review summarizes the molecular characteristics, epidemiology and possible treatment of SBP caused by CRE.

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a common complication in patients with cirrhosis. It is defined as ascitic fluid infection in the absence of alternative surgically treatable sources of intra-abdominal infection^[1]. SBP diagnosis relies on ascitic fluid polymorphonuclear cell count greater than or equal to 250 cells/mm³. Microbiological culture, either from ascitic fluid or the bloodstream, enables identification of the etiological pathogen^[2,3]. Approximately 2.5% of all hospitalizations of patients with cirrhosis are for SBP, and the short-term mortality is about 25%^[4]. In-hospital mortality remains a significant burden to the healthcare system, especially in patients with concurrent risk factors such as older age, female gender, hepatic encephalopathy, coagulopathy, variceal hemorrhage, sepsis, pneumonia and acute kidney injury^[5].

Historically, the most frequent etiological agents remain gram-negative bacteria (GNB), especially *Enterobacteriaceae* spp. Although in recent times gram-positive bacteria (GPB) appear to be on the rise^[6,7]. Today, SBP due to multidrug-resistant (MDR) bacteria represents a growing and complex healthcare problem. Infections caused by MDR-bacteria carry a high mortality rate in the cirrhotic patient^[8]. This is likely due to difficulty in establishing an effective antibiotic regimen along with a depressed immune system^[9].

SBP due to MDR bacteria proves to be a clinical challenge^[10,11], and clinicians should consider reported resistance profiles for the decision-making process in deciding empiric antibiotic regimens^[12]. Third generation cephalosporins that for decades have been used as the treatment of choice for community acquired-SBP should no longer be used as first-line therapy^[13]. Carbapenem antibiotics, introduced in the 1980s, have long been considered the most active agents against MDR-GNB. Unfortunately, over the last decade carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as SBP causing bacteria^[14] and have shown a higher mortality rate than infections caused by bacteria sensitive to carbapenem antibiotics^[15]. Current antibiotic guidelines for the treatment of SBP are insufficient^[9,16], and rapid de-escalation of empiric antibiotic treatment is not widely recognized^[17]. This review summarizes the molecular characteristics, epidemiology, and possible treatment of SBP caused by CRE.

THE BURDEN OF CARBAPENEMASE-PRODUCING ENTEROBACTERIACE IN SPONTANEOUS BACTERIAL PERITONITIS

The health burden caused by cirrhosis corresponds to 14-26 new cases per 100000 individuals and results in 170000 deaths per year in Europe^[18]. Cirrhotic patients have a higher susceptibility to infections caused by resistant bacteria (repeat hospitalizations and antibiotic exposure for long-term prophylaxis of SBP), and the management of these patients has become a major global health concern. In addition, antimicrobial resistance has emerged as a public health crisis. In the case of SBP, gram-positive cocci (methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant

Enterococci), extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* and CRE are emerging as the causative agents^[19].

While resistant GPB can be common, the classes of resistant *Enterobacteriaceae* are much rarer and more devastating^[20]. The spread of these pathogens is difficult to control because of a potential huge intestinal reservoir^[21]. A recent single-center Italian study reported that the prevalence of extensively resistant (XDR) organisms increased from 16% between 2008-2009 to 36% between 2012-2013^[22]. In patients with decompensated cirrhosis the major determinants of prognosis are bacterial infections, especially if caused by resistant pathogens. This can be shown to increase mortality rate four-fold^[23]. The likely cause of resistant pathogens in cirrhotic patients is the inadequate long-term empirical prophylactic antibiotic treatment that they are prescribed. This results in antimicrobial resistance with life-threatening consequences. Between 11% and 45% of patients with SBP and spontaneous bacteremia are infected with organisms resistant to tigecycline, which is an antibiotic that seems to be effective in the majority of healthcare-associated and nosocomial infections^[10]. The overall proportion of MDR bacteria in patients with nosocomial SBP was 22% to 73% of cases across multiple studies^[24].

The high prevalence of MDR or XDR pathogens causing SBP are directly linked to high mortality rates. It is therefore not a surprise that we have been forced to incorporate empiric use of carbapenems. The rising global empiric administration of carbapenems has now created a selection pressure promoting the emergence of CRE^[25]. It has furthermore been proven that the efficacy of empirical antibiotic therapy in nosocomial SBP is very low, ranging from 26% to 67.6%^[26].

Piano *et al*^[14] reported that even targeted therapy proved difficult for infection resolution. They described a case of SBP due to carbapenemase-producing *Klebsiella pneumoniae* (KPC) in a 57-year-old patient that was treated with meropenem for an extended period. The KPC found *via* nasal swab was susceptible to colistin and tigecycline but did not respond to treatment and ultimately led to death within 10 d. In 2015, Li *et al*^[27] studied 31 patients affected by SBP both nosocomial and non-nosocomial acquired. Among these patients, four presented with KPC and two with *Escherichia coli* (*E. coli*) resistant to meropenem. While the *E. coli* cases were nosocomial-SBP, half of the KPC patients were found to be non-nosocomial, demonstrating spread of infection outside the nosocomial setting, which is where empiric treatment is more common.

Similar difficulties of treatment have been reported by Alexopoulou *et al*^[28] in 2016. In this study the authors analyzed data from 130 patients affected by SBP. Meropenem showed a drug resistance rate of 30.7%. The 77% of pathogens resistant to meropenem were susceptible to colistin, while the 86% of GNB were susceptible to tigecycline. Only 54% of the pathogens resistant to meropenem were susceptible to tigecycline. All but one XDR bacteria were susceptible to a possible combination of colistin and tigecycline.

That same year, Lutz *et al*^[29] described ninety-two SBP cases, three of which were *Enterococcus faecium* resistant to carbapenems. Tudorascu *et al*^[30] found cases of carbapenem-resistant *E. coli*, KPC and carbapenem-resistant *Enterobacter* spp. In Italy, Salerno *et al*^[31] reported one case of carbapenem-resistant *E. coli* and seven cases due to KPC. Béjar-Serrano *et al*^[32] in 2019 reported a case of SBP caused by carbapenemase-producing *Enterobacter cloacae* (*E. cloacae*). **Table 1** summarizes the findings of the studies mentioned above describing the total number of patients affected by SBP and the number of SBP caused by CRE. Furthermore, it describes the type of pathogen involved and if the SBP was nosocomial or non-nosocomial acquired.

MOLECULAR CHARACTERISTICS OF CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE CAUSING SPONTANEOUS BACTERIAL PERITONITIS

Enterobacteriaceae show two major types of antibiotic resistance. One mechanism involves the expression of ESBL, which render bacteria resistant to cephalosporins and monobactams. The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases, which render bacteria resistant to almost all available β -lactams including the carbapenems^[33]. These bacteria are called carbapenemase-producing CRE. Carbapenemases represent the most versatile family of β -lactamases, with a breadth of activity unrivaled by other β -lactam-hydrolyzing enzymes. Although known as “carbapenemases,” many of these enzymes recognize almost all

Table 1 Synthesis of a selection of the studies published on spontaneous bacterial peritonitis due to carbapenem-resistant *Enterobacteriaceae* producing pathogens

| Ref. | Total number SBP/CRE SBP | CRE | CRE N-SBP/Total SBP | Not-N-SBP/Total SBP |
|--|--------------------------|---|---------------------|---------------------|
| Piano et al ^[14] , 2012 | 1/1 | <i>K. pneumoniae</i> | 1/1 | 0/1 |
| Li et al ^[27] , 2015 | 31/6 | <i>K. pneumoniae</i> , <i>E. coli</i> | 2/4, 2/2 | 2/4, 0/2 |
| Alexopoulou et al ^[28] , 2016 | 130/6 | <i>K. pneumoniae</i> , <i>E. coli</i> | 5/5, 1/1 | 0/5, 0/1 |
| Lutz et al ^[29] , 2016 | 92/3 | <i>E. faecium</i> | 3/3 | 0/3 |
| Tudorascu et al ^[30] , 2016 | 64/3 | <i>K. pneumoniae</i> , <i>E. coli</i> , <i>Enterobacter</i> | 1/1, 1/1, 1/1 | 0/1, 0/1, 0/1 |
| Salerno et al ^[31] , 2016 | 56/8 | <i>K. pneumoniae</i> , <i>E. coli</i> | 5/7, 0/1 | 7/2, 1/1 |
| Béjar-Serrano et al ^[32] , 2019 | 22/1 | <i>E. cloacae</i> | 1/1 | 0/1 |

CRE: Carbapenem-resistant *Enterobacteriaceae*; N: Nosocomial; SBP: Spontaneous bacterial peritonitis; CRE SBP: Spontaneous bacterial peritonitis due to carbapenem-resistant *Enterobacteriaceae*; CRE N-SBP: Nosocomial spontaneous bacterial peritonitis due to carbapenem-resistant *Enterobacteriaceae*; Not-N-SBP: Not nosocomial spontaneous bacterial peritonitis; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *E. cloacae*: *Enterobacter cloacae*; *E. faecium*: *Enterococcus faecium*.

hydrolyzable-lactams and are resilient against inhibition by all commercially viable β -lactamase inhibitors.

Carbapenemases are classified according to the degree of homology of the respective polypeptide chains. According to Ambler classification, four classes of enzymes are recognized. Classes A, C and D include the β -lactamases with serine at their active site, whereas molecular class B β -lactamases (M β LS) are all metalloenzymes with zinc at their active-site^[34]. Currently, among the four classes of β -lactamases defined by the Ambler classification system, three have been identified to give resistance to carbapenems: (1) The class A of β -lactamases in which KPC is included; (2) The class B of metal- β -lactamases to which the imipenemase (IMP) and the Verona integron-encoded metal- β -lactamase [Verona imipenemase (VIM)] belong; and (3) The class D to which β -lactamases, such as oxacillinase oxacillin-hydrolyzing (OXA)-48, belong^[35].

These enzymes are coded starting from specific genes that can be acquired in two ways: By transfer through plasmid or by clonal bacterial strain expansion^[36]. Class A carbapenemases have a serine in the active state in position 70 and can hydrolyze carbapenems, cephalosporins, penicillins and aztreonam while being inhibited by clavulanic acid and tazobactam. The enzymes KPC-1, KPC-2, KPC-3, *Guiana-Extended-Spectrum* (GES)-4, GES-5 and GES-6 have been found mainly in *Klebsiella pneumoniae*. *Serratia marcescens* (S. *marcescens*) enzyme (SME)-1, SME-2 and SME-3 have been found in *S. marcescens*; NMC-A and KPC-3 have been found in *E. cloacae*, and GES-5 has been found in *E. coli*^[34]. These enzymes are summarized in Table 2.

KPC and GES are associated with mobile elements. None have been reported yet for the SME genes^[37,38]. Figure 1 illustrates the different kind of genes and mobile elements related to each class of carbapenemase with the site of action, the inhibitor substances and the antimicrobials hydrolyzed for each class of enzymes.

Class B enzymes are characterized by resistance to beta-lactamase inhibitors. They share hydrolytic activity with Class A carbapenemases but are not effective against aztreonam. The hydrolysis mechanism depends on the activation of the active site by zinc ions. This feature makes them highly sensitive to inhibition by ethylene diamine tetraacetic acid, which is capable of chelating zinc and other cations. Although the amino acid homology of these proteases is poor (about 23%), all the class B carbapenemases show excellent zinc binding capacity and a well-preserved active site^[39]. The B carbapenemases have been found, as described in Table 3, mainly in *Klebsiella pneumoniae* (IMP-1, IMP-1-like, IMP-4, VIM-1, VIM-2-like, VIM-4), *E. coli* (VIM-1, IMP-4, IMP-1-like), *S. marcescens* (IMP-1-like, VIM-2, VIM-2-like), *E. cloacae* (VIM-1, VIM-2, VIM-2-like, VIM-5, VIM-4, IMP-1-like, IMP-4, IMP-8) and *Citrobacter freundii* (IMP-1, IMP-1-like, VIM-2)^[34]. Shown in Figure 1, these enzymes are associated with respective genes such as VIM, NMD and IMP. Furthermore, they are associated with several mobile elements (*i.e.* IncN, IncI1, multiple types; class I integrons, IncL/M, IncA/C)^[37].

Class D enzymes include oxacillin-hydrolyzing- β -lactamases identified mainly in

Table 2 Class A carbapenemase asset found in each pathogen

| Pathogens | Class A carbapenemase | | | | | | | | | |
|----------------------|-----------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | KPC-1 | KPC-2 | KPC-3 | GES-4 | GES-5 | GES-6 | SME-1 | SME-2 | SME-3 | NMC-A |
| <i>K. pneumoniae</i> | + | + | + | + | + | + | | | | |
| <i>S. marcescens</i> | | | | | | | + | + | + | |
| <i>E. coli</i> | | | | | + | | | | | |
| <i>E. cloacae</i> | | | + | | | | | | | + |

KPC: *Klebsiella pneumoniae*; GES: Guiana-Extended-Spectrum; SME: *Serratia marcescens* enzyme; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *E. cloacae*: *Enterobacter cloacae*; *S. marcescens*: *Serratia marcescens*.

Table 3 Class B carbapenemase asset found in each pathogen

| Pathogens | Class B carbapenemase | | | | | | | | | |
|----------------------|-----------------------|------------|-------|-------|-------|------------|-------|------------|-------|-------|
| | IMP-1 | IMP-1-like | IMP-4 | IMP-8 | VIM-1 | VIM-1-like | VIM-2 | VIM-2-like | VIM-4 | VIM-5 |
| <i>K. pneumoniae</i> | + | + | + | | + | | + | | + | + |
| <i>S. marcescens</i> | | + | | | | + | + | + | | |
| <i>E. coli</i> | | + | + | | | + | | | | |
| <i>E. cloacae</i> | | + | + | + | + | | + | + | + | + |
| <i>C. freundii</i> | + | + | | | | | + | | | |

IMP: Imipenemase; VIM: Verona imipenemase; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *E. cloacae*: *Enterobacter cloacae*; *S. marcescens*: *Serratia marcescens*; *C. freundii*: *Citrobacter freundii*.

Enterobacteriaceae and *Pseudomonas aeruginosa*^[40]. Functionally they are penicillinases capable of hydrolyzing both oxacillin and cloxacillin. These enzymes are characterized by extreme variability in the amino acid sequence producing many enzyme variants that are only weakly inhibited by ethylene diamine tetraacetic acid and clavulanate^[41]. The molecular structure was analyzed by detecting a homology with class A enzymes with serine in the active site in positions varying between 70 and 73 in the S-T-F-K tetrad^[34]. The active site of the D carbapenemases is very efficient due to its small size and increased hydrophobicity due to the tyrosine and methionine residues present in position 112 and 223, respectively. The OXA carbapenemases have highly conserved structures in position 144-146 with sequence Y-G-N and in position 216-218 with sequence K-T-G. At present, 102 distinct OXA enzymes have been identified, of which at least 37 (9 broad spectrum enzymes) are to be considered carbapenemases. These 37 were then divided into 9 main subgroups based on an amino acid homology exceeding 92.5%^[42]. Subgroups 1 and 2 share the substitution F with Y in the sequence Y-G-N that does not seem to improve the hydrolyzation of the imipenem compared to the other carbapenemases.

The mechanism of action is similar to other serine-carbapenemases but carbon dioxide seems to influence the kinetics of OXA-carbapenemases. In cases of high carbon dioxide concentrations, the carboxylation of lysine occurs in position 73 activating the serine at the catalytic site^[43]. OXA carbapenemases act on penicillin, cephalosporin and imipenem with faster hydrolysis of imipenem than meropenem^[44]. These enzymes, as described in Table 4, have been found mainly in *Klebsiella pneumoniae* (OXA-48, OXA-181, OXA-163), *S. marcescens* (OXA-48), *E. coli* (OXA-48, OXA-244, OXA-181) and *E. cloacae* (OXA-48). They are associated with OXA genes and several mobile elements (*i.e.* IncL/M, Tn1999, IS1999)^[37], as reported extensively in Figure 1.

Table 4 Class D carbapenemase asset found in each pathogen

| Pathogens | Class D carbapenemase | | | |
|----------------------|-----------------------|---------|---------|---------|
| | OXA-48 | OXA-163 | OXA-181 | OXA-244 |
| <i>K. pneumoniae</i> | + | + | + | |
| <i>S. marcescens</i> | + | | | |
| <i>E. coli</i> | + | | + | + |
| <i>E. cloacae</i> | + | | | |

OXA: Oxacillin-hydrolyzing; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. coli*: *Escherichia coli*; *E. cloacae*: *Enterobacter cloacae*; *S. marcescens*: *Serratia marcescens*.

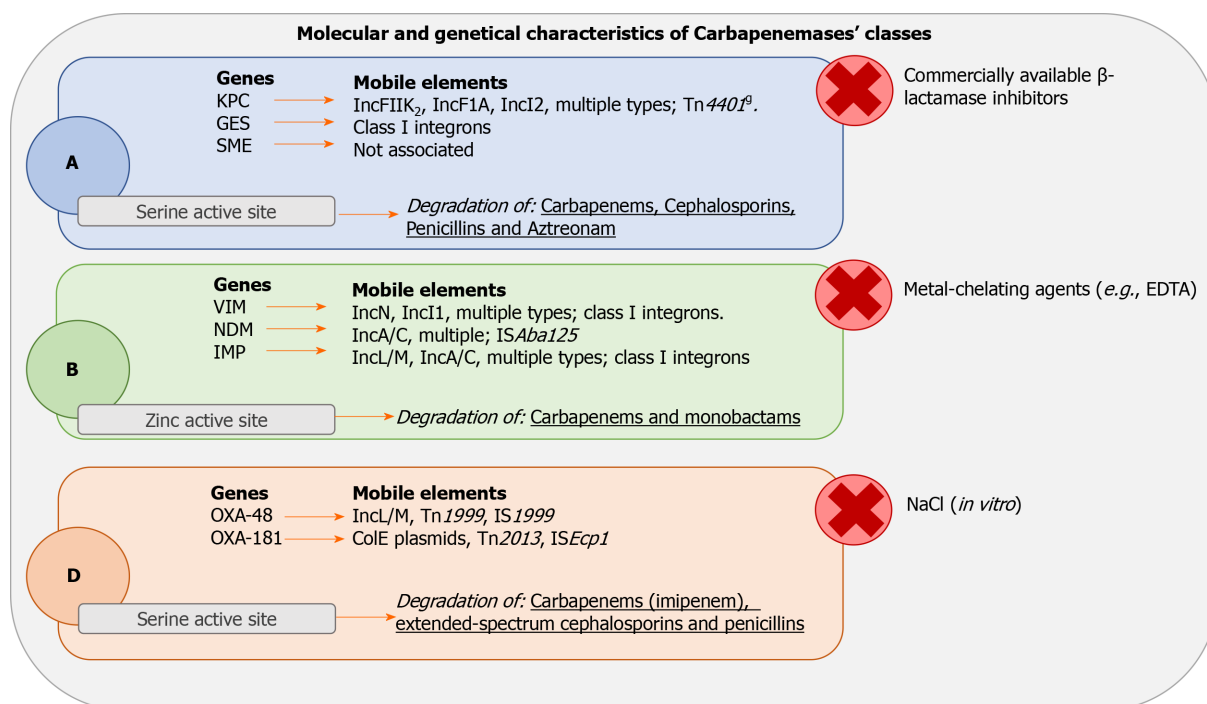


Figure 1 Molecular characteristics, genetics and activity of carbapenemases classes. A: Class A carbapenemases; B: Class B carbapenemases; D: Class D carbapenemases; GES: *Guiana-Extended-Spectrum*; SME: *Serratia marcescens* enzyme; KPC: *Klebsiella pneumoniae* carbapenemase; VIM: Verona imipenemase; NDM: New Delhi carbapenemase; IMP: Imipenemase; OXA: Oxacillinase.

ANTIMICROBIAL MANAGEMENT OF SPONTANEOUS BACTERIAL PERITONITIS DUE TO CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE

Aminoglycosides, mainly amikacin and gentamicin, have been widely utilized in the era of limited treatment options for the management of CRE^[45]. Overall, antimicrobial susceptibility for CRE varies^[46]. These antibiotic agents require high dose daily administration with therapeutic drug monitoring to optimize their use^[46-48]. Plazomicin is a newly marketed aminoglycoside. It is approved for the management of complicated urinary tract infections (cUTI) in patients with limited or no options for alternative treatment^[49]. It has activity against GNB producing ESBL, KPC and AmpC^[50,51]. Overall, it has poor activity against nonfermenting GNB^[52,53].

Colistin is an old polymyxin widely utilized for the management of serious infections due to CRE^[47,48,54,55]. Colistin resistance remains low among nonfermenting GNB but is increasing in *Klebsiella* spp. producing KPC enzymes^[56]. Its role as monotherapy or within a combination regimen is still under discussion due to the absence of reliable data^[48,57,58]. Fosfomycin is another old antibiotic utilized in the treatment of infections due to CRE in critically ill patients^[59]. It has activity against GPB and GNB, including MDR strains such as CRE. However, during monotherapy rapid emergence of antibiotic resistance has been described^[17,60,61]. High doses of tigecycline

have been widely utilized as a last-resort option for the treatment of serious infections due to CRE. It is a glycolcycline with activity against a broad range of GPB and GNB including MDR strains but not *Pseudomonas* spp. or *Proteus* spp.^[62].

Eravacycline is a synthetic fluorocycline antibiotic recently approved for the treatment of complicated intra-abdominal infections (cIAI). It has broad spectrum activity including MDR and XDR isolates with the exception of *Pseudomonas* spp. and *Burkholderia* spp. Overall, it has activity against GNB producing ESBL, KPC, AmpC, MβL and OXA enzymes^[6,7,20,50]. Moreover, eravacycline is active against the most common tetracycline-resistance mechanisms such as efflux and ribosomal protection^[63]. In IGNITE 1 and 4 clinical trials, it showed a high clinical and microbiological response with a favorable safety and tolerability profile in patients with cIAIs^[64,65]. Eravacycline also has a high oral bioavailability that can facilitate a sequential antibiotic regimen (from intravenous to oral formulation) with patients being discharged home^[66].

Among β-lactam antibiotics, ceftazidime/avibactam is a novel cephalosporin/β-lactamase inhibitor combination with activity against several GNB including strains producing ESBL, KPC, AmpC and some OXA enzymes (OXA-48)^[50]. In phase 2 and 3 clinical trials, ceftazidime/avibactam demonstrated efficacy and safety in patients with cIAIs^[67-69]. It was successfully used as salvage therapy in patients with severe infections due to CRE^[70,71]. Of note, emergence of resistance during therapy has already been described^[30]. The appropriate use of ceftazidime/avibactam in the management of CRE infections as monotherapy or part of combination regimen is still an open debate^[72].

Meropenem/vaborbactam is a novel carbapenem/β-lactamase inhibitor combination with activity against GNB producing ESBL, KPC and AmpC but not MβL and OXA enzymes^[50-52,73]. Meropenem/vaborbactam was approved for the treatment of bacteremic cUTI, cIAIs, hospital-acquired pneumonia including those associated to mechanical ventilators (hospital-acquired pneumonia and ventilator associated pneumonia) and for the treatment of all infections due to GNB where treatment options were limited. In TANGO 1 and 2 clinical trials, meropenem/vaborbactam was associated with high clinical and microbiological success^[74,75]. In a sensitivity analysis of the TANGO 2 clinical trial among patients without prior antibiotic failure, meropenem/vaborbactam showed a significant higher clinical cure rate at the test-of-cure visit and a lower day-28 all-cause mortality than the best available therapy^[75].

In a multicenter retrospective cohort study, meropenem/vaborbactam was found to have similar clinical success to ceftazidime/avibactam (69% *vs* 62%; *P* = 0.49)^[76]. Although the propensity of meropenem/vaborbactam for development of resistance is lower than ceftazidime/avibactam, mechanisms of antibiotic resistance are described (porin mutations and increase in the blaKPC expression)^[77,78]. Interestingly, an *in vitro* study showed a synergistic effect of meropenem/vaborbactam plus a ceftazidime/avibactam combination against susceptible KPC strains but also against both meropenem/vaborbactam and ceftazidime/avibactam-resistant KPC isolates^[79].

Imipenem/cilastatin/relebactam is another novel carbapenem/β-lactamase inhibitor combination with activity against GNB producing ESBL, KPC and AmpC enzymes^[50-52]. Imipenem/cilastatin/relebactam was approved for the management of cUTIs and cIAIs in adult patients with limited or no available treatment options. In a phase 3 clinical trial (RESTORE-IMI 1), imipenem/cilastatin/relebactam was found as an effective and well-tolerated treatment agent for CRE infections^[80].

Aztreonam/avibactam is a monobactam and β-lactamase inhibitor combination in the late form of development. It has activity against GNB producing ESBL, KPC, AmpC, MβL and some OXA enzymes (OXA-48)^[50-52]. Cefiderocol is a siderophore cephalosporin recently approved for the treatment of cUTIs in adults. It has a broad spectrum of activity against GNB, including MDR *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*^[50]. The approved drugs used to treat these CRE producing pathogens causing SBP are displayed with their advantages and disadvantages in Table 5.

Many more agents are in several phases of development: Cefepime/taniborbactam (phase 3), cefepime/enmetazobactam (phase 3), sulbactam/durlobactam (phase 3), sulopenem/etzadroxil/probenecid (phase 3), tebipenem pivoxil hydrobromide (phase 3), BOS-228 (phase 2), OP0595/RG6080 (phase 1), QPX-2015/QPX-7728 (phase 1), SPR206 (phase 1), SPR741 (phase 1), TP-6076 (phase 1) and WCK 5222 (phase 1).

CONCLUSION

SPB due to CRE is a major concern for hepatologists. Overall, CRE infections are

Table 5 Advantages and disadvantages of the antimicrobials used to treat spontaneous bacterial peritonitis due to gram-negative bacteria producing carbapenem-resistant *Enterobacteriaceae*

| Antimicrobial agent | Advantages | Disadvantages | Ref. |
|--|--|--|---------|
| Aminoglycosides (<i>i.e.</i> Plazomicin) | Good activity against GNB producing ESβL, KPC, AmpC but not MβL enzymes | Heterogeneous susceptibility high dose (toxicity) | [49] |
| Polimixins (<i>i.e.</i> Colistin) | Low resistance emergence | Low efficacy for <i>Klebsiella</i> spp. producing KPC enzymes | [56] |
| Fosfomicyn | Moderate activity against MDR-CRE | Rapid emergence of antibiotic resistance | [59] |
| Glycylcycline (<i>i.e.</i> Tigecycline) | Good activity against MDR-CRE | High dose (toxicity) | [62] |
| Fluorocycline (<i>i.e.</i> Eravacycline) | Broad spectrum activity (even if MDR and XDR pathogens). Active against the most common tetracycline-resistance mechanisms. High oral bioavailability. Safety and tolerability | Not active on <i>Pseudomonas</i> spp. and <i>Burkholderia</i> spp. | [63-65] |
| β-lactams/β-lactamase inhibitors (<i>i.e.</i> ceftazidime/avibactam) | Good activity against GNB producing ESβL, KPC, AmpC, OXA-48 and MβL. Safety and tolerability | Frequent emergence of antibiotic resistance | [67] |
| Carbapenem/β-lactamase inhibitors (<i>i.e.</i> meropenem/vaborbactam or Imipenem/cilastatin/relebactam) | Good activity against GNB producing ESβL, KPC and AmpC. Outcome improvement | Not active on GNB producing OXA-48 and MβL | [79] |
| Monobactam/β-lactamase inhibitor (<i>i.e.</i> aztreonam/avibactam) | Good activity against GNB producing ESβL, KPC, AmpC and OXA-48 | Recently approved | [50-52] |
| Siderophore cephalosporin (<i>i.e.</i> Cefidecol) | Broad spectrum of activity against GNB, including MDR <i>Enterobacteriaceae</i> , <i>Pseudomonas aeruginosa</i> and <i>A. baumannii</i> | Recently approved | [50] |

GNB: Gram-negative bacteria; ESβL: Extended-spectrum β-lactamase; CRE: Carbapenem-resistant *Enterobacteriaceae*; KPC: *Klebsiella pneumoniae*; MβL: Molecular class B β-lactamases; MDR: Multidrug resistant; XDR: Extensively resistant.

associated with an increased risk of morbidity and mortality. Current antibiotic guidelines for the treatment of SBP caused by CRE are insufficient. This review summarizes the current molecular characteristics, epidemiology and possible treatment regimens for CRE causing SBP. Many new antibiotics are being introduced into clinical practice and others are still in the preclinical and clinical phases of development. Further research of these novel agents is required for appropriate use (microbiological activity and pharmacokinetic/pharmacodynamic parameters). A multidisciplinary approach (hepatologists, infectious diseases specialists, intensivists, microbiologists, pharmacists) is essential for the adequate placement of these newer anti-infective agents in therapy. In order to optimize antimicrobial treatments and preserve the antibiotic armamentarium, a careful knowledge of local microbiological epidemiology and antibiotic-resistant rates along with detailed antimicrobial stewardship programs must be applied.

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Molecular heterogeneity in intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is a heterogeneous primary liver cancer, and currently there exist only a few options of targeted therapy. Histopathologically, iCCA is sub-classified according to morphology (mass forming type, periductal infiltrating type, and intraductal growing type) and histology (small duct type and large duct type). According to different histopathological types, clinical features such as risk factors and prognosis vary. Recent developments in genomic profiling have revealed several molecular markers for poor prognosis and activation of oncogenic pathways. Exploration of molecular characteristics of iCCA in each patient is a major challenge in a clinical setting, and there is no effective molecular-based targeted therapy. However, several recent studies suggested molecular-based subtypes with corresponding clinical and pathological features. Even though the subtypes have not yet been validated, it is possible that molecular features can be predicted based on clinicopathological characteristics and that this could be used for a more rational approach to integrative clinical and molecular subclassification and targeted therapy. In this review, we explored the genomic landscape of iCCA and attempted to find relevance between clinicopathologic and molecular features in molecular subtypes in several published studies. The results reveal future directions that may lead to a rational approach to the targeted therapy.

Key Words: Cholangiocarcinoma; Mutation; Gene expression; Pathway; Target therapy; Molecular

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Core Tip: Intrahepatic cholangiocarcinoma (iCCA) is a histopathologically and molecularly heterogeneous tumor. Recent developments in genomic profiling have revealed several molecular markers for poor prognosis and activation of oncogenic pathways. Exploration of molecular characteristics of iCCA in each patient is a major

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challenge in a clinical setting, and there exists no effective molecular-based targeted therapy. Therefore, the analysis of relevance between molecular and clinicopathological features is very important. The present analysis showed that the molecular subtypes of iCCA have distinct clinicopathologic features and prognostic differences. For developing effective targeted and personalized therapies based on clinical and molecular understanding, future additional large scale studies are needed.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor that arises from biliary epithelium in any portion of the bile duct. Intrahepatic cholangiocarcinoma (iCCA) arises from small peripheral bile duct to second-order segmental bile duct. Risk factors, clinical symptoms, type of surgical resection, and prognosis of iCCA are different from those of extrahepatic CCA (eCCA; Klatskin tumor and distal bile duct cancer)^[1,2]. iCCA is the second most common primary malignant liver tumor and accounts for 10%-15% of hepatobiliary neoplasms^[3]; however, recently, there has been an increase in the incidence and associated significance of pathogenic, clinical, and therapeutic challenges^[4]. Based on morphological and gross appearance, iCCA can be further classified into three subtypes: Mass forming (MF), periductal infiltrating (PI), and intraductal growing (IG). The prognosis of the subtypes differ according to gross morphology^[5]. Due to pathologic heterogeneity and lack of specific symptoms, iCCA is hard to diagnose at the early stages, and most of the patients are at an advanced stage at the time of diagnosis. Therefore, prognosis after curative surgical resection is dismal, and efficacy of chemotherapy or targeted therapy is limited^[6].

Recent molecular analyses revealed several markers for poor prognosis and activation of oncogenic pathways (KRAS mutation, human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGFR) signaling)^[6,7]. In addition, various recurrent mutations and fusions have been reported, including *IDH1* and *IDH2*, *BRAF*, *TP53*, and *FGFR2* genes^[7,8]. These molecular findings demonstrate a more integrative analysis of clinical and molecular alterations in iCCA. However, there exist a few other challenges. First of all, the molecular characteristics of tumor heterogeneity are not yet clear. Second, integrative relevance between clinical and molecular characteristics is not enough; and finally, most prevalent oncogenic alterations in CCA are still undruggable. Understanding the molecular characteristics in these heterogeneous tumors may derive specific biologically meaningful subtypes that can be used to define more rational potential targeted therapy.

This review provides an overview of the genetic characteristics and heterogeneity of iCCA with a focus on molecular subtypes and their relevance with the clinicopathological phenotype. Furthermore, the role of molecular markers to stratify patients based on their prognosis and response to therapies is discussed.

HISTOPATHOLOGIC CLASSIFICATION OF iCCA

Histological classification of iCCA is important for understanding the molecular heterogeneity of iCCA. Several investigations have revealed that a whole range of phenotypical traits of hepatocytes, cholangiocytes, and progenitor cells was seen in primary liver cancer [hepatocellular carcinoma (HCC) and iCCA]. It has been suggested that iCCA originated from biliary tree stem cells located within the peribiliary gland as well as hepatic progenitor cells within canals of Hering^[2]. Hepatic bipotent progenitor cells along the small intrahepatic bile duct possibly differentiate not only into hepatocytes but also into cholangiocytes, which can lead to iCCA^[9,10]. Consequently, two different histological types of iCCA may develop: One originating from hepatic stem cell-derived lineages with stem-like molecular characteristics similar to those in HCC or combined HCC-CCA and the other originating from biliary

tree progenitor stem cell-derived cholangiocytes found along the large intrahepatic bile duct with characteristics similar to perihilar or extrahepatic CCA^[2,11-13]. Histologically, iCCA is defined as an adenocarcinoma formed by columnar and cuboidal epithelial cells^[1,14].

Based on the histological findings, conventional iCCA can be classified into two main subtypes. Small bile duct type iCCA may derive from small intrahepatic bile ducts; hepatic progenitor cells present as small-sized tubular or acinar adenocarcinoma with scant mucin production^[11,14-16]. Small bile duct type is either represented as peripheral type or cholangiolar type^[17,18]. Meanwhile, large duct type arises from biliary tree progenitor stem cell and is constituted by mucin-producing columnar tumor cells in large segmental bile ducts or papillary architecture^[11,14-17,19]. Large duct type has been represented as a perihilar type or bile duct type in other studies^[17,18]. The gross and histological features of large bile duct type iCCA are similar to those of perihilar CCA and distal CCA. In addition, the majority of PI and IG has large bile duct type^[11,17,18]. However, the MF type, which is the most prominent morphologic type, is more heterogeneous as it comprises of both small duct type and large duct type^[17,18].

Although the two histological subtypes belong to iCCA, their clinical and molecular features are quite different. While viral hepatitis and cirrhosis are the risk factors of small duct type, cholangitis and parasite infection are the main cause of large duct type^[17,18]. Both subtypes have different precursor lesions and show different survival outcomes^[18]. Furthermore, they show different immunophenotypes like the abundant expression of mucin families, S100P, and anterior gradient homolog 2 in the large duct type, and N-cadherin and neural cell adhesion molecule 1 in the small duct type^[11,17,18,20]. These histopathological heterogeneities based on cell origin are critical for understanding the heterogeneity of iCCA as well as heterogeneous molecular characteristics of iCCA.

MOLECULAR ALTERATIONS IN iCCA

Recent technological advancements have helped in understanding the mutational landscape of iCCA. Mutations in common driver oncogenes and suppressor genes are summarized in [Table 1](#). Due to a small number of samples compared to other cancer and pathological heterogeneity, the prevalence of the mutation is variable across studies. However, several key driver somatic mutations commonly seen in other tumors, such as *KRAS*, *BRAF*, *TP53*, *BAP1*, and *ARID1A*, are also frequently identified in iCCA. Other driver genes like *BRAF*, *PIK3CA*, *GNAS*, *EGFR*, and *ERBBR/HER2* have also been identified in iCCA, but at a much lower frequency in most of the cohorts^[6,21-23]. The presence of *EGFR*, *TP53*, and *KRAS* mutation is known as poor prognostic factor^[6,21,24]. Mutation of *TERT* promoter and *ALB* gene, which are frequently seen in HCC, are also detected in CCA, but only in iCCA or combined HCC and CCA samples with less frequency^[23]. Meanwhile, isocitrate dehydrogenase (*IDH*)1 and *IDH2* mutations have been reported in 10%-20% of iCCA cases^[23]. Interestingly, a large extent of *IDH* mutation has been observed in iCCA and not in eCCA and rarely identified in HCC^[23,25]. *IDH* mutation is associated with a better prognosis^[26]. In one large scale study, *IDH1/2* mutations were identified to be associated with improved overall survival^[27]; however, as the incidence of *IDH* mutation is not frequent, survival impacts of *IDH* mutation is not yet clear^[28]. In iCCA, frequency of fibroblast growth factor receptor 2 (*FGFR2*) fusion is reported as 10%-15%^[29,30]. *FGFR2* pairs with some genes such as *TACC1*, *BICCI*, *PRKACA*, *AHCYL*, and *PRKACB*. These fusions result in the constitutive activation of *FGFR2* and its oncogenic functions^[31]. The *FGFR* related pathway is involved in cellular migration and proliferation. Patients with *FGFR2* fusion show good prognosis, which suggests that *FGFR2* fusions can be a prognostic marker as well as potential target for therapy^[21,32]. Altered genes involving chromatin remodeling, such as *BAP1*, *ARID1A*, and *PBRM1*, are also frequently found in iCCA^[27]. Meanwhile, germline DNA mismatch repair deficiency (Lynch syndrome) has been reported to be associated with CCA^[33]. There exists a report that deleterious germline mutations in breast cancer gene 1/2, *RAD51D*, *MutL* homolog 1, and *MutS* homolog 2 were detected in 11% of CCA patients^[21].

Genomic alteration in CCA is highly heterogeneous, like pathologic features. Several studies identified different gene alterations between iCCA and eCCA^[21,34]. While alterations in *IDH1/2*, *BRAF*, *FGFR2*, *BAP1*, and *NRAS* are frequently found in iCCA, *TP53*, *KRAS*, *SMAD4*, and *BRAF* mutations are common in eCCA^[35]. Interestingly, some of the altered genes commonly found in eCCA such as *KRAS*,

Table 1 Frequency of genetic alteration in intrahepatic cholangiocarcinoma

| Pathway | Gene | Frequency of alteration |
|---|----------------|-------------------------|
| NADPH metabolism | <i>IDH1/2</i> | 4-36 |
| Chromatic remodeling | <i>BAP1</i> | 9%-25% |
| | <i>ARID1A</i> | 11-36 |
| | <i>PBRM1</i> | 11-17 |
| Cell cycle regulation and DNA damage response | <i>CDKN2A</i> | 7 |
| | <i>CDK6</i> | 7 |
| | <i>TP53</i> | 3-38 |
| | <i>BRCA1/2</i> | 4 |
| PI3K signaling | <i>PIK3CA</i> | 4-6 |
| | <i>PTEN</i> | 1-11 |
| Ras/Raf/MEK/ERK | <i>EGFR</i> | 2.2 |
| | <i>KRAS</i> | 9-24 |
| | <i>NRAS</i> | 3.6 |
| | <i>BRAF</i> | 3-22 |
| FGF | <i>FGFR2</i> | 4-38 |

DNA: Deoxyribonucleic acid; ERK: Extracellular signal-regulated kinase; MEK: Mitogen-activated protein kinase kinase; NADPH: Nicotinamide adenosine dinucleotide phosphate; PI3K: Phosphoinositide-3-kinase.

SMAD4, and *TP53* were also shared by large duct types of iCCA. Whereas, small duct iCCA has frequent *IDH1/2* mutations and *FGFR2* gene fusion^[31,36]. Therefore, pathological characteristics and genetic alterations appear to be closely related to each other.

EPIGENETIC PROFILE OF iCCA

Epigenetic mechanisms of iCCA include histone modification, DNA methylation, and noncoding RNAs. In CCA, hypermethylation at the promoters of tumor suppressor genes has been reported^[37]. iCCA is a highly epigenetic regulated tumor type.

DNA methylation is an early molecular lesion of carcinogenesis; tumor suppressor promoter hypermethylation of tumor suppressor gene leads to transcriptional modification and inactivation, and hypomethylation of oncogenes results in activation^[38]. Most of the genes that were altered by CpG methylation belonged to wingless-related integration site (WNT), transforming growth factor beta, PI3K, MAPK, and NOTCH signaling pathways in iCCA^[39]. Like other cancers, promoter hypermethylation of tumor suppressor genes, such as *DAPK*, *SOX17*, and *RUNX3*, has been commonly reported^[40]. It is known that IDH mutations result in hypermethylation and induce silencing of *ARID1A*^[8].

MicroRNA (miRNA) plays a crucial role in diverse cellular processes and regulates gene function. Several pieces of research revealed that overexpression of miR-21 inhibits *TIMP3* and *PDCD4* and sequentially leads to cancer progression^[41]. Besides, miR-191, miR-200, miR-141, miR-204, miR-214, and miR-221 are involved in CCA development^[42]. Among these miRNAs, miR-21, miR-191, and miR-26a were identified as poor prognostic markers^[43]. Meanwhile, the high expression of several lncRNA (*H19*, *NEAT1*, *PVT1*, *CKDN2B-AS1*, and *HUILC*) has been reported to be associated with poor survival of CCA^[44,45].

However, most of the epigenetic mechanisms of iCCA have not been studied sufficiently, and their role as biomarkers and potential targeted therapies should be extensively investigated.

EXPRESSION PROFILE AND FUNCTIONAL GENOMIC PATHWAY OF iCCA

Several studies based on microarray or NGS revealed the expression profile and oncogenic pathway of iCCA. The major key oncogenic molecules, including tumor necrosis factor, transforming growth factor, extracellular regulated-signal kinase, epidermal growth factor, RAS, AKT, p53, NOTCH, and platelet-derived growth factor, are deregulated in iCCA. Immune response-related pathways and inflammation associated with signatures are also enriched^[6,8,22,24]. Aberrant HER2 expression is seen in about 30% of iCCA, and it is related to poor prognosis with coactivation of ERBB3 and EGFR2 as well as mesenchymal epithelial transition factor and mammalian target of rapamycin^[6]. Inflammation associated signatures are commonly activated in iCCA, but their oncologic and prognostic role is controversial^[22,24]. Activation of the WNT pathway is often seen in iCCA, and it relates to inflammatory reaction because macrophages in the stroma surrounding the tumor are required for the maintenance of activated WNT pathway^[46].

The oncogenic signatures are also found in many other cancers. However, deregulated pathways are different according to pathologic and molecular subtypes; therefore, subtype-specific activated pathways are important to assess biology.

INTEGRATIVE CLINICAL-MOLECULAR SUBTYPES OF iCCA

Based on the genomic profile, a few studies have suggested molecular subtype of iCCA beyond anatomical and histological subclassification (Table 2). The recent advances in the molecular classification allow better characterization of heterogeneity of iCCA. Furthermore, they provide insight into the integrated approach of clinical and molecular characterization of iCCA.

Although each subclassification has some heterogeneity, the molecular feature of iCCA is dichotomized two subtypes that have different survival and clinical outcomes^[6,8,22,24,47]. Generally, the poor prognostic molecular subtype is associated with the KRAS mutation. Also, BRAF, ERBB2, and HER alterations are often seen in a poor prognostic subtype. On the other hand, IDH mutation and FGFR fusion are commonly seen in the good prognostic subtype. The molecular subclasses were reported to be rather related to clinical and pathologic features. While PI type, similar to eCCA, is commonly seen in the poor prognostic subtype, the MF type is almost evenly distributed in both good and poor prognostic subtypes^[24]. Large duct type, history of cholangitis and parasite infection, and elevated levels of serum biomarkers (carcinoembryonic antigen and carbohydrate antigen 19-9) are associated with poor prognostic molecular subtype, while small duct type and history of viral hepatitis are associated with good prognostic molecular subtype^[24] (Figure 1).

Several molecular subclassifications in the reported studies provide information about molecular heterogeneity in addition to histopathological heterogeneity. The integrated clinical and molecular subclassifications would be helpful to provide a more rational approach to overcome clinical and molecular heterogeneity. Molecular profile of iCCA is helpful for early diagnosis and prognosis prediction and may potentially provide personalized treatment. However, exploration of molecular characteristics of iCCA in each patient is a major challenge in a clinical setting because of the high cost for evaluating molecular characterization. If molecular subtypes of iCCA have specific clinical and pathologic features, molecular subtypes can be predicted from clinical features. Although the subclassifications reported in several studies have a few differences based on demographic characteristics and study methods, there is still no consensus on the molecular subclass. The present review shows that clinical and molecular relevance based on molecular subclassification has been exploring and may establish integrative clinical and molecular subclassification soon. Since the number of patients is not sufficient in iCCA compared to other cancers, further large scale studies are necessary for validation and establishment of molecular classification.

Still, molecular-based target therapy is not considered to be effective in CCA due to molecular heterogeneity. However, the establishment of molecular subtypes can promote the development of effective subtype-specific therapeutic molecular targeted therapy. Lapatinib, a dual inhibitor of EGFR and HER2, has been reported to be effective in cell lines that had genetic characteristics similar to poor prognostic subtype^[6], while gemcitabine was identified to be effective in cell lines with similar expression profile to good prognostic subtype, which had enriched gemcitabine sensitive genes^[24]. Although these are the outcomes of cell line studies and not

Table 2 Integrative clinical-molecular subclassification of intrahepatic cholangiocarcinoma

| Good prognostic subclass | | Poor prognostic subclass |
|--------------------------|---|--|
| GSE26566 | | Periductal infiltrating type, perineural invasion; KRAS mutation, EGFR and HER2 signatures |
| GSE32225 | Well differentiated tumor; inflammation-related signatures | Poor differentiated tumor; RTK-related pathways (AKT, MET, RAS/RAF/MAPK); overexpression of EGFR; KRAS mutation |
| GSE32879 | | EMT-related signatures; TGFβ1, NCAM1, CD133 |
| GSE89749 | Fluke-negative; FGFR fusion; BAP1, IDH mutation | Fluke-positive; BRCA1/2, TP53 mutation; ERBB2 gain |
| GSE107943 | Small duct type (cholangiolar type); underlying hepatitis, cirrhosis; metabolism-related signatures; FGFR2 fusion | Large duct type (bile duct type); Elevated CEA, CA 19-9; underlying cholangitis; P53, inflammation-related signatures; KRAS mutation |
| TCGA ¹ | Mitochondria/metabolic-related signatures; IDH, BAP1 mutation | Inflammation-related pathways |

¹In The Cancer Genome Atlas, two subtypes have no statistical different survival.

BRCA1/2: Breast cancer gene 1/2; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; EGFR: Endothelial growth factor receptor; EMT: Epithelial to mesenchymal transition; FGFR2: Fibroblast growth factor receptor 2; HER2: Human epidermal growth factor receptor 2; IDH: Isocitrate dehydrogenase; MAPK: Mitogen-activated protein kinase; NCAM1: Neural cell adhesion molecule; TCGA: The Cancer Genome Atlas; TGFβ1: Transforming growth factor beta 1.

validated clinical data, it is hypothesized that additional applications of drug study on different subtype signaling pathways may be helpful to stratify patients for targeted approaches for the treatment of iCCA.

CONCLUSION

In the present study, we reviewed the molecular heterogeneity of iCCA in association with the clinicopathological features. Several recent studies have revealed molecular characteristics of iCCA and suggested several molecular subclassifications. Molecular study of iCCA may help identify patients at risk of developing iCCA, predicting prognosis, and targeting approach to treatment. However, molecular exploration in all patients is not feasible because of the high cost. Accordingly, analysis of relevance between molecular and clinicopathological features is considered as imperative because if clinicomolecular relevance is established, molecular characteristics can be predicted based on clinical features in each patient.

The present analysis showed that the molecular subtypes of iCCA have distinct clinicopathologic features and prognostic differences. However, integrative clinical and molecular subclassification is not yet validated. For developing effective targeted and personalized therapies based on clinical and molecular knowledge, future additional large scale studies are necessary.

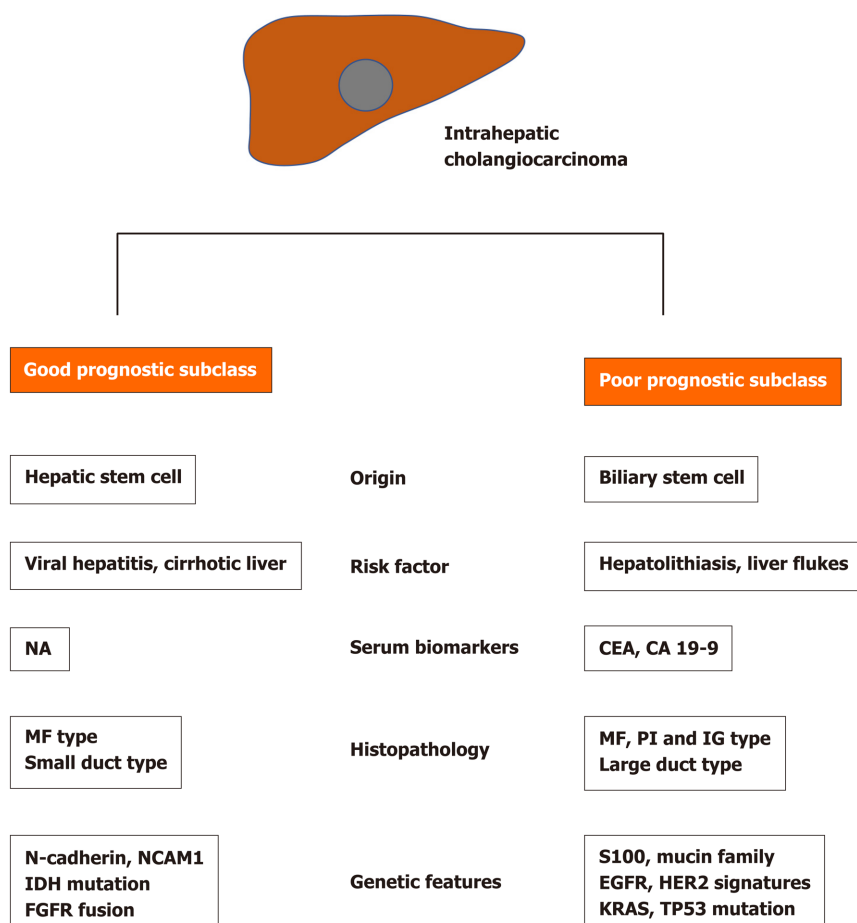


Figure 1 Summary of clinical and molecular characteristics of molecular-based subtypes of intrahepatic cholangiocarcinoma. CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; FGFR2: Fibroblast growth factor receptor 2; HER2: Human epidermal growth factor receptor; IDH: Isocitrate dehydrogenase; IG: Intraductal growing; MF: Mass forming; PI: Periductal infiltrating.

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Spectrum of esophageal motility disorders in patients with liver cirrhosis

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Abstract

Disorders of esophageal motility have been described in patients with cirrhosis in a small number of studies. In this review, we aim to provide an overview of the available evidence on esophageal motility disorders in cirrhosis and their clinical implications. This review delves into the following concepts: (1) Gastroesophageal reflux disease is common in liver cirrhosis due to many mechanisms; however, when symptomatic it is usually nocturnal and has an atypical presentation; (2) Endoscopic band ligation is better than sclerotherapy in terms of its effect on esophageal motility and seems to correct dysmotilities resulting from the mechanical effect of esophageal varices; (3) Chronic alcoholism has no major effects on esophageal motility activity other than lower esophageal sphincter hypertension among those with alcoholic autonomic neuropathy; (4) An association between primary biliary cholangitis and scleroderma can be present and esophageal hypomotility is not uncommon in this scenario; and (5) Cyclosporin-based immunosuppression in liver transplant patients can have a neurotoxic effect on the esophageal myenteric plexus leading to reversible achalasia-like manifestations.

Key Words: Esophagus; Motility; Cirrhosis; Dysmotility; Gastroesophageal reflux disease; Manometry

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Core Tip: (1) The link between liver cirrhosis and esophageal motility; (2) The association of cirrhosis with gastroesophageal reflux disease; (3) Esophageal motility

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INTRODUCTION

Disorders of esophageal motility have been described in patients with cirrhosis in a small number of studies where conventional manometry was used to assess their esophageal function. Likely, due to the severity of their liver disease, symptomatic esophageal dysmotilities are rarely investigated in routine clinical practice for this population. However, the clinical implications, especially in the setting of dysphagia, can be potentially deleterious for a patient's quality of life and nutritional status, which may contribute to increased risk of complications and hepatic decompensation. In this review, we aim to provide an overview of the available evidence on esophageal motility disorders in cirrhosis and their clinical implications.

The review will cover the following topics: (1) The link between liver cirrhosis and esophageal motility; (2) The association of liver cirrhosis with gastroesophageal reflux disease (GERD); (3) Esophageal motility disorders in liver cirrhosis patients [with and without esophageal varices (EV)]; (4) The impact of variceal treatment on esophageal motility and function; (5) The impact of some etiologies of liver cirrhosis on esophageal motility, particularly in cases of chronic alcoholism and primary biliary cholangitis (PBC); and (6) The effect of immunosuppressive therapy used in post-liver transplantation patients on esophageal motility.

HOW LIVER CIRRHOSIS CAN AFFECT ESOPHAGEAL MOTILITY

The contraction of the esophageal smooth muscle is regulated by both central and peripheral control mechanisms in a complex mechanism. Vagal preganglionic motor nerves synapse with postganglionic motor neurons in the myenteric plexus (Auerbach's plexus). Both pre- and post-ganglionic motor neurons can be excitatory or inhibitory. The amplitude of contraction is determined by a balance between intrinsic excitatory cholinergic, and inhibitory nitric oxide (NO) producing neurons. It was found that of 60%-70% of myenteric neurons are connected to vagal excitatory efferent neurons. Thus, in situations where parasympathetic hypofunction is evident, the amplitude of peristaltic contractions would be hampered^[1-3].

Liver cirrhosis is known to be associated with autonomic neuropathy (parasympathetic hypofunction and sympathetic hyperfunction), increased NO production, and gut hormonal changes that can impact esophageal motility and lead to esophageal hypomotility^[4].

Miyajima *et al*^[5] studied the autonomic nervous function in 27 patients with liver cirrhosis by using spectral analysis of heart rate variability. The results showed that decreased parasympathetic tone and increased sympathetic tone are commonly observed in patients with liver cirrhosis, particularly those in a hyperdynamic state. The cause of this autonomic dysfunction was presumed to be caused by increased NO production and increased activity of the renin-angiotensin system.

Also, Koshino *et al*^[6] conducted a neuropathological study in autopsy specimens of the esophagus of 8 cirrhotic patients with large EV, in which esophageal motility disorders were demonstrated by esophageal manometry, esophagography, and endoscopy. The histological findings were compared to those of 7 control patients without esophageal or liver disease. The study demonstrated a significant reduction of the number of ganglion cells at the myenteric plexus in the esophagus of cirrhotic patients with large varices. Thus, the investigators suggested that such

neuropathological changes may have contributed to, at least in part, the development of impaired motility of the esophagus with large varices.

LIVER CIRRHOSIS AND GERD

Generally, the two mechanisms that mainly contribute to the pathogenesis of GERD are: (1) Increased rate of transient lower esophageal sphincter (LES) relaxations; and (2) Decreased LES resting pressure (LESP) leading to stress and spontaneous reflux. Many factors in liver cirrhosis contribute to increases in LES relaxation: (1) Increased NO concentration in patients with liver disease can lead to a decrease in the LESP and increased transient LES relaxations^[7]; (2) Increased levels of some gastrointestinal (GI) hormones like vasoactive peptides and neurotensin are known to reduce the pressure of the LES^[8,9]; (3) The presence of ascites creates an increase in intra-abdominal pressure^[10,11]; and (4) Lastly, the presence of EV causes mechanical impairment of esophageal emptying, thus extending the contact time between the refluxate and the esophageal mucosa^[12].

GERD can be viewed as a motility disorder involving the LES and the lower esophagus. Detection and treatment of GERD in the setting of liver cirrhosis are particularly important, as GERD can significantly affect the quality of life of those patients. Also, persistent GERD is considered to be the main cause of esophagitis and can lead to damage to the mucosal barrier possibly creating a nidus for bleeding. The presence of esophagitis in cirrhotic patients has the potential to result in more complications given the effects of cirrhosis on clotting factors and indirectly on platelets.

The prevalence of GERD in liver cirrhosis patients was reported to be ranging between 55%-64%^[13,14]. Zhang *et al*^[13] assessed 78 cirrhotic patients without EV using esophageal manometry, simultaneous ambulatory 24-h esophageal pH, and bilirubin monitoring and esophagogastroduodenal endoscopy (EGD). The study showed that the resting LES pressure in cirrhotic patients was diminished. This manometric finding was attributed to the high levels of NO in the systemic circulation of patients with cirrhosis, leading to hypotensive LES and increased GERD. Moreover, the same study reported a higher prevalence of reflux esophagitis (37%) compared to healthy controls. The study also demonstrated that mixed acid and bile reflux was the main pattern of reflux in cirrhotic patients with a stepwise increase of mixed reflux along with the severity of liver function damage. However, assessment of the symptom burden in those patients revealed that typical GERD symptoms were reported in just (32%) of patients with cirrhosis, with the symptoms being predominantly nocturnal.

Another study of 1280 patients with the chronic liver disease found that patients with the most severe cirrhosis had asymptomatic manifestations and/or atypical GERD symptoms^[15]. In this study, typical GERD symptoms only accounted for around 10% of cirrhotic patients.

Therefore, adding questions about atypical GERD symptoms should be a part of the cirrhotic patient's history and possibly can be incorporated into variceal screening indications. If present, GERD work-up should be done with EGD, or at least empirical GERD treatment might be considered.

EV AND MOTILITY

Liver cirrhosis without varices

Two notable studies in the literature examined the effects of liver cirrhosis (without the effect of varices) on the motility of the esophagus. A Chinese research group assessed seventy-eight cirrhotic patients with no EV confirmed by upper endoscopy *vs* thirty healthy control volunteers^[13]. The study showed that cirrhotics without EV had significantly abnormal LESP, peristaltic amplitude, peristaltic duration and velocity, in comparison to those in the control group. LESP was significantly lower in patients with severe liver function damage, and had a negative correlation with Child-Pugh score ($P < 0.01$, $r = -0.625$). These results confirmed that liver cirrhosis itself is another important factor impacting esophageal motility.

Another study by Passaretti *et al*^[16], assessed esophageal motility in 45 patients with cirrhosis and EV, 15 patients with cirrhosis without EV, and 20 healthy controls. The study demonstrated diminished peristaltic wave amplitude in the lower esophagus.

In summary, the majority of cirrhotic patients without varices presented with

esophageal dysmotility (particularly hypomotility), and it was concluded that cirrhosis itself was an important causative factor.

Liver cirrhosis and varices

Abnormalities in esophageal motility may be further aggravated by the presence of EV. A study that included 45 patients with EV and 45 healthy controls showed decreased peristaltic wave amplitude and increased frequency of tertiary contractions in the former group, but these abnormalities were not associated with symptoms^[17].

Another study showed that the presence of varices was associated with an increase in LES length and reduced lower esophageal contraction pressure and failure of sphincter relaxation during swallowing^[18].

Passaretti *et al*^[16] compared cirrhotics with varices *vs* those without varices *vs* controls. Cirrhotics with varices showed a decreased amplitude of peristaltic waves in the lower half of the esophagus ($P < 0.01$). Resting LES pressure and duration of sphincter relaxation were similar in patients and controls. The study proved that the presence of EV is associated with more pronounced esophageal motility disorders and that maybe the mechanical effect of the presence of varices. However, the clinical significance of this observation is unclear as these disorders are rarely associated with retrosternal pain or dysphagia. Thus, it seemed that EV itself, independent of the cirrhosis, delayed esophageal clearance and increased the contact time between acid and mucosa.

Furthermore, another study assessed esophageal transit used radionuclide material; reported longer transit time in patients with large varices than in those with small varices (8.3 ± 1.7 s *vs* 7.2 ± 0.7 s, $P < 0.05$)^[19]. Another study using scintigraphy found that esophageal transit was prolonged in 47.3% of patients with EV and 29.2% of patients without varices ($P < 0.05$). Also, the frequency of esophageal transit alteration was related to the severity of liver disease using the Child-Turcotte-Pugh (CTP) score, ($P < 0.01$)^[20].

Flores *et al*^[21] included 74 patients suffering from liver cirrhosis and EV, without previous endoscopic treatment. All of them performed esophageal manometry while 55 patients had 24-h pH monitoring. Esophageal motility disorders have been found in 44 patients (60%). The most prevalent was the ineffective esophageal motility (IEM), observed in 28%. This showed that the majority of cirrhotic patients with treatment-naïve EV have esophageal motility disorder, mainly IEM. The clinical relevance of these manometric findings needs more research in the scope to determine the real significance of these abnormalities.

ESOPHAGEAL MOTILITY CHANGES AFTER VARICEAL TREATMENT

Band ligation

Endoscopic variceal ligation (EVL) has largely replaced sclerotherapy based on comparative effectiveness in the management of acute variceal bleeding and eradicating EV with considerably fewer complications. Also, EVL does not seem to have a significant effect on esophageal motility^[22]. Chen *et al*^[17] examined forty-five patients who had liver cirrhosis and EV before and after EVL. Another 45 matched patients without hepatic, esophageal, or systemic disease served as the control group. The study demonstrated that the LESP and contractile amplitude in the lower esophagus was significantly lower in patients before EVL ($P < 0.05$) but returned to the level of control subjects after EVL ($P > 0.05$). The percentage of tertiary waves was significantly higher in patients before and after EVL than in the control group ($P < 0.05$). However, no significant swallowing disturbance was noted in patients after EVL. The study further confirmed that the presence of varices affects esophageal motility. Interestingly, EVL normalized esophageal motility and did not induce any motility abnormality.

Another study showed similar results where manometric abnormalities detected before banding were corrected after the procedure^[23]. However, a different study demonstrated an inverse correlation between the frequency of EVL sessions and esophageal peristaltic amplitudes^[24].

Regarding the relationship between EVL and GERD, de la Peña *et al*^[25] assessed the presence of GERD in twenty-six cirrhotic patients using pH monitoring before and after EVL. Five patients had excess gastroesophageal reflux before the band ligation. A further six patients developed excess gastroesophageal reflux after endoscopic treatment. The only factor implicated in the development of excess gastroesophageal reflux was the use of sclerosant at the end of treatment to ensure complete eradication.

The study concluded that EVL does not significantly provoke excess GERD if sclerosant is not used in the endoscopic technique.

Sclerotherapy

Endoscopic injection sclerotherapy (EIS), has a documented effect on esophageal structure and motility. EIS mainly impacts the function of the lower esophagus resulting in decreased amplitude and/or velocity and increased duration of the peristaltic contractions, which may be replaced by non-propagating contractions^[22]. It may also prolong acid clearance, but this is usually transient and resolves within a week. Therefore, a short course of antacid therapy is justified following EIS. A small study, assessed esophageal motility pre and post EIS, showed decreased LES pressure in the latter, but without a significant increase in GERD episodes^[26]. The hypothesized mechanism of this motility abnormality is that the sclerosing agent may induce vagal nerve injury. EIS has been also associated with structural abnormalities, mainly sclerosant-induced esophageal ulcerations and fibrotic strictures^[27], and less commonly with esophageal perforation^[28]. Strictures can be associated with dysphagia and may require endoscopic dilatation.

Bretagne *et al*^[29] assessed the effects of EIS on esophageal symptoms and function. The study included 24 cirrhotic patients 60 d after variceal eradication had been achieved (group I); nine cirrhotics with varices (group II) and 16 normal volunteers (group III) as a control group. After sclerotherapy, nine patients developed an esophageal stricture and most of them had dysphagia. The percentage of patients with abnormal peristaltic waves (non-propulsive contractions) was significantly more in the sclerotherapy group. Also, the percentage of time below pH 4 did not differ between the EIS group and the normal control group on 3 h postprandial esophageal pH monitoring.

A similar conclusion was obtained by Sauerbruch *et al*^[30], in which the study group found that sclerotherapy of EV may lead to a reduced peristaltic esophageal motility with an impaired transport function. This could contribute to the development of dysphagia or esophagitis.

Masclee *et al*^[31] tried to test the hypothesis that these complications result from vagal nerve injury due to EIS. This was done by measuring pancreatic polypeptide secretion in response to insulin-induced hypoglycemia (insulin 0.1 U/kg i.v.), a well-known stimulus for vagal nerve function. Six patients with cirrhosis and variceal bleeding were included before and 3 d after the first sclerotherapy (group A). Also, six other patients with cirrhosis after 6 mo of successful repeated EIS (group B) and 12 control subjects (group C) were tested. No significant reduction in the integrated pancreatic polypeptide secretion between or within groups. However, only a transient and reversible reduction in pancreatic polypeptide secretion were observed directly after sclerotherapy.

GERD after EIS was investigated as a possible contributing factor in the development of strictures following EIS^[32]. Twelve randomly selected patients underwent repeated EIS for the management of bleeding EV. Half of the patients developed strictures; however, there were no significant differences between stricture and non-stricture patients during 24 h of esophageal pH monitoring. It was concluded that GERD is not likely to play a major role in EIS stricture formation.

Esophageal band ligation vs sclerotherapy

A randomized controlled study comparing esophageal function in patients receiving EIS *vs* EVL following an episode of variceal bleeding. The esophageal function was assessed utilizing esophageal manometry and 24-h pH study at presentation and a month later^[33]. EIS was associated with decreased peristaltic wave amplitude, increased simultaneous contractions, and increased exposure to pH < 4. No esophageal dysfunction was observed in the EVL group. As mentioned earlier, EVL led to the partial improvement of esophageal dysmotility in 45 patients with cirrhosis at 4-6 wk following variceal obliteration^[17].

A comparison between these two endoscopic techniques was conducted by another study by Goff *et al*^[34]. Twenty-eight patients (seven with no prior treatment, eight undergoing sclerotherapy, and 12 undergoing variceal ligation) were evaluated with a symptom questionnaire and esophageal manometry. The study showed that variceal ligation therapy causes less esophageal dysfunction and has fewer local complications, as eight of the nine sclerotherapy patients had a stricture after treatment that required dilatation, whereas none of the ligation patients had strictures.

Radionuclide esophageal transit tests were used to compare esophageal emptying following EIS *vs* EVL^[35]. The results showed significant impairment of esophageal transit time with EIS but the impact was reversible. However, EVL exerts no

significant impact on esophageal emptying.

Surgical treatment of varices

Ten to 15% of patients with variceal bleeding do not respond to non-operative methods and require surgical intervention. Surgical options include shunt and non-shunt procedures. Devascularization (non-shunting) operations can serve in bridging the interim waiting period until the definitive treatment (live transplantation) is available to be done. In the United States, the options of non-surgical modalities, as well as liver transplantation, are more easily available. However, in other parts of the world where the facilities are not as well developed, devascularization procedures still have a significant role to play in the emergency management of esophagogastric variceal bleeding^[35].

We found only one study discussing the effects of surgical variceal treatment on esophageal function. Draczkowski *et al*^[36] assessed the esophageal motility in patients undergoing extensive devascularisation and esophageal transection. The study examined eight patients before and after the operation. It demonstrated no significant differences between pre and postoperative manometric findings, suggesting that the surgery does not affect a significant effect on esophageal motility. Future studies are needed to validate these findings.

IMPACT OF ALCOHOL ON ESOPHAGEAL MOTILITY

Alcohol consumption can affect the upper GI tract by multiple mechanisms relying either on direct contact of ethanol and/or its metabolite acetaldehyde with the mucosa as well as by the fermentation products of alcoholic beverages. These mechanisms can lead to: (1) Inflammation of the esophageal and gastric mucosa; (2) Impairment of motility and affection of GI sphincter pressures; and (3) Alteration of gastric acid secretion. All these effects are considered reversible and dose-dependent^[37-40].

Increased prevalence of GERD or reflux esophagitis has been reported in alcoholics^[41]. Inflammation of the esophageal mucosa induced by ethanol is caused by direct damage of the mucosal barrier which predisposes tissue to acid injury. In animal models, exposure of esophageal epithelium to HCl alone induced little or no morphological or functional changes. However, the simultaneous exposure to both ethanol and HCl resulted in significant morphologic and functional impairment^[42].

Both acute and chronic alcohol consumption affects esophageal motility. Acute ethanol administration *in vivo*, in man as well as in cats, caused a transient decrease in the LESP, amplitude of contraction of the lower esophagus, and mucosal clearance due to a primary and secondary peristalsis reduction^[43]. In a cat model, cervical vagotomy or nerve block did not prevent the effects of acute ethanol administration suggesting a direct inhibitory effect of alcohol on esophageal muscle cells^[44,45]. In an *in vitro* model, carbachol-dependent shortening of the cells was significantly diminished when esophageal smooth muscle cells were pre-exposed to ethanol, thus confirming that ethanol directly inhibits the contractile activity of the esophageal muscle cells^[44].

Conversely, the chronic effect of alcohol on esophageal motility resulted in an increased tone of the LES and reduced esophageal clearance^[43]. Yazir *et al*^[46] reported that chronic alcohol consumption in an alcohol-fed rat model, caused impairment of LES relaxation and contractile responses of both LES and muscularis mucosa layer of the esophagus.

Ferdinandis *et al*^[47] examined twenty-three chronic alcoholic subjects and 12 control subjects. Eight alcoholic subjects had heartburn and regurgitation but none had dysphagia. Ten (43%) alcoholic subjects had autonomic neuropathy and four (17%) had increased GERD. LES hypertension was observed in alcoholic subjects with autonomic neuropathy. Esophageal body motility parameters (*i.e.*, frequency, duration, amplitude, and percentage of peristaltic waves) were not significantly different between alcoholic subjects and controls.

These results of esophageal manometry on chronic alcoholic subjects, seem to show that long-term ethanol intake has no major effects on the esophageal motility activity other than LES hypertension among those with alcoholic autonomic neuropathy.

COEXISTENT PBC WITH CREST SYNDROME (REYNOLDS SYNDROME)

Esophageal motility abnormalities are well known in progressive systemic sclerosis

(scleroderma/CREST syndrome), Sjögren's syndrome, and some rheumatic diseases with sicca syndrome^[48-50]. However, there is not enough data about esophageal dysmotility in patients with PBC.

PBC is a chronic progressive autoimmune disease affecting intrahepatic bile ducts^[51]. A distinctive feature of PBC is its association with autoimmune disorders. Sjögren's syndrome and autoimmune thyroiditis appear to be the most common extrahepatic^[52].

PBC is reported to be associated with CREST syndrome in 1%-6% of cases^[51]. Márquez Galán *et al*^[53] described this entity in a mini-series of 6 patients with other characteristics and thus, it is sometimes called PACK syndrome (PBC, anti-centromere antibody, CREST syndrome, and keratoconjunctivitis sicca) or Reynolds syndrome. Tojo *et al*^[54] reported that, compared with PBC alone, patients with coexistent PBC and CREST syndrome had a higher association of EV in earlier stages of PBC, higher titers of anticentromere antibody, lower titers of antimitochondrial antibody, and a higher prevalence of HLA-DR9.

Esophageal involvement in PBC was investigated using esophageal manometry in 18 patients with PBC *vs* control group of 18 matched subjects^[55]. All patients were screened for clinical manifestations of scleroderma and the presence of Sjögren's syndrome. Four patients had scleroderma. Three patients with scleroderma had aperistalsis and diminished lower sphincter pressure. These results indicate that esophageal motility dysfunction is often present in patients with PBC who have scleroderma.

Another study detected esophageal dysmotility in 17 of 37 patients (45.9%) with PBC^[52]. The study showed that the most common esophageal motility abnormality in this group of patients was IEM (10 non-specific esophageal motility disorder and 5 patients with esophageal hypomotility).

In summary, it should be remembered that PBC can be associated with an extrahepatic autoimmune disorder such as limited cutaneous SSS (CREST syndrome). Screening for these autoimmune disorders can prevent further morbidity and keep patients viable candidates for a liver transplant.

IMPACT OF IMMUNOSUPPRESSIVES ON ESOPHAGIN POST LIVER TRANSPLANTATION PATIENTS

Cyclosporine A (CsA) is an immunosuppressive agent commonly used in liver transplant patients. It is a calcineurin inhibitor drug that exerts its immunosuppressive effect by preventing interleukin-2 (IL-2) production in T cells^[56].

Koch *et al*^[57] reported a case of a 59-year-old man who underwent liver transplantation for cryptogenic liver cirrhosis. Initial immunosuppressive therapy consisted of CsA-based immunosuppression.

Approximately 3 mo after discharge, the patient started to suffer from dysphagia usually involving solids, associated with intermittent retrosternal pain and globus sensation. A few weeks later, the case presented with vomiting/regurgitation, weight loss, and severe dysphagia for solids and liquids. Esophageal manometry revealed the pattern of achalasia with poor relaxation of the LES and simultaneous, repetitive contractions in the esophageal body. CsA was then discontinued resulting in a significant improvement of the esophageal symptoms. The dysphagia was completely resolved during the follow-up, and the patient returned asymptomatic. Esophageal manometry was repeated three months later, which showed recovery of the LES and esophageal body functions to the normal range.

CsA (calcineurin inhibitor) exerted a neurotoxic effect on the intrinsic nerves of the myenteric plexus, most likely affecting NO-producing neurons. Calcineurin is widely distributed throughout the nervous system and, experimentally, calcineurin inhibition leads to blockage of NO synthase activity, which may contribute to the reported reversible esophageal motility disorder. In conclusion, esophageal manometry should be considered early in the diagnostic workup in transplant patients on CsA-based immunosuppression and presenting with dysphagia.

CONCLUSION

(1) GERD is common in cirrhosis and usually presents with atypical symptoms; (2) EV can impact motility and band ligation is better than sclerotherapy regarding correcting

dysmotilities; (3) Chronic alcoholism has no major effects on the esophageal motility activity other than LES hypertension, on the other hand, acute ethanol consumption seems to lower LES pressure; (4) Reynolds syndrome involves an association between PBC and scleroderma. Esophageal hypomotility is expected in this setting; (5) Cyclosporin-based immunosuppression in liver transplant patients can have a neurotoxic effect on the esophageal myenteric plexus leading to reversible achalasia-like manifestations; and (6) Future studies are needed to determine the association of the liver disease with esophageal dysmotility particularly IEM. As an initial step, studying the prevalence of non-obstructive dysphagia and non-cardiac chest pain in this population will allow us to determine the utility of motility testing in this population.

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Metabolic associated fatty liver disease: Addressing a new era in liver transplantation

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Abstract

Metabolic associated fatty liver disease (MAFLD), previously termed non-alcoholic fatty liver disease, is the leading global cause of liver disease and is fast becoming the most common indication for liver transplantation. The recent change in nomenclature to MAFLD refocuses the conceptualisation of this disease entity to its metabolic underpinnings and may help to spur a paradigm shift in the approach to its management, including in the setting of liver transplantation. Patients with MAFLD present significant challenges in the pre-, peri- and post-transplant settings, largely due to the presence of medical comorbidities that include obesity, metabolic syndrome and cardiovascular risk factors. As the community prevalence of MAFLD increases concurrently with the obesity epidemic, donor liver steatosis is also a current and future concern. This review outlines current epidemiology, nomenclature, management issues and outcomes of liver transplantation in patients with MAFLD.

Key Words: Fatty liver; Metabolic associated fatty liver disease; Non-alcoholic fatty liver disease; Liver transplantation; Cirrhosis; Metabolic syndrome

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Core Tip: Metabolic associated fatty liver disease (MAFLD), previously termed non-alcoholic fatty liver disease, is the leading global cause of liver disease and is becoming the most common indication for liver transplantation. Several challenges exist in the pre-, peri- and post-liver transplant setting for patients with MAFLD, which mostly relate to comorbid medical conditions, obesity and cardiovascular risk. Donor

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liver steatosis is also an increasing concern. In this review, we summarise the current literature and provide an approach to address the current challenges of MAFLD and liver transplantation.

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INTRODUCTION

Metabolic associated fatty liver disease (MAFLD), previously termed non-alcoholic fatty liver disease (NAFLD), has emerged as the most common cause of liver disease globally^[1]. With the expanding epidemic of obesity worldwide^[2], MAFLD is becoming an increasingly burdensome condition, both clinically and economically^[3,4]. The global prevalence of MAFLD was estimated at 25% in 2013, rising from 15% in 2005^[1]. Obesity and type 2 diabetes mellitus (T2DM) coexist in 51%-60% and up to 76% of individuals with MAFLD, respectively. The peak age group affected is 45-62^[1], however it is also a disease of the older patient, with over 40% of people over the age of 60 years affected^[5].

The term MAFLD encompasses all fatty liver disease states, which aligns with the traditional view that NAFLD represents a spectrum of liver disease associated with insulin resistance, starting with pure "benign" steatosis (NAFL), through to non-alcoholic steatohepatitis (NASH)^[6], which is the inflammatory state that can lead to advanced fibrosis or cirrhosis. The community prevalence of NASH is estimated to be between 1.5% to 6.5%, however, data are sparse due to the reliance on liver biopsy for diagnosis^[7]. NASH is more rapidly progressive than benign steatosis, with fibrosis progression occurring at approximately one fibrosis stage every seven years for NASH and every 14 years for NAFL^[8]. A subgroup of "rapid progressors" has been proposed in NASH, with 21% of patients in the same study without significant fibrosis progressing to F3/F4 fibrosis over median of 5.9 years. Once cirrhosis is established, time to decompensation is variable but it is estimated that 10%-39% of patients with cirrhosis will decompensate within five years^[9-11]. The hepatocellular carcinoma (HCC) risk in MAFLD cirrhosis is estimated to be 6.7% at 5 years, and 15% at 10 years^[12]. This variability in the natural history of NAFLD has driven the recent proposition to rename this disease entity as MAFLD, which we have adopted for the majority of this review. There are currently no approved pharmacological therapies that have been proven to effectively halt disease progression in terms of decompensation or HCC development.

Liver transplant (LT) is the major therapeutic intervention in well-selected patients with MAFLD-related advanced liver disease and has a 5-year survival of 85%^[13]. The main indications for LT in MAFLD are clinical decompensation or HCC. MAFLD is the fastest rising indication for LT in the United States, the second most common indication for LT overall and the leading indication in female LT recipients^[14,15]. Similar trends have also been observed in Europe, Australia and New Zealand^[16,17]. The association of MAFLD with obesity, the metabolic syndrome, advancing patient age and cardiovascular morbidity poses several unique challenges in the setting of liver transplantation. This review aims to summarise current data regarding liver transplantation and MAFLD by exploring the pre-, peri- and post-transplant considerations in this patient group.

NOMENCLATURE

While the term NAFLD has been used since the 1980s to describe fatty liver disease in the absence of significant alcohol intake or other causes of steatogenesis^[18,19], it does not adequately describe the underlying pathogenic factors that drive the disease process. NAFLD is heterogeneous, with a multitude of factors influencing disease severity and natural history, including age, sex, ethnicity, alcohol intake, dietary habits, hormonal status, genetics and epigenetics, microbiota and metabolic status. At an individual level, disease phenotype is shaped by the dynamic interplay between genetics,

metabolic status and environment, and the predominant driver is different between individuals with the same overarching disease. This heterogeneity is clinically important when considering the natural history of disease, non-invasive assessment of fibrosis, applicability of animal modelling and the generalisability of clinical trials. Furthermore, the term NAFLD has derogatory connotations with the use of terms “alcoholic” and “fatty”, which may imply blame on the patient for their condition and create stigma. These factors have sparked a revision of the definition and nomenclature of NAFLD, with four aspects cited as main factors in support of a change^[20]; that this disease should be diagnosed by inclusion rather than exclusion, that its name should not be directly linked to alcohol, that dichotomous stratification into NASH and non-NASH can be misleading, and that considering disease heterogeneity is vital when approaching management or the design and interpretation of clinical trials.

Recent survey results from an international expert consensus panel reported that the majority panel members believed the terminology should be updated, and that the words “metabolism”, “fat”, and “liver” should be included in disease nomenclature^[21]. Metabolic associated fatty liver +/- disease (MAFL/MAFLD) emerged as the most preferred term, acting as an “umbrella term” for the heterogeneity of disease and reflecting metabolic factors as the major driver of the disease, rather than a lack of alcohol. The proposed criteria for a positive diagnosis of MAFLD are based on histological, imaging or blood biomarker evidence of hepatic steatosis, in addition to the presence of at least one of the following: Obesity, T2DM, or metabolic dysregulation^[20]. Hence, this diagnosis can exist regardless of alcohol consumption or other co-existing liver diseases. Renaming fatty liver disease and refocusing the conceptualisation of the disease process to its metabolic underpinning may help to spur a paradigm shift in the approach to its management; in research design and targets, system-wide interventions, funding and public and patient perception^[19]. For the purpose of this review, we have used MAFLD in place of NAFLD, particularly as MAFLD is yet to be accepted universally^[22] and the term NASH has been removed in the new nomenclature. The term NASH dominates the literature in the setting of LT, reflecting a progressive phenotype of disease and hence we have continued to use NASH as used in the cited studies. We acknowledge and support that this change in nomenclature to MAFLD could be valuable in highlighting the added complexities of managing metabolic disease in pre-, peri- and post-transplant settings (Figure 1).

METABOLIC ASSOCIATED FATTY LIVER DISEASE AND TRENDS IN LIVER TRANSPLANTATION

Chronic Hepatitis C virus (HCV) infection has previously been the leading global indication for LT. However, with the advent of direct acting antivirals (DAAs), this landscape is dramatically changing. It is anticipated that MAFLD related cirrhosis and HCC will become the leading indication for LT within the next decade^[23,24]. Even before the widespread use of DAAs for HCV, MAFLD was the most rapidly rising etiology in LT recipients in the United States, increasing four-fold from 2002 to 2012^[25]. NASH has now emerged as the second leading etiology of chronic liver disease for LT recipients in the United States. Examination of United Network for Organ Sharing and Organ Procurement and Transplantation (UNOS/OPTN) data from 2004 to 2013 found that new waitlist registrants with NASH increased by 170%, compared with 45% for alcohol related liver disease (ALD) and 14% with HCV^[26]. The Australia and New Zealand Liver and Intestinal Transplant Registry reports that the proportion of patients transplanted for MAFLD has increased from 8.0% to 10.2% from 2012 to 2018, compared with a reduction from 33.8% to 13.3% for HCV^[13]. European Liver Transplant Registry (ELTR) rates of LT for NASH have increased from 1.2% in 2002 to 8.4% in 2016^[27]. UNOS/OPTN data also found that NASH is now the leading indication for LT in females, increasing by 91% from 2004 to 2016^[15]. In men, NASH increased by 120% over the same period, second only to alcohol related liver disease^[15].

MAFLD has also emerged as the fastest growing cause of HCC in LT candidates. On the basis of Scientific Registry of Transplant Recipients (SRTR) data from 2002-2016, the proportion of NASH in HCC in LT candidates increased sevenfold over that time period, from 2.1% to 16.2%, while the proportion with HCV and ALD remained stable. While HCV remains the leading etiology of HCC in waitlisted candidates at 48% in 2017, NASH is now the second leading etiology at 18%, compared with 2% in 2002^[14]. Data from the UNOS/OPTN registry demonstrate similar results, with the number of

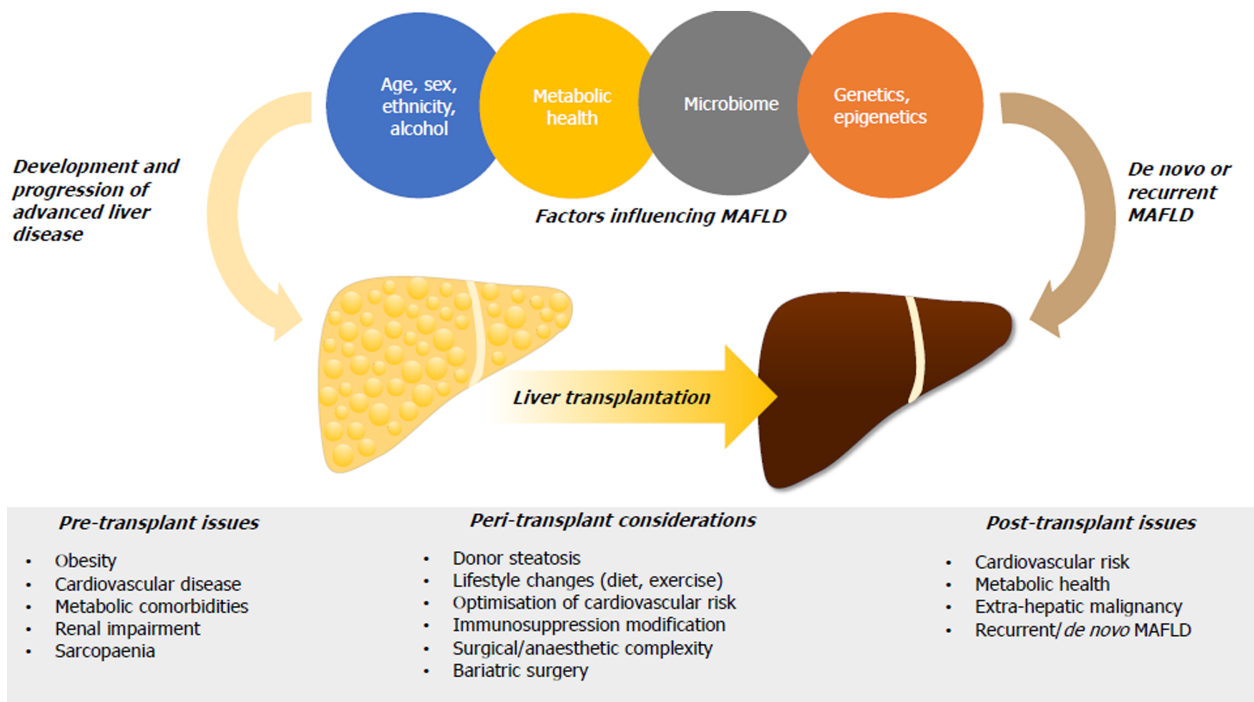


Figure 1 Metabolic associated fatty liver disease and the influence on liver transplantation. MAFLD: Metabolic associated fatty liver disease.

patients undergoing LT for HCC secondary to NASH increasing 4-fold from 2002 to 2012, representing 13.5% of patients in 2012, second only to HCV at 49.9%^[25]. In Europe from 2002-2016, 39.1% of patients transplanted for NASH had HCC compared with 28.9% of non-NASH patients^[27]. In Australia and New Zealand, similar trends have been observed with NASH associated HCC increasing from 4% of LTs performed in 2004 to 14% in 2017^[28].

MAFLD may pose specific challenges on the LT waiting list. A study using UNOS/OPTN data^[26] reported that ALD patients were least likely to survive on the waiting list at one year [likely because of higher Model for End Stage Liver Disease (MELD) scores at time of registration]. However, after adjusting for MELD, patients with ALD, HCV or a combination of the two were more likely to survive 90 d on the liver LT waitlist compared with NASH patients. Similar outcomes were also demonstrated at one year.

An important factor in considering longer term data trends in MAFLD and transplantation is the recognition that the majority of patients formerly diagnosed with cryptogenic cirrhosis (CC) likely had “burnt out” NASH^[26,29]. Whether the waitlist and post-transplant outcomes for CC can now be considered interchangeable with NASH remains controversial. Golabi *et al*^[30] used SRTR data from 1994 to 2016 to compare outcomes of patients listed or transplanted for NASH or CC in the United States. NASH and CC accounted for 12.5% of total listings; the term NASH was not used until 2004 and became more prevalent than CC by 2009. The total CC + NASH rate increased from 8.3% in 2012, to 19.5% in 2016. Interestingly, there was almost no pre-transplant diabetes recorded in any liver transplant patients prior to 2004, possibly as LT recipient selection was historically more restrictive in terms of comorbidities. After 2004, diabetes was found at rate of 40%-55% in NASH diagnoses, 30%-35% in CC diagnoses and 14%-18% in other chronic lung disease (CLD) controls. A similar trend was seen for obesity, although unlike diabetes, the rates of obesity in CC were stable pre- and post-2004. As the rates of metabolic syndrome were considerably higher in CC patients *vs* other CLD controls, the authors concluded that a large proportion of CC patients listed for LT have underlying NASH. Post-transplant diabetes was similar in the CC and NASH group, and higher than other CLD controls, inferring that the same metabolic risk underlies liver disease in CC *vs* NASH. Post-transplant outcomes were similar in patients whether CC or NASH was the listing diagnosis. In contrast, in an analysis of Australian registry data from 1994-2017, the phenotypic profiles of NASH and CC were examined, and NASH patients has a significantly higher proportion of diabetes (50% *vs* 16%), hypertension and coronary artery disease, as well as a higher mean body weight at time of LT (93.8 *vs* 68.1 kg), suggesting a low misclassification rate^[28]. With the evolution of MAFLD as a diagnosis of inclusion,

future classification of MAFLD patients in these databases could be much clearer.

PRE-TRANSPLANT ASSESSMENT

Several predisposing factors for MAFLD have been identified (Figure 1), however, not all have clinical relevance in the setting of pre-LT assessment. Established genetic polymorphisms associated with MAFLD progression such as *PNPLA3* (patatin-like phospholipase domain-containing protein) and *TM6SF2* (transmembrane 6 superfamily member 2) are not routinely screened for, but may have some influence on post-LT steatosis^[31,32]. Similarly, the role of epigenetic factors and the microbiome, beyond traditional metabolic risk factor assessment and modification, are yet to be elucidated in the assessment candidates for LT^[21].

As MAFLD often co-exists with metabolic syndrome components and is associated with increased cardiovascular risk, careful evaluation of comorbidities is paramount in pre-LT assessment. Aggressive risk factor modification should be initiated or continued on the waiting list where possible. However, there are scant data to support a specific strategy for the management of comorbidities in MAFLD patients compared to other etiologies. An approach to MAFLD and LT is presented in Table 1.

Cardiovascular disease

The presence of MAFLD carries a significant risk of cardiovascular disease, which is the most common cause of death in this group. Whether MAFLD per se is an independent driver of cardiovascular disease remains contentious^[33], the degree of hepatic fibrosis is clearly proportional to cardiovascular risk. A large meta-analysis of 16 studies with over 34000 participants found that the presence of MAFLD was associated with a 64% increased risk of fatal and non-fatal cardiovascular events over a median of seven years follow up, with risk increasing with severity of liver disease, however traditional cardiovascular risk factors were not controlled for^[34]. Another large study of European primary care databases found that after adjusting for age, sex, smoking and classic metabolic risk factors (hypertension, T2DM, high cholesterol and statin use), there was no positive association between MAFLD and myocardial infarction or stroke, concluding that cardiovascular risk assessment in adults with MAFLD should be conducted in the same way as for the general population^[35]. A single-centre retrospective study of 115 NASH patients undergoing LT, compared to 127 controls with ALD, found that patients with NASH were 4-fold more likely to have a cardiovascular event in the first year after LT, even when controlling for traditional risk factors. The majority (70%) of these events occurred in the post-operative period. There was no difference between patient, graft or cardiovascular mortality between the groups^[36].

Whether driven by MAFLD itself or traditional risk factors, patients with advanced liver disease being considered for LT are at great risk of clinically evident or sub-clinical cardiovascular disease. The aim of pre-transplant assessment is to diagnose and manage this pre-LT^[37], in particular coronary artery disease, portopulmonary hypertension, and myocardial disease. However, there is currently insufficient evidence to recommend a specific approach to pre-LT cardiovascular assessment in the MAFLD population. A combination of stress-testing, cardiac imaging and invasive angiography may be often required, however the approach to assessment is generally dictated by local protocols and cardiology expertise^[38].

Diabetes, hypertension and dyslipidemia

The presence of pre-transplant diabetes has an adverse effect on mortality, with or without MAFLD. An analysis of SRTA data between 1994 and 2013 found that 11.2% of 85194 LT recipients had pre-transplant diabetes. Diabetes pre-LT was found to be an independent predictor of LT recipient mortality with an adjusted hazard ratio of 1.21 (95% CI: 1.12-1.30)^[39]. An Australian multicentre cohort study of 617 patients undergoing LT, pre-LT diabetes was associated with reduced post-transplant survival (hazard ratio: 1.89, 95% CI: 1.25-2.86), whereas obesity, hypertension, dyslipidemia, and the metabolic syndrome itself were not. Obese diabetic patients had longer intensive care and hospital stays than non-obese diabetic or obese, non-diabetic patients. The impact of diabetes and obesity was greater in older patients and those with HCC^[40]. These results were not specific to patients with NASH as the indication for LT. Optimal glucose control is paramount in the pre- and peri-LT setting, in light of increased postsurgical complications with poor glucose control^[41].

While management of the modifiable risks of diabetes, hypertension and

Table 1 Approach to metabolic associated fatty liver disease in the transplant candidate

| Management stage | Challenges | Considerations | Approach |
|------------------|------------------------|--|--|
| Pre-transplant | Cardiovascular disease | Most common cause of death in MAFLD patients; Older patients with multiple comorbidities driving cardiovascular risk, disease may be subclinical; Pharmacologic optimisation of risk factors can be limited by liver dysfunction <i>e.g.</i> , statins, beta blockade, anti-platelets agents | Rigorous pre-transplant assessment including stress echocardiography and coronary angiography in high risk patients; Risk factor modification as per general population |
| | T2DM | Pre-LT diabetes associated with reduced survival post-LT; Poor glycemic control immediately pre-LT and peri-LT increases surgical complications | Tight glycemic control during waitlist period and peri-operative; Multidisciplinary approach to diabetic management |
| | Renal dysfunction | Multifactorial in MAFLD, with hypertension and T2DM; Even mild disease at time of LT associated with higher risk of all-cause and cardiovascular mortality | Prevent even small deterioration in renal function prior to LT; Consider simultaneous liver kidney transplant where indicated |
| | Nutrition | Pre-LT nutrition has major influence on post-LT morbidity, mortality and hospital stay; Assessment is difficult in obese patients and those with ascites; Sarcopenic obesity and myosteatosis are common. Risk factors for long term mortality | Specialist nutritional consultation prior to transplant with assessment for sarcopenia; High energy, high protein diet with enteral feeding if required |
| Peri-operative | Obesity | More common in MAFLD than other etiologies; Peri-operative challenges <i>e.g.</i> , surgical technique, wound infection and dehiscence, biliary complications; Balancing healthy weight loss in pre-LT period with muscle loss and sarcopenia; Exercise often limited by frailty and possible transient increases in portal pressure with excessive strain | Controlled weight loss in pre-LT period ensuring protein requirements met. Very low-calorie diets not recommended Bariatric surgery pre-LT or simultaneously with LT in highly selected patients. Sleeve gastrectomy preferred over laparoscopic banding or gastric bypass |
| | Donor steatosis | Donor steatosis > 30% is a risk factor for primary graft non-function and graft loss; Balancing risk of complications with steatotic donors against organ availability and demand | Assessment of hepatic steatosis at all stages of organ procurement; Future possibilities with machine perfusion and liver reconditioning |
| | Cardiovascular risk | NASH patients more likely to have cardiovascular events in the post-operative period | Careful pre-operative assessment to predict risk; Close perioperative monitoring |
| Post-transplant | Recurrent MAFLD | Due to non-dynamic genetic, metabolic and behavioural factors, 50% of MAFLD transplant recipients have recurrent MAFLD post-LT | Choice of less diabetogenic immunosuppression regimen <i>e.g.</i> , steroid free protocols, CNI sparing; Lifestyle and behavioural modification and traditional risk factor modifications <i>e.g.</i> , hyperlipidemia, hypertension as per general population |
| | <i>De novo</i> MAFLD | Contributors to new MAFLD post-LT include diabetogenic medications <i>e.g.</i> , CNI, steroids, obesity related to steroids, inactivity and return of appetite | As above |

MAFLD: Metabolic associated fatty Liver disease; LT: Liver transplant; T2DM: Type 2 diabetes mellitus; CNI: Calcineurin inhibitor.

dyslipidemia in the pre-transplant setting is vital, there is no evidence to suggest that the approach should be different in or specific to the MAFLD patient as opposed other etiologies, although the prevalence of these comorbidities may be higher^[38].

Renal dysfunction

Renal dysfunction in MAFLD is often multifactorial, with concomitant hypertension and T2DM as common risk factors for chronic kidney disease in addition to the spectrum of hepatorenal syndrome seen in end stage liver disease. Renal dysfunction is an important risk factor for post-LT cardiovascular disease and mortality, with even mild renal disease at the time of LT being associated with higher risk of all-cause and cardiovascular mortality, independent of measured confounders^[42]. This study also showed that each five-unit reduction in estimated glomerular filtration rate was associated with a 2% higher risk of all-cause mortality and 5% higher hazard ratio of cardiovascular mortality. Based on UNOS/OPTN data, NASH is the most rapidly growing indication for simultaneous liver kidney transplant (SLKT)^[43,44]. NASH and CC accounted for 22% of all SLKT in 2011, compared with 9% in 2002^[43]. While patient and liver graft outcomes are similar in NASH plus CC compared to other etiologies, the risk of kidney graft loss at five years has been reported as over 1.5 fold higher in NASH patients after controlling for recipient characteristics^[44]. In the pre-transplant setting, the goal is to prevent small deteriorations in renal function prior to transplant and to consider SLKT where indicated^[38].

Obesity

Obesity is a common pre-LT issue in patients with MAFLD and results in added complexities that include managing sarcopenic obesity, the assessment of obesity with fluid overload and considerations regarding bariatric surgery. As well as the correlation of obesity with traditional cardiovascular comorbidities, obesity presents potential peri-operative challenges that include technical surgical issues, wound infection, wound dehiscence and biliary complications^[45,46]. However, obesity does not appear to have a clear adverse effect on mortality^[47,48]. Between 2002 and 2011, 33% of LT recipients in the United States were classified as obese using body mass index (BMI) compared to 20% in the period between 1988 and 1996^[49,50]. The presence of ascites confounds BMI, and when corrected for 11%-20% of patients were reclassified to a lower BMI classification in one study of 1330 LT recipients. This study also showed that patient and graft survival were similar across all BMI categories^[47]. Interestingly, overweight and class 1 obese patient had better survival compared to those with normal BMI values, even after adjustment for both ascites and albumin levels. Setting strict BMI cut-offs for LT candidacy remains controversial, and there are limited data to guide this^[45]. Class I-III obesity alone does not currently contraindicate LT^[38,46]. Similarly, weight loss is a general goal in the Pre-LT setting, however, there are no specific targets or consensus regarding specific diet and exercise regimens. Traditional lifestyle modification used in obesity is generally safe in the pre-LT population^[51], however, excessive intrabdominal straining may lead to transient increases in portal pressure^[52]. Avoidance of very low-calorie diets (less than 1000 calories per day) is recommended. Specialist nutritionist consultation should be sought to optimise weight loss and balance this with protein and energy requirements for advanced chronic liver disease. Patients with BMI > 35 despite traditional lifestyle modification should be considered for bariatric surgery (BS)^[45].

There is ongoing debate regarding patient selection, timing and type of BS in the context of LT. In pre-transplant patients with cirrhosis, malnutrition and sarcopenia following BS can have an adverse effect on delisting and waitlist mortality. In a study of 78 patients with cirrhosis who underwent BS (predominantly Roux-en-Y bypass procedure) with matched controls, NASH was the underlying etiology for liver disease in almost half the patients. The median time from BS to LT was 7 years. Delisting or death on the waiting list was higher in the BS group (33.3% *vs* 10.1%, $P = 0.002$) and the transplantation rate was lower (48.9% *vs* 65.2%, $P = 0.03$). Despite similar BMIs between the two groups, the prevalence of malnutrition was higher in the BS group at the time of LT evaluation with 64% being malnourished *vs* 39% of the control cohort. These outcomes could be affected by selection bias in the study, where patients undergoing inappropriate BS may have decompensated as a result, and only those who went on to LT assessment were included and thus not factoring in a group where BS may have attenuated the need for LT^[53]. In a pilot of 6 cirrhotic patients, obesity-associated complications improved or resolved in all patients and nutritional parameters with similar to pre-operative levels^[54]. It remains unclear where post-transplant outcomes are improved by obesity intervention pre-transplant.

When choosing the type of BS, LT adds complexity. Gastric bands pose an infection risk in an immunosuppressed patient. Roux-en-Y may be more technically challenging post-transplant, and lack of endoscopic access to the biliary system and gastric remnant may be problematic in the setting of biliary anastomotic strictures or gastrointestinal bleeding^[55]. Therefore, sleeve gastrectomy (SG) is generally preferred in this patient population. SG can be done pre-LT, simultaneously during LT or post-LT. SG in cirrhotic patients has only been shown to be safe and efficacious in small retrospective reviews^[56] or series in Child-Turcotte-Pugh A patients^[54,57]. One small prospective study compared LT alone to simultaneous LT with SG (LT + SG) and demonstrated that patients who underwent LT + SG maintained a significantly higher percentage of total body weight loss after 3 years of follow-up. A lower prevalence of hypertension, insulin resistance, and hepatic steatosis was also demonstrated^[58]. This approach can be considered in patients who have significant risk of metabolic or cardiovascular problems post-LT. Bariatric surgery may also be performed post-transplant.

Nutrition and sarcopenia

Pre-LT nutritional status has a major influence on post-LT outcomes, including morbidity, mortality and hospital stay^[59]. Malnutrition is common in the pre-LT population and is driven by reduced dietary intake, malabsorption and altered energy metabolism^[60]. However, diagnosis can be challenging in the obese patient with decompensated cirrhosis, particularly in the presence of ascites^[61,62]. Specialist

nutritionist consultation should be undertaken in pre-LT patients, particularly as BMI and subjective global assessment are unreliable in this patient cohort^[63]. Tools such as handgrip strength are emerging as a useful bedside test in stratifying and prognosticating in sarcopenia^[64], as well as subcutaneous adipose tissue index in females^[65]. Sarcopenia is characterised by a progressive decline of skeletal muscle and strength and is independently associated with mortality in cirrhosis^[66]. In a study of 142 patients awaiting LT, 41% were sarcopenic, and this was associated with an over two-fold risk of waiting-list mortality compared to participants without sarcopenia^[63]. The coexistence of low muscle mass and increased fat mass is referred to as “sarcopenic obesity” and is estimated to affect up to 35% of patients on the LT waiting list^[67]. “Myosteatorsis” is defined as pathological fat accumulation in skeletal muscle, either intramyocellular or intermuscular, and has been reported in more than 50% of patients with cirrhosis evaluated for liver transplantation^[68]. Both sarcopenia and myosteatorsis have been shown to be independent risk factors for long-term mortality in cirrhosis^[68]. There are limited data available regarding interventions for sarcopenic obesity in cirrhosis, let alone distinguishing MAFLD patients from other etiologies of chronic liver disease. There is much work to be done to further define these conditions and to develop an evidence base to direct recommendations for nutritional, exercise and pharmacologic therapies in cirrhotic patients and those awaiting liver transplant^[67].

Malignancy

It is well established that obesity is associated with increased risk of a number of cancers, including endometrial cancer, esophageal adenocarcinoma, gastric cardia cancer and renal cell carcinoma^[69]. Metabolic syndrome has been associated with increased risk of colorectal adenoma and/or cancer^[70]. However, an independent association between MAFLD and colorectal malignancy remains contentious^[71], with a meta-analysis showing association with adenoma only, not colorectal cancer^[72]. All LT patients are evaluated for risk of malignancy and investigated or screened according to local guidelines. There is currently no evidence to support additional screening measures for extra-hepatic malignancy in pre-transplant patients with MAFLD.

POST-TRANSPLANT OUTCOMES

Post-LT survival of patients with MAFLD appears similar to non-MAFLD patients, supported by large registry studies from the United States^[24,73,74] and the ELTR^[27]. From these studies, 5-year survival was 73%-81% in NASH (+/- CC), compared with 75%-80% in non-NASH non-CC. The 10-year patient survival in NASH was 62% according to ELTR data from 2002-2016 compared with 63% in non-NASH^[27]. Survival was lower in patients transplanted for HCC with NASH (47%) compared to HCC without NASH (53%). UNOS data from 1997-2010, 10-year patient survival was 75% in NASH+CC compared with 73% in non-NASH non-CC^[73]. In the European cohort, cardiovascular mortality was the second most common cause of death (after infection) with no difference between NASH and non-NASH groups. Increasing age, MELD and extremes of BMI independently predicted death in patients transplanted for NASH without HCC^[27]. A limitation of the comparator non-MAFLD groups in large registry studies is that they mostly do not include data after the widespread use of DAAs for HCV, suggesting that more recent outcomes may be different. Small studies have reported increased 30-d and 1-year mortality in NASH LT-recipients, attributed mainly to infection^[30,75]. There is no evidence that patients who have undergone LT for MAFLD are at any greater risk of extra-hepatic malignancy post-transplant than other indications and therefore routine post-LT malignancy screening in line with local protocols is recommended.

Given the underlying genetic and environmental factors that drive MAFLD (Figure 1), it is unsurprising that MAFLD may recur post-transplant. It is estimated that 50% of MAFLD transplant recipients have recurrent MAFLD, 75% of which have NASH^[76,77], however less than 10% develop advanced fibrosis^[76,78]. The true incidence and risk factors for recurrent and *de novo* MAFLD post-transplant is difficult to elucidate due to significant heterogeneity in studies, as reported in a recent systematic review^[79]. The 1-, 3-, and ≥ 5 -year incidence rates were found to be 59%, 57%, and 82% for recurrent MAFLD and 53%, 57, and 48% for recurrent NASH, however there was low confidence in this result due to significant heterogeneity and high risk of bias in the included studies. Multivariate analysis demonstrated that post-LT body mass index and hyperlipidemia were the most consistent predictors of outcomes^[79].

De novo MAFLD after transplant

Many factors contribute to post-LT MAFLD, including post-transplant diabetes, obesity and medication, but the prevention of post-LT MAFLD is similar other indications for LT such as HCV or ALD. From an aforementioned systematic review, the mean 1-, 3-, and ≥ 5 -year incidence rates for *de novo* MAFLD were 67%, 40%, and 78% and 13%, 16%, and 17% for *de novo* NASH^[79].

Risk factors for both recurrent and *de novo* MAFLD include weight gain, diabetes, hyperlipidemia, hypertension and possibly female sex^[79-82]. High dose corticosteroids are associated with hepatic steatosis and metabolic syndrome post-LT^[83], as they are in the general population. Possible approaches to risk reduction include early steroid minimisation or steroid-free induction immunosuppression^[82,84]. Sirolimus based immunosuppression has been recently associated with *de novo* MAFLD post-LT^[85]. Calcineurin inhibitors such as tacrolimus and cyclosporin are also diabetogenic but their effect on *de novo* MAFLD has not been studied. Golabi *et al*^[30] noted in their study of SRT data from 1994 to 2016 that the incidence of post-LT diabetes is declining, likely because of the use of less diabetogenic immunosuppression.

Donor genetic polymorphisms associated with MAFLD (*PNPLA3*, *TM6SF2*) have been implicated in *de novo* steatosis post LT^[31,32], however, this is yet to have significant clinical management implications. Similarly, the influence of epigenetics and the microbiome in *de novo* MAFLD after LT requires further research to identify if and how these factors may differ from the pre-LT setting.

Hepatic steatosis and potential liver donors

The degree of steatosis in a potential donor liver graft is an important factor in organ selection and affects liver allograft function. Hepatic steatosis (HS) can be either macro- or microvesicular based on the size of the triglyceride droplets in the hepatocyte; with the former having a greater effect on graft quality. When macrovascular steatosis exceeds 60%, discarding the graft is recommended because of the high rate of primary graft non-function^[86,87]. Moderate to severe steatosis ($> 30\%$) is also a risk factor for graft loss and early allograft dysfunction and careful assessment of donor and recipient factors is required if such organs are to be considered for use^[88]. Mechanisms associated with poor graft function are not well defined, but include higher susceptibility to ischemia/reperfusion injury^[89], toxic cytokine formation, Kupffer cell activation, sinusoidal microcirculatory disorder and injury, which can be compounded in donation after circulatory death donor livers^[90]. The degree of HS can be assessed at different stages of organ procurement; with prognostic scores based on donor factors, imaging before harvest and biopsy after procurement. Surgeons mostly rely largely on visual inspection of the liver to assess the degree of HS, which places considerable pressure on the procurement team to make a rapid decision, with significant consequences if inaccurate. With the increasing number of donor organs affected by HS in line with the obesity epidemic, the risk of complications from steatotic donors must be weighed up against organ availability and waitlist mortality while awaiting a subsequent offer. In a recent study of 13362 waitlisted patients who accepted a steatotic donor offer ($> 30\%$ macrosteatosis on biopsy), only 53.1% were subsequently transplanted; 23.8% died and 19.4% were removed from the waitlist^[91]. In the 759 recipients who received a steatotic graft, peri-operative mortality was higher in the first month but the mortality risk was 62% lower beyond this^[91]. Candidates with MELD score of 6-21 who accepted a steatotic graft had a 7.88-fold higher mortality risk in the first month posttransplant, whereas MELD 35-40 candidates had a 68% lower mortality risk^[91]. In selected patients, the risk of a graft with HS may outweigh the risk of waitlist mortality. Ex-situ machine perfusion is a promising therapy that may recondition steatotic livers for transplantation and may play a key role in addressing this issue in the future^[88,92].

CONCLUSION

MAFLD is likely to become the leading global indication for liver transplantation within the next decade. This changing epidemiology brings the challenges of managing ageing, comorbid patients on the waiting list, through the peri-transplant period and in the long term. However, post-LT outcomes in MAFLD patients appear similar to non-MAFLD indications which implies that with good recipient selection, the outlook for MAFLD patients undergoing LT is optimistic. The rising prevalence of MAFLD has implication for both living and deceased donor livers, and balancing graft quality with organ demand will be an ongoing issue for transplant programs. As the

conceptualisation of MAFLD evolves, so will the ability to better predict disease behaviour and progression, to tailor treatment and to observe patterns of outcomes in liver transplantation across the patient spectrum and therefore address the multiple challenges posed by this disease.

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Liver injury in COVID-19: The hepatic aspect of the respiratory syndrome — what we know so far

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Abstract

The 2019 novel coronavirus disease (COVID-19) pandemic due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed a serious threat to global public health. Although primarily, the infection causes lung injury, liver enzyme abnormalities have also been reported to occur during the course of the disease. We conducted an extensive literature review using the PubMed database on articles covering a broad range of issues related to COVID-19 and hepatic injury. The present review summarizes available information on the spectrum of liver involvement, the possible mechanisms and risk factors of liver injury due to SARS-CoV-2 infection, and the prognostic significance of the presence of liver injury. Hopefully, this review will enable clinicians, especially the hepatologists, to understand and manage the liver derangements they may encounter in these patients better and provide guidance for further studies on the liver injury of COVID-19.

Key Words: COVID-19; Hepatitis; Infectious disease; Liver injury; SARS-CoV-2; Management

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Core Tip: Hepatic injury in coronavirus disease 2019 (COVID-19) has been widely observed. Although the pathogenic mechanisms still remain unclear, it is believed to be due to interplay of multiple factors. In this review, we have tried to discuss the pathophysiological mechanisms of hepatic injury in the context of COVID-19 and have proposed a management outline of such injury.

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INTRODUCTION

An outbreak of novel corona virus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) infection occurred in Hubei province of Wuhan, China in December, 2019 and since then it has assumed the form of a pandemic spanning 213 countries with more than 26.3 million confirmed cases worldwide and causing 869000 deaths as of September 3, 2020^[1]. The syndrome caused by the virus SARS-CoV-2 has been named coronavirus disease 2019 (COVID-19). The virus can cause an acute respiratory illness which resolves quickly but may also lead to massive alveolar damage with respiratory failure. Although the presenting complaints are chiefly respiratory, the gastrointestinal system and the liver, in particular have also been found to be affected^[2]. This is evident in a large series of 5700 cases from New York where the authors reported liver enzymes abnormalities in more than half of the cases^[3].

CLINICAL PROFILE OF PATIENTS OF COVID-19

SARS-CoV-2 primarily causes a respiratory syndrome heralded by fever, myalgia, sore throat, runny nose and dyspnea in severe cases. COVID-19 has been reported to have a slight male predilection of around 73% and a median age of presentation of 49 years^[4]. Amongst other atypical symptoms, diarrhea has also been reported to occur as a presenting complaint in the absence of respiratory symptoms. What is also concerning is that 32% of the patients required intensive care unit (ICU) admission, of which 15% expired. Another study reported by Wang *et al*^[5] that included 138 patients also reported similar clinical characteristics. It has also been reported that many of the patients belonging to the elderly age group who become severely ill have evidence of underlying illness such as cardiovascular disease, liver disease, kidney disease, or malignancies^[3].

A significant proportion of the patients with COVID-19 have also been found to have a number of digestive disorders like anorexia, nausea, vomiting, diarrhea and abdominal pain^[6]. The involvement of the gastrointestinal system, and the hepatobiliary system in particular, is significant in view of the fact that the case studies and data from The Fifth Medical Centre of PLS General Hospital, Beijing, China indicate that 2%-11% of patients with COVID-19 had liver comorbidities and 14%-53% cases reported abnormal levels of alanine aminotransferase and aspartate aminotransferase (AST) during disease progression^[2]. Also, it was observed that the synthetic function of the liver was affected, being evident in the fact that prothrombin time prolongation was more significant in patients with digestive symptoms^[6].

CORONAVIRUSES AND LIVER INJURY

Similar to the current pandemic, studies had showed that liver injury also occurred in patients with SARS-which broke out two decades ago—manifesting mainly as transaminitis along with increases in serum bilirubin and decreased serum albumin^[7].

It was found that, apart from causing alveolar injury, SARS also caused direct hepatocyte injury by using angiotensin converting enzyme 2 (ACE2) receptor for

cellular entry, which is abundantly expressed in the liver^[8]. Further confirmation of this came from reverse transcription polymerase chain reaction (RT-PCR) based evidence of SARS-associated coronavirus in the liver tissues despite electron microscopy failing to identify viral particles^[9].

Similarly, Middle East respiratory syndrome (MERS) caused by Middle East respiratory syndrome coronavirus (MERS-CoV) was characterised by fever, which also progressed to respiratory and multiorgan failure, in severe cases. These patients also had hyperbilirubinemia, hypoalbuminemia and transaminitis. In contrast to the SARS-CoV, MERS-CoV was found to have specific affinity for the Dipeptidyl Peptidase-4 (DPP-4) receptor which is abundantly expressed in the liver and thus was presumed to have gained entry into hepatocytes^[10]. In this case, too, viral particles could not be demonstrated in the liver tissue of patients with MERS^[11].

In a study involving 1099 patients with laboratory-confirmed COVID-19 from 552 hospitals in China, nausea or vomiting, or both, and diarrhoea were reported in 55 (5.6%) and 42 (3.8%) patients respectively^[12]. Interestingly, SARS-CoV-2 RNA was first detected in a stool specimen of the first reported COVID-19 case in the United States^[13].

It has been shown that SARS-CoV-2 shares 82% genomic sequence similarity with SARS-CoV and has also been found to have affinity for the ACE2 receptor^[6,14]. Therefore, it is not at all surprising that 14%-53% of patients with COVID-19 have abnormalities in transaminase levels^[2]. In the past too, liver impairment was reported in 60% of patients with SARS^[9,15].

Liver dysfunction in COVID-19 is manifested by abnormal transaminase levels and this has also been linked to the severity of the disease as well as the outcome. For example, in one study published by Huang *et al*^[4], elevation of AST was observed in eight (62%) of 13 patients in the ICU compared with seven (25%) of 28 patients who did not require care in the ICU. In another comparatively bigger cohort of 1099 patients from 552 hospitals in 31 provinces in China, patients with more severe disease had abnormal liver aminotransferase levels than did patients with less severe disease^[12]. In one more study, 8 patients who had a diagnosis of COVID-19 confirmed by computed tomography (CT) scan while in the asymptomatic phase had significantly lower incidence of AST abnormality compared to patients diagnosed after the onset of symptoms^[16]. All these figures indicate that liver injury manifested by transaminitis is probably more prevalent in severe cases than in mild cases of COVID-19.

RISK FACTORS FOR LIVER INJURY

There are not many studies addressing the risk factors associated with liver injury in COVID-19. It is quite natural that a thorough and comprehensive evaluation of possible risk factors that can cause or exacerbate liver injury has not been possible in such a short span of time.

An important observation in a study by Li *et al*^[17] was that among patients with abnormal liver function, patients with moderate and severe disease were more likely to have liver injury, accounting for 58.8% and 66.7% respectively. This indicates that hepatic injury is more common and is more severe during the period of critical care, where the patient is exposed to multiple insults in the form of medications, hemodynamic instability and cytokine storm. This is in addition to the fact that a significant number of patients in China have underlying hepatitis B infection.

The authors, in the same study, performed multivariate logistic regression analysis to study the association of several factors that included age, drinking history, baseline albumin, lactic acid, C-reactive protein (CRP), neutrophils, lymphocyte and myoglobin. It was found that CRP levels greater than 20 mg/L and a lymphocyte count less than $1.1 \times 10^9/L$ were independently related to alanine aminotransferase (ALT) elevation. This study, importantly, excluded the effects of drugs that might cause hepatic injury and suggested that cytokine storm may be the major mechanism behind lymphopenia and elevation in CRP, heralding liver injury.

In addition, herbal medicines, which are a major cause of liver injury in the developing nations^[18], complicate the problem further as they have been indiscriminately used in these cases in China. A dataset processed by Ou *et al*^[19] from between 2011 and 2014 in China identified Chinese herbal medicine as the primary cause of liver injury in 36% of the patients investigated in their study. Therefore, in such a background of widespread consumption of herbal products which is an established risk factor for liver injury, patients with COVID-19 seem to be at high risk for exacerbation of hepatic injury due to a combination of multiple factors.

PATHOGENESIS OF LIVER INJURY

As discussed earlier, it has been observed that SARS-CoV-2, by virtue of its affinity for ACE2 receptor gains access to hepatocytes and causes direct liver injury. However, what is puzzling is that the ACE2 expression of bile duct cells is reportedly much higher than that of liver cells, and is comparable to alveolar type 2 cells in the lungs^[20]. As bile duct epithelial cells are known to play important roles in liver regeneration and immune response^[21], it is believed that the liver injury in COVID-19 patients may be due to the damage to bile duct cells by the virus, and not the liver cells, and in such a scenario, it would be expected that COVID-19 patients should have evidence of cholestatic liver injury. However, the normal ALP levels and elevated AST/ALT levels do not favour the injury hypothesis, and our understanding with regards to liver injury related to SARS-CoV-2 continues to evolve. Besides, histopathological analysis of liver tissue from a deceased patient also did not show the presence of viral inclusion bodies in the liver^[22].

It is in this context that other factors apart from direct viral cytopathic injury need to be considered. In addition to direct hepatotoxicity, it is clear that immune dysregulation does occur in COVID-19 in varying forms. Systemic Inflammatory Response Syndrome (SIRS) and accompanying release of interleukins and other mediators of inflammation lead to a form of cytokine storm that can cause and exacerbate hepatocellular injury^[23]. Furthermore, a metanalytic study investigating the relationship between liver damage and COVID-19 suggests that SARS-CoV-2 may produce a relevant hepatic damage probably through the immune interactions requiring the action of intrahepatic cytotoxic T cells and Kupffer cells^[24].

Another factor that needs to be kept in mind while dealing with hepatic dysfunction in COVID-19 is the possibility of pneumonitis associated hypoxia and fall in mean arterial pressure that can cause ischemic hepatitis. Besides, the fact that patients in ICU had higher levels of transaminases might also be due to the higher degrees of hypoxia requiring mechanical ventilation. Levels of AST and ALT in thousands are an established feature of ischemic hepatitis, and hypoxia may in fact exacerbate the liver injury along with other causes of liver dysfunction.

Drug induced liver injury during COVID-19 related illness should be considered in the differential diagnosis considering many of the unapproved drugs are being tested either empirically or are undergoing clinical trials. While there is no specific treatment for COVID-19 till date, there has been a frenetic attempt to use various existing combinations of antivirals, immunomodulators, antibiotics and steroids in addition to 'hepatoprotective' drugs. The indiscriminate use of such cocktails might, in fact, be perpetuating hepatic dysfunction observed in these cases. Biopsies performed post mortem in SARS-CoV-2 infection revealed microvascular steatosis and mild lobular and portal activity, indicating that the injury could have been caused by drug-induced liver injury in addition to the SARS-CoV-2 infection^[25]. Use of steroids, antibiotics and antivirals have all been associated with liver injury. It has also been reported that the liver injury observed in COVID-19 patients might be caused by lopinavir/ritonavir, which is being used as an antiviral for the treatment of SARS -CoV-2 infection^[25]. The drug Remdesivir, widely used in COVID-19, has been reported to cause hepatocellular injury and derangement of liver function^[26,27]. Use of biologics like Tocilizumab has been associated with Hepatitis B reactivation^[28]. Azithromycin which is commonly prescribed in COVID-19 is a known cause of idiosyncratic liver injury while hydroxychloroquine may also rarely cause idiosyncratic hepatotoxicity^[29,30]. Therefore, it is amply clear that the sheer number of medications being tried in COVID-19 is highly likely to cause liver injury ranging from asymptomatic transaminitis to greater degrees of hepatotoxicity. The APASL expert panel consensus recommendations advise careful investigation of drug induced liver injury in COVID-19 and specifically mention against using certain drugs like Remdesivir in patients with decompensated chronic liver disease and ALT elevations more than 5 times the upper limit of normal^[27]. Close monitoring of liver function tests has been advised. In addition, it has also been suggested that use of herbal products and nutraceuticals in COVID-19 may interfere with the body's natural immune mechanisms and hence the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) has advised against using these substances derived from plants like poplar, birch, willow, goldenrod, curcuma *etc.*^[31,32]. A summary of the spectrum of drugs used in COVID-19 likely to induce liver injury is shown in **Figure 1**.

To add to this growing concern over liver injury in COVID-19, the presence of chronic liver disease adds to the burden of the problem worldwide. The prevalence of liver disease varies globally and so do the etiologies. Viral hepatitis, alcohol related liver disease and non-alcoholic fatty liver disease are prevalent in epidemic

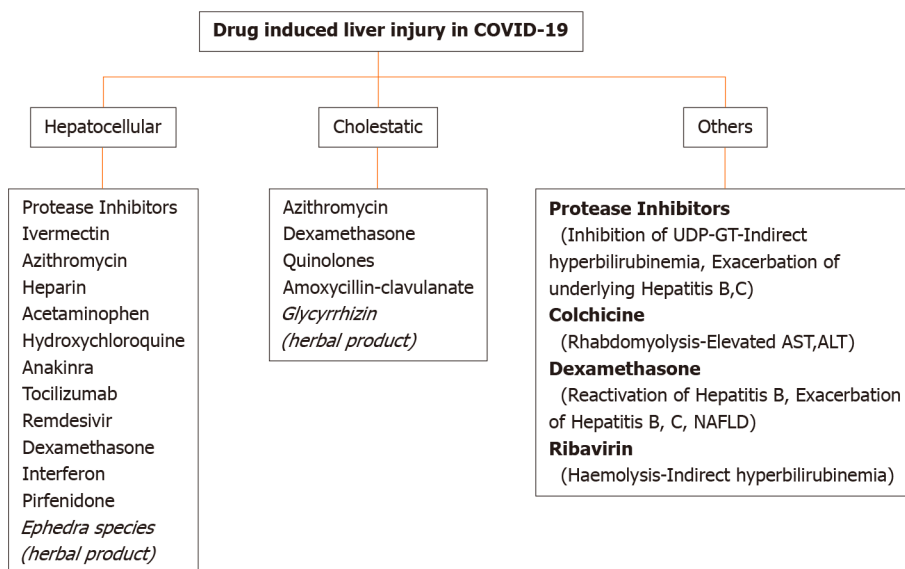


Figure 1 Spectrum of medications used in coronavirus disease 2019 likely to cause liver injury. COVID-19: Coronavirus disease 2019; UDP-GT: UDP-glucuronosyltransferase; NAFLD: Nonalcoholic fatty liver disease.

proportions worldwide and the impact of COVID-19 in patients with these pre-existing diseases is largely unknown. A recent case series from China reported that decompensated cirrhosis may be a risk factor for a poor outcome in patients with COVID-19^[33]. The use of complementary, herbal and indigenous drugs that can exacerbate liver injury, especially in countries like China where the infection originated and in India, where the incidence of new cases has been steadily rising - poses great challenges.

The presence of co-morbidities makes the pathogenic processes complex and at the moment, there are very few studies that have looked at this problem. A systematic review and meta-analysis by Yang *et al*^[34] has shown that the most prevalent comorbidities were hypertension and diabetes, followed by cardiovascular diseases and respiratory system disease. It is well known that both hypertension and diabetes are commonly treated with ACE inhibitors. The expression of ACE2 is substantially increased in patients with type 1 or type 2 diabetes, who are treated with ACE inhibitors and angiotensin II type-I receptor blockers (ARBs). This results in an upregulation of ACE2. Consequently, it has been hypothesised that the increased expression of ACE2 in different organs like the lung and the liver might facilitate infection with SARS-CoV-2. Thus, it has been assumed that diabetes and hypertension treatment with ACE2-stimulating drugs increases the risk of developing severe and fatal COVID-19^[26]. Further, these studies suggest that patients with co-morbidities having SARS-CoV-2 infection might have multiple pathogenic mechanisms of host injury and inflammatory response proceeding simultaneously. Given the high burden of co-morbidities, especially among the elderly population, this area needs to be meticulously investigated. The potential mechanisms of liver injury in COVID 19 are summarized in [Figure 2](#).

PATTERN OF LIVER INJURY IN SARS-COV-2 INFECTION

Liver injury in SARS-CoV-2 infection has a variable incidence of 14%-53% as indicated by elevated AST and ALT levels. There is also an accompanying mild increase of serum bilirubin levels^[35]. However, from the existing data, the pattern of bilirubin rise (direct *vs* indirect) and its relationship to the disease process is not very clear. Also, no correlation between the rise in transaminases and bilirubin levels have been found. Hypoalbuminemia has also been found to occur with the serum albumin levels around 2.63-3.09 mg/dL according to one study^[4]. A recent study including 417 patients with COVID-19, 318 (76.3%) had abnormal liver test results and 90 (21.5%) had liver injury during the hospitalization. Ninety-one (21.8%) developed severe disease and 326 (78.2%) had mild disease during hospitalization ([Figure 2](#))^[36]. A recent study has noted that AST-dominant aminotransferase elevation is common in COVID-19, mirrors

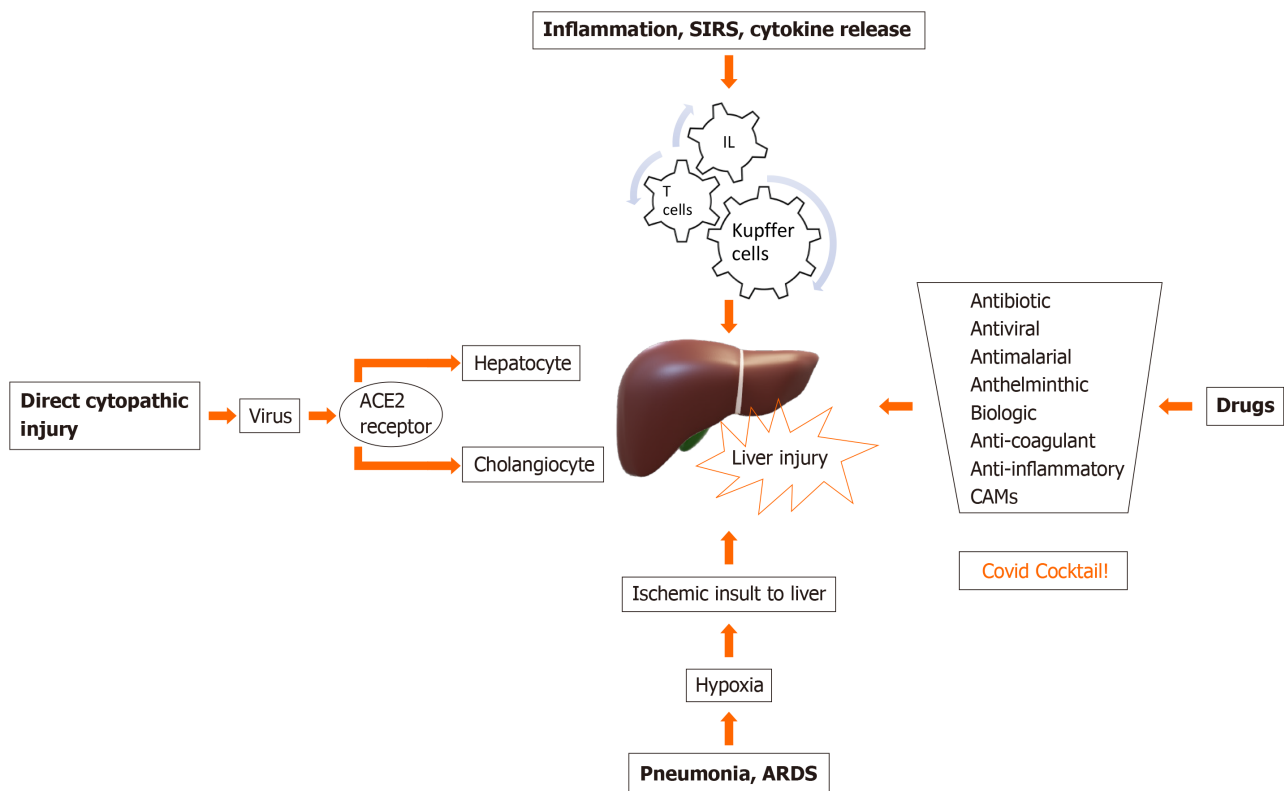


Figure 2 Potential mechanisms of liver injury in coronavirus disease 2019. ACE2: Angiotensin converting enzyme 2; ARDS: Acute respiratory distress syndrome; CAMs: Complementary and alternative medicines; IL: Interleukin.

disease severity, and appears to reflect true hepatic injury^[37].

Interestingly, COVID-19 infection presenting as acute non-icteric hepatitis which preceded the development of fever and respiratory symptoms has recently been reported in a patient^[38]. This particular case report assumes importance in view of the fact that the patient did not have typical features of COVID 19 at presentation and was worked up as a case of acute viral hepatitis. All possible causes of acute hepatitis were ruled out. After 2 d, she developed respiratory signs and symptoms and was found positive for SARS CoV-2. She responded to treatment with supportive measures and hydroxychloroquine^[38]. Furthermore, a case of COVID-19 hepatitis in a living donor liver allograft recipient whose donor subsequently tested positive for COVID-19 has been reported with unique histopathological findings^[39].

The proportion of patients developing liver injury in severe COVID-19 has been found to be significantly higher than that in patients with milder disease. In a metanalytic study by Mantovani *et al*^[24], patients with severe COVID-19 disease tended to have higher levels of liver enzymes, as well as a greater activation of coagulative and fibrinolytic pathways. While the elevation in AST and ALT have been around three to four times and has varied across studies, one study reported elevations in AST and ALT to be in the range of thousands. In another study, although serum gamma glutamyl transpeptidase (GGT) was found to be increased in severe cases, serum alkaline phosphatase (ALP) level was in the normal range in both mild and severe cases^[20]. **Table 1** summarizes the pattern of liver dysfunction in COVID 19 patients reported in various studies^[35,40-70].

SARS-COV-2 INFECTION IN THE SETTING OF PRE-EXISTING LIVER DISEASE

Patients with pre-existing chronic liver disease have a wide spectrum of immune dysfunction starting from cytopenia to cytokine storm. The proportion of COVID-19 patients with pre-existing liver conditions ranged from 2% to 11% in one study^[2]. In patients with chronic hepatitis B and C infection, it is not known as to what kind of effect co-infection with SARS-CoV-2 might have. Such patients who remain as inactive

Table 1 Summary of the pattern of liver injury reported in coronavirus disease 2019 in various studies

| Ref. | Number of patients | Pattern of liver injury | Pre-existing liver diseases | Comments | Place of study |
|--|--------------------|---|---|--|--|
| Richardson <i>et al</i> ^[3] | 5700 | Elevated AST: 58.4%; elevated ALT: 39% | Cirrhosis: 0.4%; chronic hepatitis B: 0.1%; chronic hepatitis C: 0.1% | | Northwell Health System, New York, United States |
| Huang <i>et al</i> ^[4] | 41 | 15(31%) | 1 (2%) | Elevated AST observed in 62% of patients in ICU compared with only 25% of patients not in ICU | Wuhan, China |
| Wang <i>et al</i> ^[5] | 138 | Mild elevation of AST and ALT | 4 (2.9%) | - | Wuhan, China |
| Guan <i>et al</i> ^[12] | 1099 | Elevated AST: 22.2%; elevated ALT: 21.3%; elevated total bilirubin: 10.5% | 23 (2.3%) | AST elevated in 18.2% of non-severe disease but in 39.4% of severe disease; ALT elevated in 19.8% with non-severe disease and 28.1% of severe disease | 552 hospitals in 30 provinces, autonomous regions, and municipalities in mainland China |
| Shi <i>et al</i> ^[16] | 81 | Transaminitis in 43 (53.1%) | 7 (8.6%) | Patients with subclinical infection had lower AST | Wuhan, China |
| Fan <i>et al</i> ^[25] | 148 | Abnormal LFT in 50.7%; elevated ALT in 18.2%; elevated AST in 21.6% | | Higher proportion (56.1%) with liver injury received lopinavir/ ritonavir than those without liver injury (25%) | Shanghai Public health Clinical Centre, China |
| Chen <i>et al</i> ^[35] | 99 | 43(43%); elevated AST: 35%; elevated ALT: 28%; elevated total bilirubin: 98% | | One patient had severe liver injury with ALT 7590U/L | Wuhan, China |
| Cai <i>et al</i> ^[36] | 417 | During hospitalisation, rise in liver enzymes > 3 times of upper limit seen; elevated ALT: 49 (23.4%); elevated AST: 31 (14.8%); elevated total bilirubin: 24 (11.5%); elevated GGT: 51 (24.4%) | | 318 (76.3%) had abnormal liver biochemistries and 90 (21.5%) had liver injury during hospitalization; 91 (21.8%) developed severe disease and 326 (78.2%) had mild disease during hospitalization; use of lopinavir/ritonavir increased the odds of liver injury by 7-fold | Shenzhen, China |
| Tabata <i>et al</i> ^[40] | 104 | Elevated AST: 17.3%; elevated ALT: 16.3% | - | - | Diamond Princess Cruise, Japan |
| Huang <i>et al</i> ^[41] | 36 | Elevated ALT: 13.33%; elevated AST: 58.06%; elevated Total bilirubin: 12.90% | | All fatal cases only | Wuhan, China |
| Zhang <i>et al</i> ^[42] | 82 | Liver dysfunction in 64 (78%) | 2 (2.4%) | All fatal cases only | Wuhan, China |
| Yang <i>et al</i> ^[43] | 52 | 15 (29%) | | No difference in incidence of liver injury between survivors and non-survivors | Wuhan, China |
| Cao <i>et al</i> ^[44] | 128 | | | Transaminitis present only in severe disease | Xiangyang, China |
| Xu <i>et al</i> ^[45] | 62 | Elevated AST in only 16.1% | 7 (11.0%) | No patient had elevated ALT while a sixth had elevated AST | Zhejiang Province, China |
| Cai <i>et al</i> ^[46] | 298 | 44 (14.8%) | 8 (2.7%) | Transaminitis 4 times commoner in severe disease (36.2%) compared to mild disease (9.6%) | Shenzhen, China |
| Kujawski <i>et al</i> ^[47] | 12 | Elevated AST: 58.3%; elevated ALT: 58.3% | 8.3% had HBV and 8.3% had fatty liver disease | | Center of Disease Control California, Illinois, Arizona, Massachusetts, Washington, Wisconsin, |

| | | | | | |
|--|-----|--|-----------------------------|--|---|
| | | | | | United States |
| Arentz <i>et al</i> ^[48] | 21 | Median AST: 273 (range 14-4432); median ALT: 108 (range 11-1414) | 4.8% had cirrhosis of liver | | Kirkland, Washington, United States |
| Jin <i>et al</i> ^[49] | 651 | Liver injury seen in 13 out of 74 with GI symptoms vs 51 out of 577 without GI symptoms | | Rate of increased AST, but not ALT, was significantly higher in patients with GI symptoms than in those without GI symptoms | Zhejiang Province, China |
| Qi <i>et al</i> ^[50] | 267 | Elevated AST: 7.2%; elevated ALT: 7.5%; elevated bilirubin: 2.2% | | Elevated AST seen in 9 out of 217 patients with non-severe disease and 10 out of 50 patients with severe disease. Elevated ALT seen in 10 out of 217 patients with non-severe disease and 10 out of 50 patients with severe disease. Elevated bilirubin in 3 out of 217 patients with non-severe disease and 3 out of 50 patients with severe disease | Chongqing, China |
| Omrani-Nava <i>et al</i> ^[51] | 93 | Elevated AST: 29.2%; elevated ALT: 30.3%; elevated ALP: 17%; elevated total bilirubin: 10.2%; elevated direct bilirubin: 45.8% | | Risk of being transferred to the intensive care unit strongly associated with the elevated levels of AST and direct bilirubin | Sari, Amol, Mazandaran Province, Iran |
| Mao <i>et al</i> ^[52] | 214 | Median AST 26 (8-8191); median ALT 26 (5-1933) | | Liver enzymes were significantly higher in severe cases compared to non-severe cases | Wuhan, China |
| Xu <i>et al</i> ^[53] | 45 | Elevated AST/ALT: 37.8%; median Bilirubin: 0.91 (IQR 0.61-1.3) | | | |
| Tian <i>et al</i> ^[54] | 24 | Elevated AST: 8.33 %; elevated ALT: 4.17 % | 4.17 % had cirrhosis | | Shandong, China |
| Chen <i>et al</i> ^[55] | 291 | Elevated AST: 15.1%; elevated ALT: 10.3%; elevated bilirubin: 9.3% | 5.2% chronic liver disease | Elevated AST in 5 out of 29 cases in mild illness, 23 out of 212 cases in moderate illness and 16 out of 50 cases in critically ill. Elevated ALT in 4 out of 29 in mild illness, 16 out of 212 cases in moderately ill and 10 out of 50 cases in critically ill. Elevated bilirubin in 4 out of 29 cases in mild illness, 17 out of 212 in moderately ill and 6 out of 50 in critically ill | Hunan Province, China |
| Wang <i>et al</i> ^[56] | 18 | Elevated AST or ALT in 25% | | | Zhengzhou, Henan Province, China |
| Yan <i>et al</i> ^[57] | 168 | Elevated AST: 17.3%; elevated ALT: 8.0% | | Elevated AST seen in 7 out of 75 patients with non-severe disease and 11 out of 29 patients with severe disease; Elevated ALT seen in 5 out of 81 patients with severe disease and 4 out of 31 patients with severe disease | Hainan, China |
| Lin <i>et al</i> ^[58] | 95 | Elevated AST: 4.2%; elevated ALT: 5.3% | | | Zhuhai, Guangdong Province, China |
| Zhao <i>et al</i> ^[59] | 77 | Elevated AST: 26.0%; elevated ALT: 33.8% | | Elevated AST seen in 11 out of 57 non severe patients and 9 out of 20 severe patients; Elevated ALT seen in 17 out of 57 patients with non-severe disease and 9 out of 20 patients with severe disease | Beijing, China |
| Chen <i>et al</i> ^[60] | 274 | Elevated AST: 30.7%; elevated ALT: 21.9%; median bilirubin: 0.6 (IQR 0.4-0.8) | 4 % were HbsAg positive | | Wuhan, China |
| Rubin <i>et al</i> ^[61] | 54 | Elevated AST: 42.59%; elevated ALT: 40.7% | 1.8 % were HBV infected | AST: mean/SD-73.4 ± 61.8 (females); 45.1 ± 19.5 (males) ALT: mean/SD- 69.6 ± 65.2 (females); 43.9 ± 25.8 (males) | Stanford University School of Medicine, California |
| Cholankeril <i>et al</i> ^[62] | 116 | Deranged LFT in 26 out of 65 cases (40%). Higher levels of AST compared to ALT. Median bilirubin- 0.4 (IQR 0.3-0.7) | 2.6% chronic liver disease | 22 of the 26 patients with liver enzyme elevations had normal baseline liver enzymes | Stanford University Hospitals California, United States |
| Yao <i>et al</i> ^[63] | 40 | Elevated AST: 40%; elevated ALT: 52.5% Elevated Bilirubin: 25% | | Out of 22 critical cases, 17 had hepatic dysfunction. Out of 18 noncritical cases, 5 had hepatic dysfunction | Xi'an, Shaanxi Province, China |

| | | | | | |
|-----------------------------------|------|---|---------------------------------|---|----------------------------------|
| Zhao <i>et al</i> ^[64] | 75 | Elevated AST: 18.7%; elevated ALT: 20%; elevated Bilirubin: 16% | 5.3 % had chronic liver disease | | Hefei, Anhui Province, China |
| Ai <i>et al</i> ^[65] | 102 | Elevated AST: 25.5%; elevated ALT: 19.6% | | | Xiangyang, China |
| Ma <i>et al</i> ^[66] | 81 | Deranged AST/ALT: 38.2% | | | Wuhan, China |
| Xu <i>et al</i> ^[67] | 355 | Elevated AST: 28.7%; elevated ALT: 25.6%; elevated Total bilirubin: 18.6% | | | Wuhan, China |
| Shi <i>et al</i> ^[68] | 416 | Median AST: 30 (IQR 22-43); median ALT: 28 (IQR 18-46) | 1% had HBV infection | | Wuhan, China |
| Luo <i>et al</i> ^[69] | 1141 | Among 183 patients, median AST: 65.8 ± 12.7, median ALT: 66.4 ± 13.2 | | | Wuhan, China |
| Qi <i>et al</i> ^[70] | 21 | Elevated AST: 38.1%; elevated ALT: 23.8%; elevated GGT: 23.8% | All patients | Most common etiology of chronic liver disease was chronic hepatitis B infection | 16 designated hospitals in China |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase; IQR: Interquartile range; HBV: Hepatitis B virus; LEF: Liver function tests; GI: Gastrointestinal; ICU: Intensive care unit; SD: Standard deviation.

carriers or in the immunotolerant phase might have reactivation of the virus and hepatic injury. Mantovani *et al*^[24], in their metanalytic study, have suggested that patients with pre-existing chronic liver disease may be more susceptible to liver damage from SARS-CoV-2. Implications of underlying non-alcoholic fatty liver disease (NAFLD) has been recently evaluated. The authors have noted that patients with NAFLD had higher risk of disease progression [6.6% (5/126) *vs* 44.7% (34/76), $P < 0.0001$], higher likelihood of abnormal liver function from admission to discharge [70% (53/76) *vs* 11.1% (14/126), $P < 0.0001$] and longer viral shedding time (17.5 ± 5.2 d *vs* 12.1 ± 4.4 d, $P < 0.0001$) when compared with non-NAFLD subjects^[71]. Patients with autoimmune hepatitis and primary biliary cholangitis on steroids and other immunosuppressive therapy might be at higher risk of developing severe disease and may pose dilemma in treatment, considering the fact that use of medications like antivirals and antibiotics might worsen liver injury. This issue also requires further investigation. An interesting case series of three patients of COVID-19 with chronic liver disease showed that while two of the patients with Child-Pugh C disease died, the patient with Child-Pugh class B did not^[33]. In addition, the patient with the highest Model for End-stage Liver Disease (MELD) score survived compared to the ones with lower MELD scores who did not, possibly indicating that clinical decompensating events may be more important in predicting outcome of patients with COVID-19 and pre-existing cirrhosis^[33].

In a multicentric study in China evaluating the clinical course and risk factors for mortality of COVID-19 patients with pre-existing cirrhosis, most patients were found to have compensated cirrhosis while chronic HBV infection was found to be the most common aetiology^[70]. The study did not report any significant differences between

survivors and non-survivors in terms of age, sex, comorbidities, aetiology of cirrhosis, Child-Pugh class, MELD score, interval between onset and admission, or onset symptoms of COVID-19^[70]. Further, COVID-19 patients who died had lower total lymphocyte and platelet counts, and higher direct bilirubin levels than patients who survived, while the frequency of acute respiratory distress syndrome (ARDS) and gastrointestinal (GI) bleeding were higher in non-survivors compared to survivors^[70]. Importantly, the cause of death in most patients was respiratory failure rather than acute on chronic liver failure^[70]. Further studies need to be carried out across different population groups to determine the exact impact of SARS-CoV-2 co-infection in patients with chronic liver disease.

SARS-COV-2 INFECTION IN THE SETTING OF LIVER TRANSPLANTATION

Taking a cue from the previous SARS outbreak of 2002, it has been hypothesised that liver transplantation might involve a risk of transmission of viral infection from donor to recipient. Hence, donor screening and testing might prove to be extremely crucial^[72]. However, at this juncture, there is a lack of evidence to justify this hypothesis. A case series of patients with COVID-19 had several patients with various comorbidities, but none of them had been a transplant recipient^[12]. A recent study from Italy reported experience from a single transplant centre, and noted 3 deaths out of their 111 Long-term liver transplant survivors (transplanted more than 10 years ago) compared to none of the three infected with SARS-CoV-2 transplanted within the last 2 years. All three who died were male, older than 65 years, receiving antihypertensive drugs, overweight (body mass index > 28 kg/m²), with hyperlipidaemia, and diabetes (median Hemoglobin A1c of 6.9%)^[73]. In the present circumstances, in the event of a liver transplantation, the hepatologists have to follow the guidance issued by the Transplantation Society^[74], as well as local health department guidelines for isolating, quarantining, testing, and monitoring returned travellers from endemic areas. Recent AASLD guidance reports that there is a significant false negative rate and transplant programs should consider symptoms of COVID-19 to be strongly suggestive of infection despite negative testing. Transplantation in SARS-CoV-2-positive recipients is currently not recommended.

PREVENTION AND TREATMENT

Liver injury in COVID-19 infection can be fleeting and mild and enzyme levels can normalise spontaneously. As of now, there is no specific therapy for liver injury in COVID-19 infection. In addition to managing the respiratory syndrome in COVID-19, monitoring of the liver function tests (LFT) should be done and all factors known to cause or exacerbate liver injury should be taken into consideration while treating the patient. A proper history especially with regard to recent or long-term intake of herbal preparations and hepatotoxic drugs must be taken. Drugs known to cause liver injury must be used with caution and LFT should be repeated at regular intervals. All the studies concerning COVID 19 and liver injury are from China and one thing common in these studies is the use of a cocktail of drugs that include steroids, antivirals, antibiotics and compounds like ammonium glycyrrhizinate which should be viewed with caution as some of these are known to cause hepatic injury. Especially in the setting of hypoxia and cytokine storm, use of these drugs can exacerbate any existing hepatic insult. Special caution is warranted for patients with pre-existing liver disease. At the moment, there is lack of robust data to support the use of specific hepatoprotective agents. However, in cases of severe liver injury, liver protective agents have been used in COVID-19. **Figure 3** succinctly delineates the diagnostic approach to COVID-19 patients with abnormal liver biochemistries, investigations to be performed and monitoring of such patients.

In the face of this pandemic, management of patients with chronic liver disease has also posed problems. Recently, the AASLD and EASL have come up with recommendations for patients with compensated as well as decompensated liver disease. In case of compensated liver disease, AASLD recommends limiting outpatient visits and to consider seeing in person only new adult and paediatric patients with urgent issues and clinically significant liver disease (*e.g.*, jaundice, elevated ALT or AST > 500 U/L, recent onset of hepatic decompensation), to continue treatment for hepatitis B and hepatitis C if already on treatment, to continue monitoring in those on or off therapy for hepatocellular carcinoma (HCC), to continue surveillance in those at

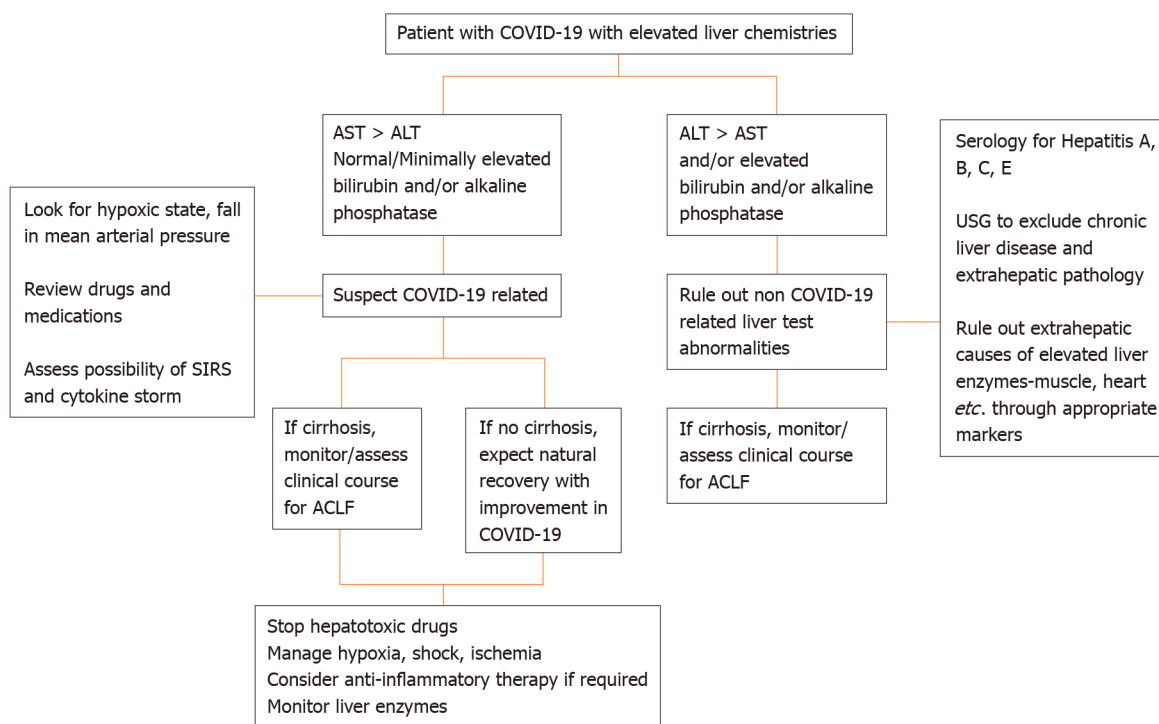


Figure 3 Approach to a coronavirus disease 2019 patient with liver dysfunction. COVID-19: Coronavirus disease 2019; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ACLF: Acute-on-chronic liver failure; USG: Ultrasonography.

risk for HCC and to proceed with HCC treatments when able rather than delaying them due to the pandemic^[75]. In those with decompensated liver disease, AASLD recommends evaluating only patients with HCC or those patients with severe disease and high MELD scores who are likely to benefit from immediate liver transplant listing^[75]. In autoimmune liver disease too, EASL recommends against reducing immunosuppressive therapy unless indicated in special circumstances^[76].

LIVER DYSFUNCTION AND PROGNOSIS

The prognostic variables of hepatic dysfunction in COVID-19 are still being worked out. As previously mentioned, patients with more severe disease have abnormal levels of transaminase levels, suggesting that elevated transaminase levels may be viewed as a prognostic factor and these patients need to be treated with caution. However, the causality association of this factor might be subject to bias. In a retrospective study from Wuhan by Zhou *et al*^[77], several factors were identified that might be associated with higher mortality in adults who were hospitalised with COVID-19. Older age, d-dimer levels greater than 1 µg/mL, and higher Sequential Organ Failure Assessment (SOFA) score on admission were associated with higher odds of in-hospital death. Also, levels of interleukin 6 (IL-6), high-sensitivity cardiac troponin I, and lactate dehydrogenase (LDH) were elevated and lymphopenia was more common in severe COVID-19 illness. Preliminary data suggests the reported death rate varies depending on the study and country. However, the mortality rate estimates are based on the number of deaths relative to the number of confirmed cases of infection, which is not representative of the actual death rate. Extrapulmonary involvement like hepatic and renal injury could indicate more severe inflammatory responses and might have a bearing on the mortality rates. Furthermore, it is not clear at this point of time whether the algorithms and scores that are commonly used to assess prognosis in patients with acute hepatic failure are applicable to liver injury in COVID-19, considering the multiple factors involved in the pathogenesis. This, therefore, warrants extreme degree of caution and an individually tailored approach while dealing with such patients.

CONCLUSION

SARS-CoV-2 infection has taken the world by storm. The pandemic is yet to reach a plateau, with the incidence rising and newer populations getting infected. However, physicians, researchers and scientists have left no stone unturned to understand the pathogenesis of this multi-system afflicting disease, find out effective therapies and contain the pandemic. While the disease primarily affects the respiratory system, liver injury does pose problems in the management of COVID-19 patients. Both direct virus-mediated cytopathic effects and indirect immune mediated, drug induced or hypoxic states are probably responsible for causing and perpetuating the liver injury. However, a word of caution: Transaminitis in patients with COVID-19 should not be overly investigated. Only in those patients where there is suspicion of cholestatic pattern of injury, investigations like ultrasonography and magnetic resonance cholangiopancreatography may be performed. Besides, although it may sound slightly premature, in view of the recent case report, clinicians should also, in this era of COVID-19 infection, keep in mind that acute non-icteric hepatitis may be the virus's initial presentation prior to the development of respiratory symptoms^[38]. As new evidence trickles in and more facts come to light, we will be in a better position to understand and tackle liver injury in COVID-19. Intensive monitoring and individually tailored approach are the need of the hour to treat patients with severe liver injury or patients with pre-existing liver diseases.

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Basic Study

Cyclin-dependent kinase inhibitors p21 and p27 function as critical regulators of liver regeneration following 90% hepatectomy in the rat

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Abstract

BACKGROUND

Liver reduction is the main curative treatment for primary liver cancer, but its use remains limited as liver regeneration requires a minimum of 30% functional parenchyma.

AIM

To study the dynamics of the liver regeneration process and consequent behavior of cell cycle regulators in rats after extended hepatectomy (90%) and postoperative glucose infusions.

METHODS

Post-hepatectomy liver failure was triggered in 84 Wistar rats by reducing their liver mass by 90%. The animals received a post-operative glucose infusion and were randomly assigned to two groups: One to investigate the survival rate and the other for biochemical analyses. Animals that underwent laparotomy or 70% hepatectomy were used as controls. Blood and liver samples were collected on postoperative days 1 to 7. Liver morphology, function, and regeneration were studied with histology, immunohistochemistry, and western blotting.

RESULTS

Postoperative mortality after major resection reached 20% and 55% in the first 24 h and 48 h, respectively, with an overall total of 70% 7 d after surgery. No

additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared in accordance with them.

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apparent signs of apoptotic cell death were detected in the extended hepatectomy rat livers, but hepatocytes displaying a clear cytoplasm and an accumulation of hyaline material testified to changes affecting their functional activities. Liver regeneration started properly, as early events initiating cell proliferation occurred within the first 3 h, and the G1 to S transition was detected in less than 12 h. However, a rise in p27 (Kip1) followed by p21 (Waf1/Cip1) cell cycle inhibitor levels led to a delayed S phase progression and mitosis. Overall, liver regeneration in rats with a 90% hepatectomy was delayed by 24 h and associated with a delayed onset and lower peak magnitude of hepatocellular deoxyribonucleic acid synthesis.

CONCLUSION

This work highlights the critical importance of the cyclin/cyclin-dependent kinase inhibitors of the Cip/Kip family in regulating the liver regeneration timeline following extended hepatectomy.

Key Words: Major hepatectomy; Liver failure; Liver regeneration; Post-hepatectomy liver failure; p21; p27

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Core Tip: There is a current pandemic of obesity and diabetes and the chronic liver damages they cause, and the outcomes of patients undergoing liver mass reduction for malignant diseases are poor. To design efficient strategies that limit the risk of post-hepatectomy liver failure, we used a rat model to clarify the causes of death after enlarged liver resection. Compared with standard 2/3 hepatectomy, enlarged resection resulted in a loss of hepatocyte functional activities and impaired regenerative capacities, which were associated with an overexpression of p21 and p27 inhibitors. The use of extracorporeal support device with p21 and p27 should be considered for the management of severe liver failure following extended hepatectomy.

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INTRODUCTION

Because the liver is able to regenerate its mass, surgical resection is used routinely as a curative treatment to manage primary liver cancers and hepatic metastases (*i.e.* those of colorectal and breast cancers^[1]). This is a relatively safe procedure for patients and the only efficient treatment for these tumors. The principal challenge faced by surgeons is estimating the volume of liver that can be resected without increasing the risk that the patients will develop post-hepatectomy liver failure (PHLF), a disorder related to the small-for-size syndrome (SFSS) that occurs when too little liver is transplanted. Therefore, to ensure full patient safety and avoid post-resection liver failure, a minimum of 30% functional hepatic parenchyma is required^[2].

Hepatocellular carcinoma (HCC), which is the most common primary liver cancer, occurs predominantly in patients with an underlying chronic liver disease. The setting of chronic inflammation involves steatosis, fibrosis, or cirrhosis^[3]. Fewer than 30% of HCC patients are eligible for surgery, mainly because of the lesions resulting from chronic inflammation. This situation is becoming even more problematic in the context of the current epidemic of diabetes and obesity that affects 25% to 30% of the global population and is associated with the development of metabolic syndrome and its continuum of chronic liver disorders such as steatosis, fibrosis, non-alcoholic steatohepatitis (NASH) and cirrhosis. When liver reduction is considered as a treatment for liver metastases, the preoperative chemotherapies used to reduce the primary colon or breast tumors and metabolized by the liver can aggravate the clinical

picture. All patients should be deemed at risk of developing post-resection liver failure. To prevent or limit fatalities and complications after liver resection, a preoperative evaluation of hepatic functional status is necessary, and the criteria used to define patient eligibility include the Child-Pugh score, the indocyanine green retention rate at 15 min, magnetic resonance imaging^[4], and the determination of liver stiffness using FibroScan®^[5]. Despite these precautions, it remains difficult to judge recovery after surgery, and the incidence of PHLF still exerts a major impact on 2-year survival following resection^[6]. Because PHLF shares a common clinical picture and outcome with SFSS (jaundice, ascites, coagulopathy, encephalopathy, *etc.*), both syndromes are considered as a single entity.

The cellular and molecular mechanisms giving rise to PHLF remain unclear, but several causal factors are considered to be important. The excessive portal blood inflow and resulting intrahepatic shear stress that occur after the transplantation of a graft that is too small have been shown to play a central role in the development of SFSS^[7,8]. For this reason, hemodynamic modulation of the portal vein is proposed to ensure successful adult-to-adult living-donor liver transplantation^[9]. As demonstrated by Bucur *et al*^[10] the use of a portal ring to modulate blood inflow can improve liver regeneration after surgical resection in a porcine model^[10]. Similar results have been observed using splenectomy to control hemodynamic parameters^[11]. An accumulation of liver injuries post-resection has also been suggested as a factor leading to post-operative mortality^[12]. Part if not all these injuries are associated with the sustained activation of Küpffer cells because of elevated endotoxin levels in the liver after surgery^[13] and the massive oxidative stress that results. Reducing oxidative stress has been shown to enhance markedly the regenerative capacity of the liver in an experimental model of acute liver failure^[14-16]. Likewise, preconditioning reduces ischemic reperfusion injuries and improved rat survival after hepatectomy performed on a liver affected by steatosis^[17]. However, Lehmann *et al*^[18] showed that failure of regeneration may occur in the absence of serious liver damage affecting the small remnant liver using an improved technique of extended hepatectomy in mice^[18]. That study also reported a delay in liver regeneration because of retarded progression through the cell cycle^[18]. They showed that extended liver resection positively regulated p21, a cyclin-dependent kinase inhibitor (CKI) at both the G1/S and the G2/M transitions. In addition, p21 deficiency enhances regenerative capacity of multiple tissue types including complete rescue and regeneration of injured liver^[18-21]. If confirmed, this finding is important as it will open the way to new therapeutic regimens targeting p21. This is even more important given that patients undergoing liver resection routinely receive intravenous glucose infusions to manage hypoglycemia, and such infusions have been shown to inhibit post-resection liver regeneration in a p21-dependent manner in mice^[22].

To create acute liver failure in rats, we performed an extended hepatectomy (eHx) with removal of 90% of the liver mass. We then studied the dynamics of the liver regeneration process and consequent behavior of cell cycle regulators in rats after eHx and post-operative glucose infusions.

MATERIALS AND METHODS

Animal model

All animal procedures were approved by the CE2A-03 CNRS-Orléans Ethics Committee. Male Wistar rats ($n = 119$) aged 10 wk and weighing 200-230 g were housed at the CNRS-SEAT animal care facility (Université Paris-Sud, Villejuif) and kept on a 12 h day/night cycle with free access to food and water. The number of rats used was in compliance with institutional ethical rules and consistent with common practice in the fields of post-hepatectomy liver regeneration. All the rats were anesthetized by isoflurane inhalation and then underwent a midline incision after sterilization of the area. For a standard 70% hepatectomy, the left lateral and left and rights parts of the median lobes of the liver were resected. For an extensive 90% resection, the left lateral, median, and both right lobes were carefully removed, leaving the two caudate lobes and liver tissues surrounding the vena cava. To prevent hypoglycemia, a subcutaneous injection of 5 mL of 30% glucose solution was administered immediately after liver resection, and then the animals had free access to 20% glucose solution and rat chow *ad libitum*. Because the administration of glucose might affect the kinetics of liver regeneration, it was injected in all the rats undergoing a 70% or 90% resection. Intraperitoneal injections of bromodeoxyuridine (BrdU) were given to all surviving animals 2 h prior to sacrifice at a dose of 50 mg/kg.

Serum biological analysis

Blood samples were obtained just prior to organ harvest at sacrifice, spun immediately to collect the serum, and frozen until use. Biochemical parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin] were determined using an Olympus AU400 automat (Centre d'Exploration Fonctionnelles Intégrées, Institut Claude Bernard, Paris, France).

Histological, immunofluorescence, and immunohistochemistry analyses

The livers thus collected were fixed overnight at 4 °C in a 4% formalin solution before being embedded in a paraffin block. For histological analysis, liver sections (4 µM) were dewaxed in xylene, rehydrated through graded alcohols, and stained with hematoxylin & eosin. For immunofluorescence, 4 µmol/L liver sections were dewaxed in xylene, rehydrated through graded alcohols, and pressure cooked in a 10 mmol/L citrate buffer at pH 6 for 10 min. For BrdU staining, the 5-bromo-2'-deoxy-uridine Labelling and Detection Kit I (Roche, Basel, Switzerland) was used according to the supplier's recommendations. Tissue auto-fluorescence was reduced by applying 10 mmol/L cupric sulfate in a 50 mmol/L acetate buffer pH 5 solution for 60 min at room temperature. To visualize nuclei, Hoechst 33342 solution was added to the mounting medium at a concentration of 0.1 mg/mL. For phospho-histone H3 staining, dewaxed rehydrated sections were incubated for 45 min at 37 °C with a primary antibody (Cell Signaling, Danvers, MA, United States), washed in phosphate buffer saline (PBS) (3-times for 5 min), and incubated for 30 min at 37 °C using Alexa fluor® 594 donkey anti rabbit immunoglobulin G (Invitrogen, Carlsbad, CA, United States). The sections were then washed in PBS and mounted using Hoechst 33342 containing mounting medium at a concentration of 0.1 mg/mL. For immunohistochemistry, dewaxed rehydrated sections were blocked for endogenous peroxidase, incubated with primary anti-caspase 3 antibody (Cell Signaling) or anti-signal transducer and activator of transcription 3 (STAT3) antibody (Santa Cruz Biotechnology, Dallas, TX, United States), washed, and incubated with secondary anti-rabbit immunoglobulin G-horseradish peroxidase according to the manufacturer's instructions (DAKO, Jena, Germany). The sections were counterstained with alcian blue (Sigma, St Louis, MO, United States) before mounting the coverslips. Positively labelled cell counting was performed in 10 random microscopic fields. Cell proliferation, cell death, and cell cycle were assessed by measuring the ratio of the numbers of BrdU-, p-Histone H3-, STAT3-, and caspase 3-positive nuclei to the total nucleus count.

Immunoblot analysis

Whole-cell lysates were prepared in ice cold buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mmol/L Na₃VO₄, 20 mmol/L NaF, 1 µg/mL aprotinin, 10 µg/mL pepstatin, 10 µg/mL leupeptin, and 1 µM phenylmethylsulfonyl fluoride. Protein concentrations were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Aliquots of 30 µg were denatured by boiling in Tris-Glycine SDS buffer (Invitrogen), separated by 12% SDS and transferred onto nitrocellulose membranes (Whatman, Dominique Dutscher, Brumath Cedex, France) by electroblotting. The membranes were blocked in 5% non-fat dry milk in 0.1% Tween 20 Tris-buffered saline for 1 h and probed with primary antibodies against cyclin E1, cyclin A2, cyclin B1, p27, p21, STAT3, p-STAT3, retinoblastoma protein (Rb), p-Rb, and actin (Santa Cruz Biotechnology).

Statistical analysis

Normal distribution of the data was analyzed by the Shapiro-wilk test and homogeneity of variances by the Levene test. All groups were normally distributed, and a two-tailed Student's *t*-test was used to assess statistical differences between the groups. The statistics were performed with StatView 5.0 freeware (SAS Institute Inc., Cary, NC, United States), and differences with *P* < 0.05 were considered significant. All data are presented as means over several independent experiments ± standard error of the mean. Survival curve was constructed by the Kaplan-Meier method (log-rank test).

RESULTS

Apoptosis is not the primary cause of liver dysfunction after subtotal (90%) hepatectomy in the rat

To determine the direct cause of death in liver failure after massive hepatectomy, 84 Wistar rats underwent 90% liver resection (eHx) and were randomly divided into two groups: One (20/84) to analyze rat survival over time and (64/84) another for biochemical analyses. For the second set of experiments, some rats were sacrificed every 24 h for 7 d. To prevent deaths linked to hypoglycemia, the rats were injected post-operatively with glucose solution and subsequently offered free access to a glucose solution. Control groups of either rats that underwent laparotomy (sham) or rats that underwent 70% liver resections (pHx) were treated similarly. As depicted in **Figure 1A**, the outcomes after liver surgery differed considerably between the groups, with a 30% survival rate after eHx (6/20) compared to 100% ($n = 11$) after pHx. This rate is consistent with previous studies. Peak mortality was seen within the first 48 h and accounted for 78% (11/14) of all deaths, 28% (4/14) during the first 24 h and a further 50% (7/14) between 24 h and 48 h. Before they died, the animals were hypoactive and displayed signs of liver failure such as jaundice and coma.

Blood was collected from surviving animals at the time they were sacrificed in order to measure markers for liver function and liver damage. Hepatic enzymes (ALT, AST) and bilirubin levels were significantly elevated 24 h after surgery when compared to sham animals (**Figure 1B-D**). At the same time point, markers levels remained moderate in the pHx rats but were significantly higher in the eHx animals, suggesting an accumulation of parenchymal injuries with an impairment of liver function in this group. The serum levels of both enzymes declined over time to reach a standard level 96 h after surgery, while conjugated bilirubin levels remained elevated in the eHx group even after 7 d indicated that liver functional activities were still impaired at that time (**Figure 1D**).

Despite the clinical picture of acute liver failure, the histological analysis of hematoxylin and eosin-stained liver sections did not reveal any signs of extensive apoptotic and necrotic cell death (**Figure 1E**). This result was confirmed by immunohistochemistry that showed almost no caspase 3-positive cells within the tissue sections (data not shown), indicating that apoptosis was not a major inducer of hepatic failure after excessive hepatectomy in this experimental surgical setting. Nevertheless, a pattern of parenchymal abnormalities was observed over time following resection, and these changes were much more pronounced in the eHx group. Hypertrophic hepatocytes associated with a clear cytoplasm testifying to fluid and lipid infiltration were detected at 24 h and 48 h after eHx. Lipid droplets were visible after 48 h in pHx animals but not until 96 h postoperatively in eHx sections. In addition, globular red hyaline material within hepatocytes was detected in eHx livers, evidencing alterations to protein synthesis and secretion processes (**Figure 1**, white arrowhead). Post mortem histological analysis of the rat livers that could be collected just after death revealed similar but much more developed changes. As for the rats sacrificed at each time-point, their livers did not display any signs of massive cell death or massive hemorrhagic parenchyma, but hypertrophic hepatocytes associated with a clear cytoplasm corresponding to fluid and lipid infiltration and an accumulation of globular red hyaline material were widely detected in the tissue sections (**Figure 2**).

Extended hepatectomy delayed cell cycle progression through S phase

We found numerous mitotic figures histologically in pHx livers as soon as 24 h after surgery (**Figure 1E**) but not before the 48 h time point in eHx tissues (**Figure 1E**). Immunohistochemical analyses revealed that although both pHx and eHx rats reached a maximum of BrdU incorporation (**Figure 3A**) and phospho-histone H3 Labelling (**Figure 3B**) at 48 h post-surgery, only pHx rats displayed labelled cells at the 24 h time point. **Figure 3C** shows determinations of the liver weight to body weight (LW/BW) ratio at various post-operative time points. A significant rise in the LW/BW ratio was noted 48 h after surgery in rats that had undergone pHx and at the 72 h point in eHx animals. These findings establish that hepatocyte proliferation and liver mass restoration were delayed in eHx rats.

Liver regeneration after pHx is a well-known mechanism that involves the sequential activation of cytokines and growth factor-related pathways. This cascade of events leads to a peak of deoxyribonucleic acid synthesis 24 h after surgery in the rat (for a review, see^[23]). To evaluate proper implementation of the mitogenic program, western blot analyses were performed on frozen liver specimens from rats that had

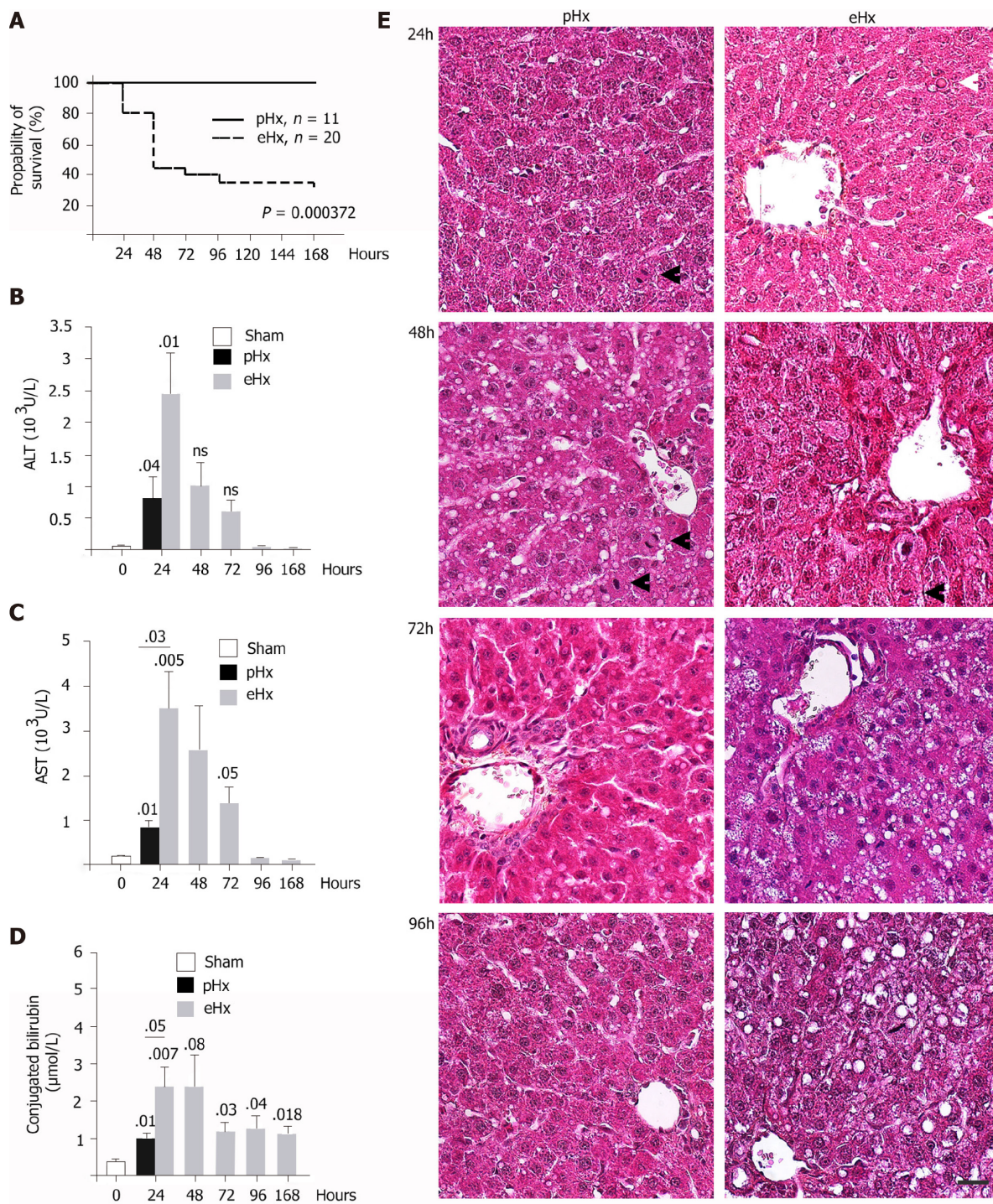


Figure 1 Survival rates and post-operative liver injury after enlarged liver resection. Male Wistar rats were subjected to 70% [hepatectomy (pHx)] or 90% [extended hepatectomy (eHx)] followed by harvest of blood samples and the remnant liver tissues at the indicated time points after surgery. A: Kaplan-Meier survival plots; B: Serum levels of alanine aminotransferase (ALT); C: Serum levels of aspartate aminotransferase (AST); D: Conjugated bilirubin from hours 24 to 168 post-eHx (n = 45); E: Representative liver images stained with hematoxylin, eosin, and alcian blue at the indicated time points post-eHx; black arrowheads indicate mitotic figures, and white arrowheads point to globular red hyaline material. Scale bar: 50 μ m. Sham: Laparotomy control group.

undergone enlarged hepatectomy and were sacrificed 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h post-surgery. As STAT3 is activated rapidly during liver regeneration in an interleukin 6-dependent manner and drives hepatocytes to switch from a quiescent state into a proliferative wave, STAT3 activation was verified (Figure 4A and B). We found STAT3 activation quickly 3 h post-surgery (Figure 4A and B). Peak STAT3 activation was observed at the 6 h time point and then gradually returned to standard levels 48 h post-resection (Figure 4A and B). These data therefore indicated that the priming of rat hepatocytes had occurred and that they correctly re-entered the cell cycle after eHx, despite the infusion of glucose.

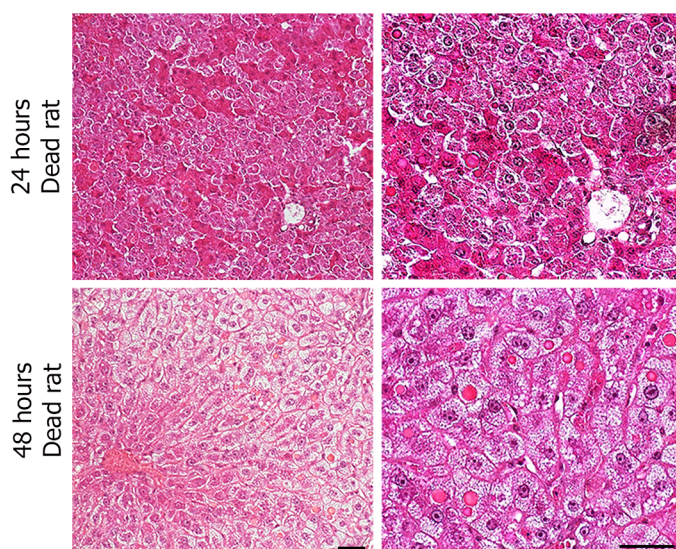


Figure 2 Severity of hepatocyte damage on the onset of liver regeneration. Histological analysis of representative liver sections harvested within minutes of death and stained with hematoxylin, eosin, and alcian blue from rats that died 24 h and 48 h post-resection. Note the presence of enlarged hepatocytes containing cytoplasmic hyaline inclusions. Scale bar: 50 μm /L.

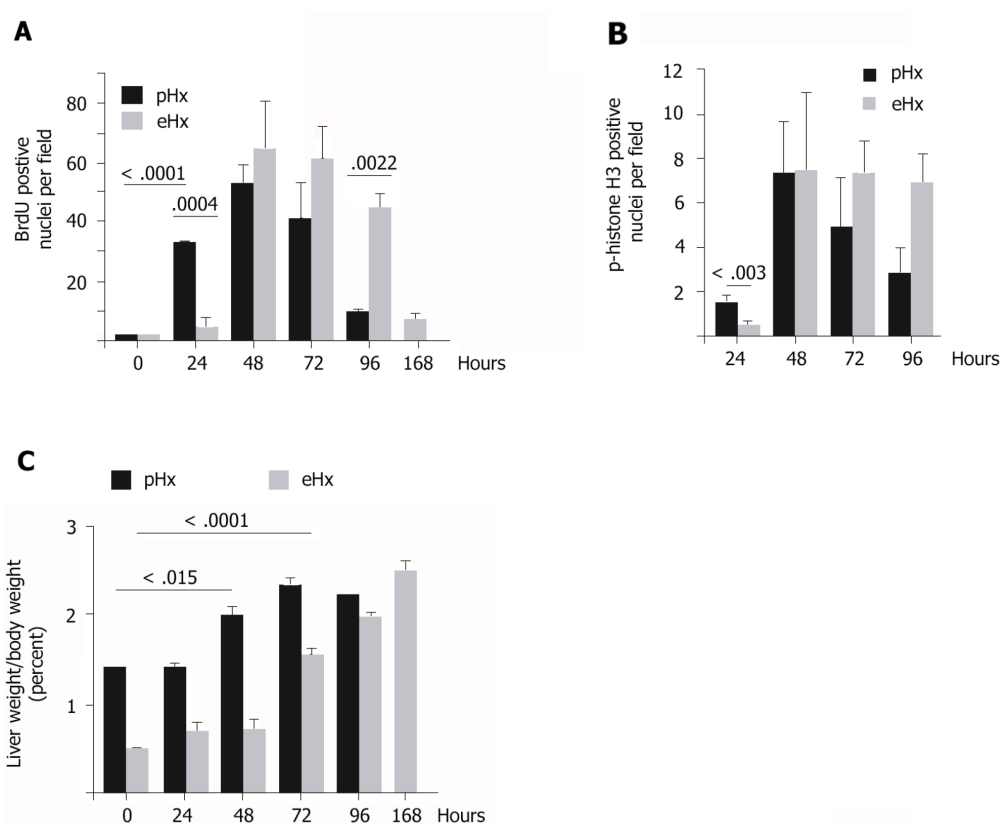


Figure 3 S-phase progression is markedly delayed after extended hepatectomy. Animals were sacrificed at the indicated time points after 70% [hepatectomy (pHx)] or 90% [extended hepatectomy (eHx)], and remnant liver tissues were harvested for immunofluorescence analysis. A: Bromodeoxyuridine (BrdU) incorporation; B: Phospho-histone H3 Labeling of mitotic cells. Cell counting was performed in 10 random microscopic fields on 6 rats in the eHx group and 3 rats in pHx; C: Mean liver to body weight ratios after pHx and eHx. eHx: Extended hepatectomy; pHx: 70% hepatectomy.

We next studied cell cycle checkpoint proteins p-Rb in G1 (Figure 4C), cyclin E1 for the G1 to S transition (Figure 4D), cyclin A2 for the S to G2 transition (Figure 4D), and cyclin B1 for the G2 to mitosis transition (Figure 4D). Levels of p-Rb were significantly upregulated during the first 3 h after resection, remained high for 24 h, and then normalized by the 48 h time point (Figure 4C). The cyclin E1 level rose significantly

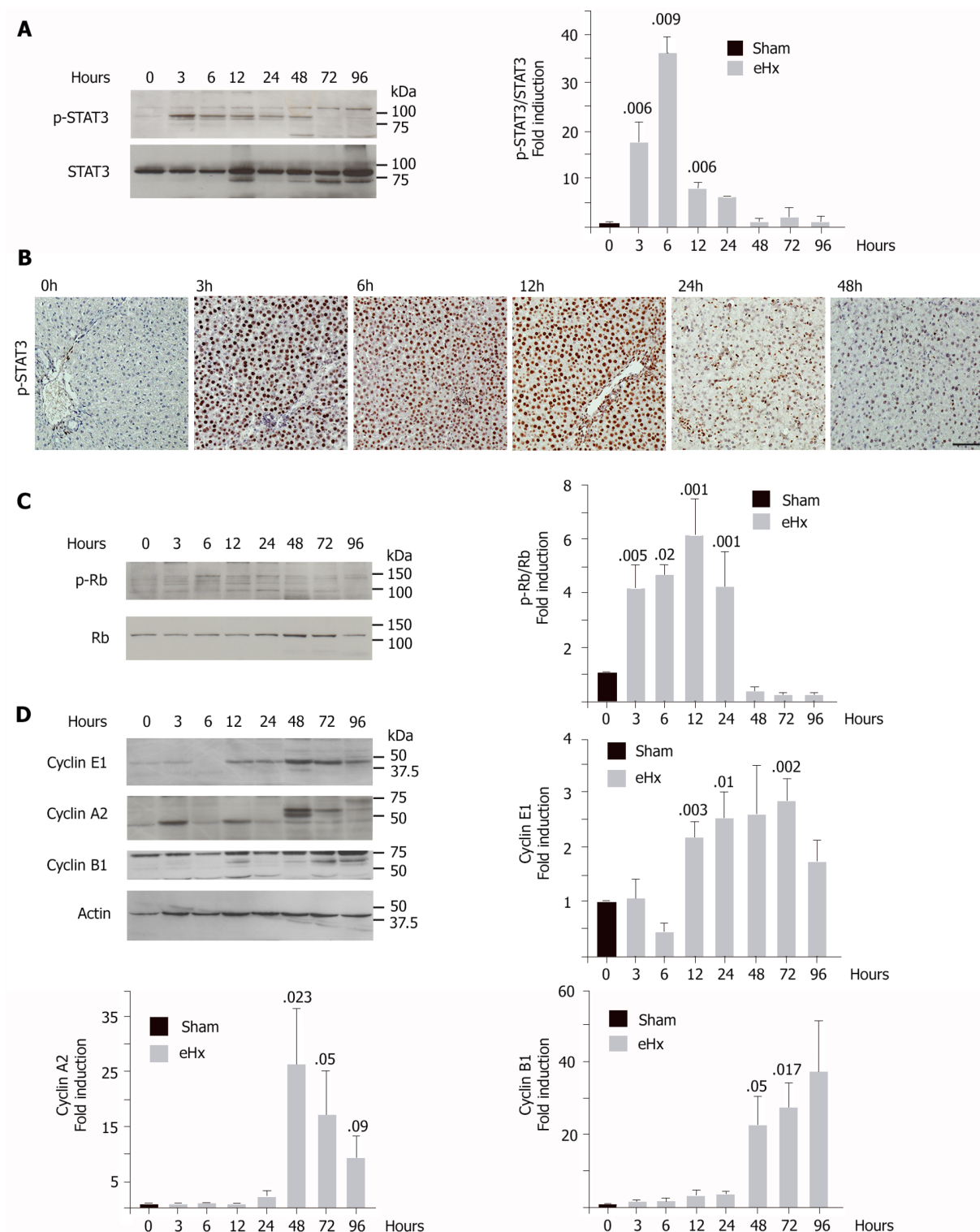


Figure 4 Expression and activation of cell cycle proteins during liver regeneration. Extracts from rat liver tissues harvested at the indicated time points after extended hepatectomy (eHx) were subjected to western blot and immunohistochemistry. A and B: Signal transducer and activator of transcription 3 (STAT3) activation; C: Retinoblastoma protein (Rb) inactivation; D: Cyclin E1, A2, and B1 expression. Bar graphs represent the mean densitometry value \pm standard error of the mean $n = 6$ rats per time point. Sham: Laparotomy control group.

after 12 h and remained elevated until the 72 h time point (Figure 4D). Levels of cyclin A2 and B1 remained stable at very low levels for the first 24 h post-surgery, rose markedly after 48 h, and then remained high until the 96 h time point (Figure 4D). Taken together, our results showed that hepatocytes entered the cell cycle correctly, but the absence of any detection of cyclin A2, 24 h after surgery, established a delayed S phase progression.

Altered expression of p21 and p27 in the small liver remnant during regeneration

As depicted in Figure 5, our model of eHx rapidly induced an upregulation of the p21 and p27 CKI in hepatocytes, which resulted in decreased regeneration. The p21 protein was undetectable in quiescent rat liver. Its level rose gradually just after surgery to reach an initial peak at 12 h post-resection that corresponded to a 11.6 ± 4.5 -fold amplification ($P < 0.05$). P21 displayed a second peak of expression at 48 h after resection, with a 32 ± 9.3 -fold ($P = 0.007$) amplification *vs* baseline. Our data also pointed to a prolonged expression of p27 during the first 24 h, the level reaching 1.7 ± 0.25 -fold ($P = 0.03$) as early as 3 h post-eHx, indicating that p27 acts as a rapid brake to S-phase progression. P27 levels then normalized after 24 h. A second wave of p21 expression was detected afterwards.

DISCUSSION

Liver resection offers a chance of a cure in patients presenting with primary and secondary liver cancers and is currently the gold standard treatment for these malignancies. If performed on appropriate patients, liver reduction is a safe operation. Highly selective criteria based on preoperative assessments of both the extent of the disease and the liver function need to be met in order to minimize post-operative complications. Some surgical strategies have been developed to increase the number of patients who are eligible for resection, such as portal vein embolization, which enables expansion of the portion of healthy liver prior to resection, or two-stage liver resection. However, most patients diagnosed with primary or secondary liver cancer remain ineligible for surgery. The principal challenge for clinicians in the coming years is to find alternative treatments for patients who are denied surgical reduction. This issue is all the more important given the worldwide progression of obesity and diabetes that is causing chronic inflammatory liver disorders, even though the impact of liver resection in obese patients remains controversial^[24-26]. An increased risk of developing liver failure post-resection was demonstrated when performed in patients and mouse models of steatosis^[27], NASH^[28] and cirrhosis^[29]. To ensure the safety of patients and avoid liver failure post-resection, a minimum of 30% functional hepatic parenchyma is required^[3]. However, there is as yet no complete understanding of the mechanistic details of hepatocellular failure below this critical mass.

The development of novel pharmaceutical strategies to help patients recover from extended liver resection requires full identification and characterization of the causes of morbidity-mortality, and thus the reasons why the remnant liver lobes failed to regenerate. A search in the bibliography on this topic generally produces papers that refer to multivariate analyses performed on large cohorts of patients who underwent liver resection and lists the predictive factors that will enable a better stratification of patients prior to surgery^[30-32]. These factors include diabetes, steatosis, chemotherapy-associated steatohepatitis, patient age and gender, and, of course, the volume of liver to be removed. However, such a review highlights two principal reasons for post-resection-induced morbidity-mortality: An insufficient number of functioning hepatocytes to achieve proper synthesis, excretion and detoxification, and excessive portal blood inflow that leads to sinusoidal dilatation and necrosis. The use of a portal ring to control portal blood inflow has been shown to improve liver regeneration following surgical resection in a pig model^[10]. Post-operative biochemical parameters were improved in pigs with a portal ring but no significant difference was noted regarding the mortality rate, probably because of the small sample size of eight pigs *per* condition^[10]. Further studies are necessary in both animals and humans to clarify the benefits of this approach. To compensate for the loss of liver function, extracorporeal hepatic support devices have been evaluated in patients presenting with acute post-operative liver failure. These devices, such as MARS®, Prometheus®, and SPAD, are albumin-linked hemodialysis systems that improve the biochemical parameters of patients but fail to improve survival rates^[33]. A study combining the use of both liver support and portal ring devices needs to be envisaged in the future so as to determine the effects on perioperative outcomes and long-term survival. However, the results reported at present show that improvements of liver function and portal flow were insufficient to improve survival following major liver resection, suggesting that a different and underestimated mechanism is also responsible for post-resection lethal failure.

This assumption is in line with findings of different studies, including those of Lehmann *et al.*^[18], who showed an impairment of the regenerative capacity of the small remnant liver linked to a p21-dependent cell cycle block in a mouse model^[18].

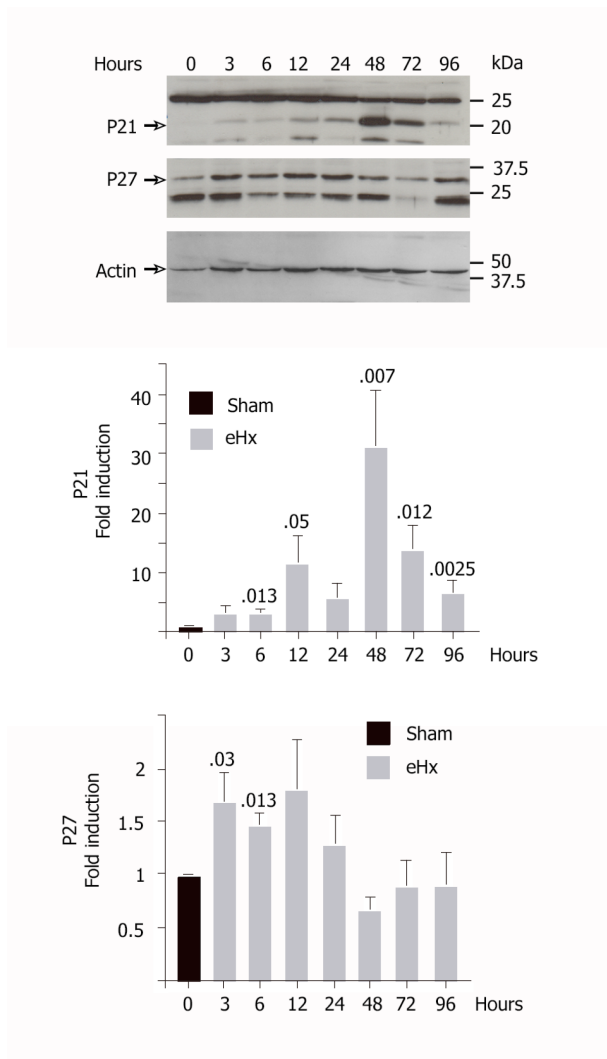


Figure 5 Increased expression of p21 and p27 in regenerating rat liver. The expression of p21 and p27 was investigated using western blot performed on snap-frozen liver samples collected at the indicated time points post-extended hepatectomy (eHx). Six rats were studied for each time point. A representative image is presented in the upper part of the figure, and the mean values for 6 rats per condition are shown as bar graphs. Sham animals correspond to the laparotomy group. Sham: Laparotomy control group.

Inhibiting p21 in transgenic animals partially restored the regenerative capacity of the liver and improved the survival rate^[18], and a treatment with a senescence-inhibiting drug improved liver regeneration after partial hepatectomy by disrupting aberrantly prolonged p21 expression in mice^[34].

To investigate further the contribution of CKI in the failed liver regeneration, we examined the earliest events to occur in response to experimentally hepatic insufficiency induced by 90% hepatectomy in the rat. We showed that the delayed liver regeneration of the small remnant liver is associated with altered expression of p27 and p21, being detected as early as 3 h and 12 h post-operatively, respectively. The priming of quiescent hepatocytes occurred correctly, as depicted by STAT3 activation coincident with Rb phosphorylation as early as 3 h post-resection, reflecting entry into the cell cycle. But extended hepatectomy resulted in significant delay in S-phase progression and mitosis, which was compensated in surviving animals by increased deoxyribonucleic acid synthesis at later time points, eventually leading to restored liver mass and functional activity. Our results therefore highlighted the critical importance of the cyclin/cyclin-dependent kinase inhibitors of the Cip/Kip family in regulating the liver regeneration timeline following 90% hepatectomy. To this is added a large number of molecular signals that were switched on or off to guarantee a timely hepatocyte entry and progression into the cell cycle^[35,36]. However, the choice of the experimental conditions (hepatectomies ranging from 80% to 95% of total liver weight, glucose supplementation, species-specific features, housing conditions, and diet) affects many of these signaling pathways, accounting for the noticeable differences between the studies.

In our model, peak mortality after eHx was reached within the first 48 h and accounted for 71% of all deaths. Among these, 29% occurred within less than 24 h, and these deaths could not be attributed to cell cycle changes as an increase in liver mass was first detected after 24 h in the control group. Although histological analysis of the liver tissues from dead animals did not reveal any massive liver injuries, hepatocytes displayed a clear cytoplasm with numerous accumulations of globular red hyaline material, testifying to an impairment of liver function that involved the protein excretion process. Our findings were also in accordance with conjugated bilirubin levels that remained elevated in the eHx group (even after 7 d) while ALT and AST returned to normal levels after 96 h.

CONCLUSION

In conclusion, the loss of hepatocyte functional activities and a hindrance to the regenerative capacities of the remnant lobe both contribute to mortality following major liver resection. The use of extracorporeal support devices along with inhibitors of p21 and p27 now needs to be evaluated in terms of managing liver failure after extended hepatectomy. This combination may facilitate access to curative surgical treatments for primary or secondary cancer for patients who are not eligible according to current standards.

ARTICLE HIGHLIGHTS

Research background

Liver reduction is routinely performed as curative treatment of primary liver cancer and liver metastases, but its use remains limited as liver regeneration requires a minimum of 30% functional parenchyma.

Research motivation

As such, less than 30% of patients with hepatocellular carcinoma are eligible for surgery, and this is connected to the underlying chronic inflammation and the preoperative chemotherapies. Post-surgery accumulation of liver injuries, excessive portal blood inflow, and oxidative stress are the main causal factors suspected to give rise to liver failure, but the molecular mechanisms that block liver regeneration remain unclear.

Research objectives

Our objective was to monitor, step by step, the molecular events in relation to liver regeneration after extended liver resection and so to clearly delineate the blocking points that prevent liver regeneration.

Research methods

Post-operative liver failure was modelled in the rat by 90% liver resection. Animals undergoing simple laparotomy and 70% hepatectomy were used as control. All animals received glucose infusion to avoid post-operative hypoglycemia. Animals were sacrificed every 3 h for the first 24 h and every 24 h for the following 7 d. Blood and liver samples were collected at the time of sacrifice and used to investigate liver function, morphology, and regeneration by biochemical methods.

Research results

Twenty-nine percent of all deaths occurred in the first 24 h in link with massive liver injuries and impaired liver function. For all other deaths, the temporal sequence of events that prime liver regeneration after 90% liver resection occurred properly, but S phase progression and mitosis were delayed by 24 h in conjunction with the rise in p27 (Kip1) and p21 (Waf1/Cip1) cell cycle inhibitor levels.

Research conclusions

The cyclin/cyclin-dependent kinase inhibitors of the Cip/Kip family are critical regulators of the liver regeneration following extended hepatectomy.

Research perspectives

The use of extracorporeal support devices along with inhibitors of p21 and p27 should be evaluated to manage liver failure after extended hepatectomy.

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Basic Study

Pivotal role of long non-coding ribonucleic acid-X-inactive specific transcript in regulating immune checkpoint programmed death ligand 1 through a shared pathway between miR-194-5p and miR-155-5p in hepatocellular carcinoma

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Conflict-of-interest statement: The

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Abstract

BACKGROUND

Anti-programmed death therapy has thrust immunotherapy into the spotlight. However, such therapy has a modest response in hepatocellular carcinoma (HCC). Epigenetic immunomodulation is a suggestive combinatorial therapy with immune checkpoint blockade. Non-coding ribonucleic acid (ncRNA) driven regulation is a major mechanism of epigenetic modulation. Given the wide range of ncRNAs that co-opt in programmed cell-death protein 1 (PD-1)/programmed death ligand 1 (PD-L1) regulation, and based on the literature, we hypothesized that miR-155-5p, miR-194-5p and long non-coding RNAs (lncRNAs) X-inactive specific transcript (XIST) and MALAT-1 are involved in a regulatory upstream pathway for PD-1/PD-L1. Recently, nutraceutical therapeutics in cancers have received increasing attention. Thus, it is interesting to study the impact of oleuropein on the respective study key players.

authors have no conflicts of interests.

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AIM

To explore potential upstream regulatory ncRNAs for the immune checkpoint PD-1/PD-L1.

METHODS

Bioinformatics tools including mircorna.org and InCeDB software were adopted to detect targeting of miR-155-5p, miR-194-5p and lncRNAs XIST and MALAT-1 to PD-L1 mRNA, respectively. In addition, Diana tool was used to predict targeting of both aforementioned miRNAs to lncRNAs XIST and MALAT-1. HCC and normal tissue samples were collected for scanning of PD-L1, XIST and MALAT-1 expression. To study the interaction among miR-155-5p, miR-194-5p, lncRNAs XIST and MALAT-1, as well as PD-L1 mRNA, a series of transfections of the Huh-7 cell line was carried out.

RESULTS

Bioinformatics software predicted that miR-155-5p and miR-194-5p can target PD-L1, MALAT-1 and XIST. MALAT-1 and XIST were predicted to target PD-L1 mRNA. PD-L1 and XIST were significantly upregulated in 23 HCC biopsies compared to healthy controls; however, MALAT-1 was barely detected. MiR-194 induced expression elevated the expression of PD-L1, XIST and MALAT-1. However, overexpression of miR-155-5p induced the upregulation of PD-L1 and XIST, while it had a negative impact on MALAT-1 expression. Knockdown of XIST did have an impact on PD-L1 expression; however, following knockdown of the negative regulator of X-inactive specific transcript (TSIX), PD-L1 expression was elevated, and abolished MALAT-1 activity. Upon co-transfection of miR-194-5p with siMALAT-1, PD-L1 expression was elevated. Co-transfection of miR-194-5p with siXIST did not have an impact on PD-L1 expression. Upon co-transfection of miR-194 with siTSIX, PD-L1 expression was upregulated. Interestingly, the same PD-L1 expression pattern was observed following miR-155-5p co-transfections. Oleuropein treatment of Huh-7 cells reduced the expression profile of PD-L1, XIST, and miR-155-5p, upregulated the expression of miR-194-5p and had no significant impact on the MALAT-1 expression profile.

CONCLUSION

This study reported a novel finding revealing that opposing acting miRNAs in HCC, have the same impact on PD-1/PD-L1 immune checkpoint by sharing a common signaling pathway.

Key Words: Hepatocellular carcinoma; X-inactive specific transcript; MiR-155-5p; MiR-194-5p; Programmed cell-death protein 1/Programmed death ligand 1; Immune checkpoint

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Core Tip: Due to the immune rich milieu of hepatocellular carcinoma (HCC), it is a good candidate for immune-based therapies. In this study, our aim was to identify potential upstream epigenetic regulators of immune checkpoint programmed cell-death protein 1/programmed death ligand 1 in HCC which could be regarded as therapeutic targets. The findings of this study revealed the re-questioning of the role of certain non-coding ribonucleic acids in HCC. Here we deduced a novel shared upstream regulatory signaling pathway for programmed cell-death protein 1/programmed death ligand 1 immune checkpoint between paradoxically acting tumor suppressor miR-194-5p and onco-miR-155-5p, in HCC through X-inactive specific transcript expression modulation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes a global burden and is one of the leading causes of cancer mortality^[1]. A myriad of therapeutic modalities is available for HCC including tumor resection or ablation, transarterial chemoembolization, liver transplantation and treatment with tyrosine kinase inhibitors^[2]. Nevertheless, HCC is a highly therapy resistant disease and is frequently diagnosed at an advanced stage; thus, the identification of a novel therapeutic modality is essential^[3].

Recently, tumour immunotherapy has been thrust into the spotlight to inhibit tumour progression, relapse and metastasis. Immunotherapeutic techniques comprise both activation of tumour specific immune responses as well as enhancement of cellular or humoral immunity thus causing disruption of immune tolerance^[4]. HCC immunotherapy has greatly changed due to extensive ongoing immunological studies which have incorporated immunotherapy into the HCC treatment armamentarium^[5]. The rationale behind such a revolutionary therapeutic technique is the fact that HCC develops in an inflammatory milieu brimming with tumour infiltrating lymphocytes boosting HCC immunogenicity^[6].

Immune checkpoint inhibitors have been featured as a sensational paradigm shift in cancer immunotherapy^[7]. Physiologically, immune checkpoints are co-inhibitory molecules that act as “brakes” in the immune system to avoid an exaggerated response and restore its activity to a normal level^[8,9]. Programmed cell-death protein 1 (PD-1) is one of the highly expressed immune checkpoints on T-cells in most solid tumours^[10]. PD-1 was originally described by Ishida *et al*^[11] in 1992 as a cell death inducer, a discovery that paved the way for Noble prize winning immune checkpoint inhibitor studies in 2018. Tumour immune surveillance evasion can then occur upon engagement of PD-1 with its ligand, Programmed death ligand 1 (PD-L1), expressed on tumour cells leading to effector T-cell exhaustion and dysfunction^[12,13]. PD-1/PD-L1 immune checkpoint blockade has shown considerable survival benefits in patients with different metastatic tumours^[14-17]. In 2017, the Food and Drug Administration approved Nivolumab, a human immunoglobulin G monoclonal antibody against PD-1, for patients with advanced HCC, due to durable responses observed in these patients^[18].

Accumulating evidence has shown that PD-1/PD-L1 immune checkpoint is epigenetically regulated through immunomodulatory non-coding ribonucleic acids (ncRNAs) as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in several cancers including colorectal cancer^[19], lung cancer^[20] and pancreatic cancer^[21]. Furthermore, our research group demonstrated the epigenetic regulation of PD-1/PD-L1 in breast cancer^[22]. Nevertheless, such immunomodulatory loops orchestrating PD-1/PD-L1 expression and activity are still under investigation in HCC.

Due to the breakthrough established in next generation sequencing which enabled the profiling of the whole transcriptomic expression at the molecular level, our understanding of biological systems has improved^[23]. Such studies have revealed the expression deregulation of a multitude of ncRNAs^[24].

Based on bioinformatics analysis, the miRNAs, oncomiR and miR-155-5p, and tumor suppressor miR-194-5p were predicted to target PD-L1 transcriptome as well as the candidate lncRNAs, X-inactive specific transcript (XIST) and MALAT-1. Moreover, lncRNAs XIST and MALAT-1 were predicted to target PD-L1 transcript where both lncRNAs have demonstrated their role in HCC pathogenesis in several studies.

Therefore, it is interesting to study the expression profile of PD-L1 in Huh-7 cells relative to the expression manipulation of candidate ncRNAs in order to explore novel potential upstream regulatory ncRNAs for PD-L1 in HCC and the capacity of these ncRNAs as therapeutic targets. In addition, it is of value to determine the clinical relevance of the proposed regulatory signaling pathways for PD-L1 in HCC patients by assessing the expression pattern of PD-L1 as well as the lncRNAs XIST and MALAT-1 in HCC tissues.

The trend towards integrating phytochemicals in cancer therapy is being augmented worldwide, especially with increased tolerance and resistance to traditional cancer therapeutic modalities. The olive tree (*Olea europaea* L.) which belongs to the Oleaceae family is native to tropical and warm temperate regions^[25]. Several studies have postulated that the olive plant has anti-inflammatory^[26] and anti-cancer activities^[27]. Such activities are mainly attributed to the unique polyphenolic content of the olive plant.

Oleuropein is one of the highly abundant phenolic compounds in olive leaves^[28]. It is reported to have a plethora of beneficial health benefits that are attributed to a compilation of pharmacological action including anti-oxidant^[29], anti-inflammatory^[30], and anti-angiogenic^[31] activities which pave the way for its interesting anticancer

activity^[32]. Oleuropein has been demonstrated to have an anti-inflammatory and immunomodulatory effect *via* down-regulation of MAPKs and NF- κ B signaling pathways as well as controlling the production of inflammatory mediators such as IL-6 and TNF- α cytokines, MMP-1 and MMP-3 levels^[33]. Interestingly, Ruzzolini *et al*^[34] revealed the promising potential of oleuropein as an adjuvant therapy against BRAF melanoma, by manipulating the pAKT/pS6 pathway. Moreover, a recent study demonstrated the potential indirect modulatory impact of oleuropein on PD-L1 in esophageal cancer, by manipulating the expression of hypoxia-inducible factor-1^[35]. Nevertheless, to the best of our knowledge, the immunomodulatory impact of oleuropein on HCC has not been extensively studied. Hence, the impact of this promising compound on our study key players was determined.

MATERIALS AND METHODS

Bioinformatics analysis

To detect possible microRNAs targeting 3'UTR of PD-L1 mRNA, microrna.org (www.microrna.org) bioinformatics target prediction software was used. Based on the binding scores and number of hits, miRNAs with good scores were chosen. Diana tools software (<http://carolina.imis.athena-innovation.gr>) was used to analyze potential binding of miR-194 and miR-155 to the 3'UTR region of lncRNAs XIST and MALAT1. The InCeDB (Database of Human Long Noncoding RNA Acting as Competing Endogenous RNA) prediction software algorithm (<http://gyanxet-beta.com/lncedb/>) was used to analyze potential binding of lncRNA XIST and MALAT-1 to PD-L1.

Patients and tissue samples

The present study included 23 patients with HCC, who underwent liver transplant surgery in the Kasr El Einy Hospital (Cairo University, Cairo, Egypt). Four samples of cirrhotic tissues were taken from a subset of these patients with focal HCC lesions. As per the pathology report of these patients, summarized in Table 1, almost 70% of patients had > 1 focal lesion. Ten liver biopsies were obtained from healthy donors. Ethical approval for this study was issued by the Institutional Review Board of Cairo University. In addition, all participants provided written informed consent. The institutional ethics committees approving this research comply with the principles set forth in the international reports and guidelines of the Helsinki Declaration and the International Ethical Guidelines for Biomedical Research Involving Human Subjects, issued by the Council for International Organizations of Medical Sciences.

Cell culture

Huh-7 cells were purchased from Vacsera Egypt. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza, Germany, cat. no. 12-604F), supplemented with 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% fetal bovine serum (Applied Biosystems; Thermo Fisher Scientific Inc., cat. no. 10270098) and Mycozap (1:500; Lonza, cat. no. LT07-818) at 37°C in a 5% carbon dioxide atmosphere.

Transfection of miR and siRNAs oligonucleotides

Twenty-four hours prior to transfection, 1.5×10^4 or 2.8×10^4 Huh-7 cells (40%-80% confluency) per well were seeded in a 96-well plate or 24-well plate, respectively. The cells were incubated under normal growth conditions (37°C and 5% carbon dioxide). The Huh-7 cell line was transfected with miScript™ miRNA mimics/inhibitors of miR-155-5p (Syn-hsa-miR-155-5p miScript miRNA Mimic, Qiagen, cat. no. MSY0000646 and Anti-hsa-miR-155-5p miScript miRNA Inhibitor, Qiagen, cat. no. MIN0000646) and miR-194 (Syn-hsa-miR-194-5p miScript miRNA Mimic, Qiagen, cat. no. MSY0000460 and Anti-hsa-miR-194-5p miScript miRNA Inhibitor, Qiagen, cat. no. MIN0000460). Transfections with siRNAs for each of XIST (Hs_XIST_3 FlexiTube siRNA, Qiagen Germany, cat. no. SI03654483), the negative regulator of X-inactive specific transcript (TSIX), (Hs_TSIX_7 FlexiTube siRNA, Qiagen Germany, cat. no. SI04708795) and MALAT-1 (Hs_MALAT1_1 FlexiTube siRNA, Qiagen Germany, cat. no. SI03670541) were also carried out. Co-transfections of each of the miR-155 and miR194 mimics were carried out with the siRNAs of each of the three lncRNAs MALAT-1, XIST and TSIX, respectively. All transfection experiments were performed in triplicate using HiPerfect Transfection Reagent (Qiagen Germany, cat. no. 301705) according to the manufacturer's instructions, and experiments were repeated three times. Cells that

Table 1 Clinical assessment of 23 patients with hepatocellular carcinoma

| Parameter | Value |
|----------------------------------|--------------|
| Age (yr) | 49 ± 13.5 |
| Aspartate aminotransferase (U/L) | 100.5 ± 65.8 |
| Alanine aminotransferase (U/L) | 85.6 ± 95.6 |
| Alkaline phosphatase (U/L) | 110.2 ± 60.7 |
| Serum albumin (g/dL) | 4.6 ± 1.5 |
| Serum α fetoprotein (ng/mL) | 155.7 ± 22.3 |

Data are presented as the mean ± SD. Male: Female = 2:1. All patients were positive for hepatitis C virus antibody.

were exposed only to the transfection reagent were designated mock cells; cells transfected with miR-155 or miR-194 mimics were designated miR-155 cells and miR-194 cells, respectively; cells transfected with the miR-155 or miR-194 inhibitors were designated as anti-miR-155 cells and anti-miR-194 cells, respectively; cells transfected with XIST siRNAs were designated as XIST siRNA cells; cells transfected with MALAT-1 siRNAs were designated as MALAT-1 siRNA cells; cells transfected with TSIX siRNAs were designated as TSIX siRNA cells; cells co-transfected with miR-155 and XIST siRNA were designated as miR-155/siXIST; cells co-transfected with miR-155 and MALAT-1 siRNA were designated as miR-155/siMALAT-1; cells co-transfected with miR-155 and TSIX siRNA were designated as miR-155/siTSIX; cells co-transfected with miR-194 and XIST siRNA were designated as miR-194/siXIST; cells co-transfected with miR-194 and MALAT-1 siRNA were designated as miR-194/siMALAT-1; cells co-transfected with miR-194 and TSIX siRNA were designated as miR-194/siTSIX; Cells were lysed 48 h post-transfection and total RNA was extracted for further analysis.

Plant material and fractionation

Olive leaves were collected from northern Sinai, Egypt and authenticated by Mrs. Therasa Labib, Taxonomist, Orman Botanical Garden, Egypt. Voucher specimen number (00396) was deposited at the Herbarium of the Pharmaceutical Biology Department, Faculty of Pharmacy and Biotechnology, German University in Cairo. Exhaustive extraction of olive leaves was carried out using 70% aqueous-ethanol, followed by re-suspension of the residue in H₂O and fractionation against petroleum ether, chloroform and ethyl acetate to yield 17 g, 6.5 g and 4.5, g respectively. The ethyl acetate polar fraction was applied over an open column (64 cm L × 5.5 cm ID) packed with silica (250 g) as stationary phase. A CHCl₃:CH₃OH:H₂O gradient was used for the elution process to ensure purification of the sub-fractions.

Isolation of oleuropein

The sub-fraction of interest (30 mg) was obtained using CHCl₃:CH₃OH:H₂O in a ratio of 3:4:3, then injected into a preparative high performance liquid chromatograph (Waters 600 E multisolvent delivery system, Waters 600 E pump and Waters 2998 PDA) which was employed using Lichrospher 100 RP-18 (250 mm × 10 mm i.d.; 10 μm) (Merck KGaA, Darmstadt, Germany). The mobile phase used was composed of 0.2% H₃PO₄ (v/v), methanol and acetonitrile in a ratio of 96:2:2. NMR spectra were obtained using a Bruker Avance 500 spectrometer (Bremen, Germany) 5 mm-Zgrad probe, operating at 500.13 MHz for ¹H and 125.77 MHz for ¹³C. The purity of oleuropein was confirmed using analytical HPLC (Agilent Technologies, Waldbronn, Germany), equipped with a PDA detector G 1314 C (SL). Chromatographic separation was carried out on a Superspher 100 RP-18 (75 mm × 4 mm i.d.; 4 μm) column (Merck, Darmstadt, Germany) using mobile phases: (A) 2% acetic acid (pH 2.6) and (B) 80% methanol. A gradient starting from 5% B to 50% B was employed for the elution process with 100 μL/min flow rate at 30°C and compared *vs* standard material (Sigma Aldrich) using HPLC. Confirmation of oleuropein identity was carried out by comparing its spectral data to the obtained literature^[36].

Oleuropein treatment to HuH-7 cells

A stock solution of oleuropein 100 mmol/L was prepared by dissolving 0.108 g in 2 mL of free DMEM. A solution of 80 μmol/L concentration that was previously

reported as LC50 on Huh-7 cells^[37] was prepared using this stock.

RNA isolation from liver biopsies and Huh-7 cell line

RNA was isolated from Huh-7 cells and liver biopsies using the TRIzol™ LS Reagent (Applied Biosystems; Thermo Fisher Scientific Inc., cat. no. 10296010) extraction protocol.

Quantified real-time polymerase chain reaction

Total RNA extracted was reverse-transcribed into single-stranded complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific Inc., cat. no. 4368814). The relative expression of miR-155 as well as miR-194 to that of RNU6B (housekeeping gene), in addition to PD-L1 mRNA, XIST and MALAT-1 lncRNAs to that of β -2-microglobulin (β 2M; a housekeeping gene) were quantified with TaqMan RT-quantitative polymerase chain reaction [quantified real-time polymerase chain reaction (qRT-PCR); Applied Biosystems Assay IDs: 002287, 000493, 0001093, Hs01079824_m1, Hs00273907_ml and Hs00984230_m1 and Hs01060665_g1, respectively] using StepOne™ Systems (Applied Biosystems Life Technologies). The PCR for miR quantification included 1 μ L TaqMan Small RNA Assay (20 X) specific for each of miR-155 or miR-194 or RNU6B and 1.33 μ L cDNA from each miR-155 or miR-194 or RNU6B RT reactions, respectively. Taqman target gene assay expression assay (1 μ L) specific for each of PD-L1, XIST and MALAT-1 as well as 4 μ L of the respective cDNA were used for quantification. The RT-qPCR run was performed in the standard mode, consisting of two stages: A first 10 min stage at 95°C where the Taq-polymerase enzyme was activated, followed by a second stage of 40 amplification cycles (15 s at 95°C and 60 s at 60°C). Relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method. All PCR reactions, including controls, were run in triplicate.

Statistical analysis

All data were expressed in relative quantitation. For the purpose of comparison between two different studied groups, the Student's unpaired *t*-test was used. Data were expressed as mean \pm SD error of the mean. A *P* value less than 0.05 was considered statistically significant. ^d*P* < 0.0001, ^c*P* < 0.001, ^b*P* < 0.01, ^a*P* < 0.05. Analysis was performed using GraphPad Prism 7.02.

RESULTS

In silico analysis

According to miRANDA software and the miRDB database, a total of 146 miRNAs were predicted to target PD-L1 mRNA. Both miR-155 and miR-194 were predicted to bind to the 3'UTR region of PD-L1 mRNA using miRANDA software and Targetscan software, while binding of miR-194 and miR-155 to the 3'UTR region of lncRNAs XIST and MALAT1 was predicted using Diana tools software. MALAT1 and XIST were predicted to target PD-L1 mRNA according to LncCeDB software algorithms.

Expression profile of PD-L1 in liver tissues

The expression profile of PD-L1 was assessed in HCC patients, and adjacent cirrhotic biopsies in a subset of patients together with 10 donor healthy controls, using qRT-PCR. PD-L1 was significantly elevated in both HCC biopsies (*P* = 0.0065) and cirrhotic biopsies (*P* = 0.0251) in comparison to healthy controls (Figure 1).

Expression profile of lncRNAs; XIST and MALAT-1 in HCC tissues

The expression profile of the endogenous lncRNAs XIST and MALAT-1 was examined in HCC patients and adjacent cirrhotic biopsies in a subset of patients together with 10 healthy donors using qRT-PCR. HCC patients showed a significant upregulation of XIST expression (*P* = 0.048) compared to healthy controls. MALAT-1 expression in HCC patients was barely detected (*P* = 0.043) and a significant upregulation was found in the cirrhotic tissues (*P* = 0.0136) (Figure 2).

Manipulation of endogenous miR-194-5p and miR-155-5p expression in Huh-7 cells.

Transfection efficiency of miR-194-5p and miR-155-5p oligonucleotides: In order to manipulate the expression of miR-194-5p and miR-155-5p in Huh-7 cells, the cells were transfected with each of the respective miRNA mimics and antagomirs, respectively.

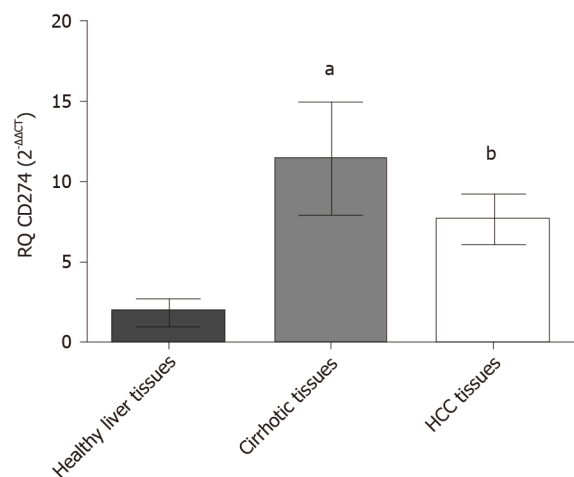


Figure 1 Relative expression level of programmed death ligand 1 in liver tissues. Endogenous programmed death ligand 1 expression profile was analyzed in hepatocellular carcinoma patients, cirrhotic and healthy controls using quantified real-time polymerase chain reaction and normalized to B2M as an internal control (housekeeping gene). Screening of programmed death ligand 1 showed that it was enhanced in cirrhotic biopsies (^a $P < 0.05$) and hepatocellular carcinoma biopsies (^b $P < 0.01$) compared to healthy controls. HCC: Hepatocellular carcinoma.

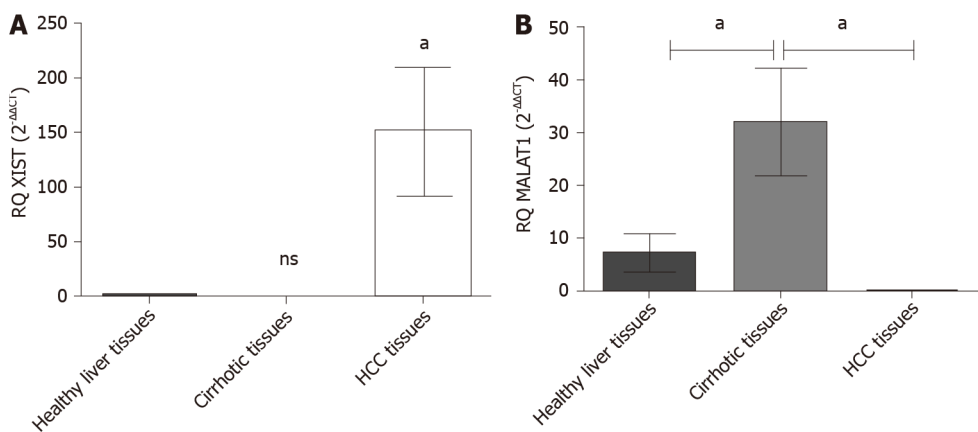


Figure 2 Expression profile of lnc-ribonucleic acid X-inactive specific transcript and MALAT-1 in hepatocellular carcinoma tissues. Endogenous X-inactive specific transcript and MALAT-1 lnc-ribonucleic acids expression profile was analyzed in hepatocellular carcinoma (HCC) patients and healthy controls using quantified real-time polymerase chain reaction and normalized to B2M as an endogenous control. A: X-inactive specific transcript lnc-ribonucleic acid showed a significant upregulation in HCC biopsies ($P = 0.048$); and B: MALAT-1 was significantly down regulated in HCC biopsies ($P = 0.043$); however, it showed elevated expression in cirrhotic biopsies ($P = 0.0136$). ^a $P < 0.05$. HCC: Hepatocellular carcinoma.

Efficient transfection was assessed 48 h post-transfection using qRT-PCR, and both miR-194-5p (Figure 3A) and miR-155-5p (Figure 3B) were markedly increased in mimicked cells compared to mock cells, ($P = 0.0026$) and ($P < 0.0001$), respectively.

Impact of miR-194-5p and miR-155-5p on PD-L1 transcript expression in Huh-7 cells: Mimicking of both miRNAs miR-155 and miR-194 in Huh-7 cells showed an up-regulation of PD-L1 expression ($P = 0.0219$) ($P = 0.0209$), respectively, compared to the mock untransfected cells (Figure 4). However, antagonizing both miRNAs resulted in a significant downregulation of PD-L1 transcript expression compared to mock untransfected cells.

Impact of miR-155-5p and miR-194-5p on lncRNAs XIST and MALAT-1 expression in Huh7 cells: Following ectopic expression manipulation of each of the respective miRNAs in Huh-7 cells, the lncRNAs XIST and MALAT-1 expression profiles were assessed using qRT-PCR and normalized to B2M an endogenous housekeeping gene. (A) Mimicking of miR-194-5p and miR-155-5p resulted in an upregulated expression profile of XIST compared to the mock untransfected cells, ($P = 0.0026$, $P = 0.0477$), respectively, as shown in Figure 5A; (B) Meanwhile, as shown in Figure 5B, mimicking of miR-194-5p and miR-155-5p had a paradoxical impact on the MALAT-1 expression profile. Mimicking of miR-194-5p induced the expression of MALAT-1 ($P = 0.0135$)

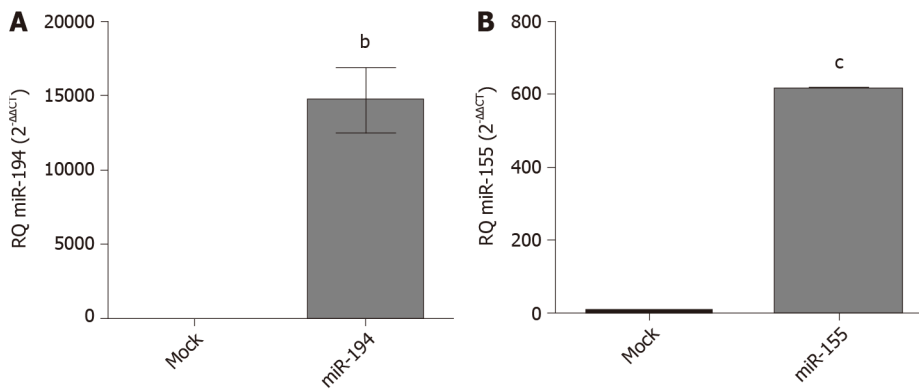


Figure 3 Transfection efficiency in Huh-7 cells with miR194-5p and miR-155-5p oligonucleotides. Using quantified real-time-polymerase chain reaction, the transfection efficiency was determined in both mimicked and mock cells 48 h post-transfection for each of the respective mi-ribonucleic acids. MiR-194-5p and miR-155-5p were normalized to RNU6B as an endogenous control. A: Mimicking of miR-194-5p; and B: miR-155-5p resulted in an increase in each of the respective miRNAs, ($P = 0.0026$) and ($P < 0.0001$), respectively. The expression levels were compared with the unpaired Student's *t*-test. ^b $P < 0.01$ and ^c $P < 0.001$.

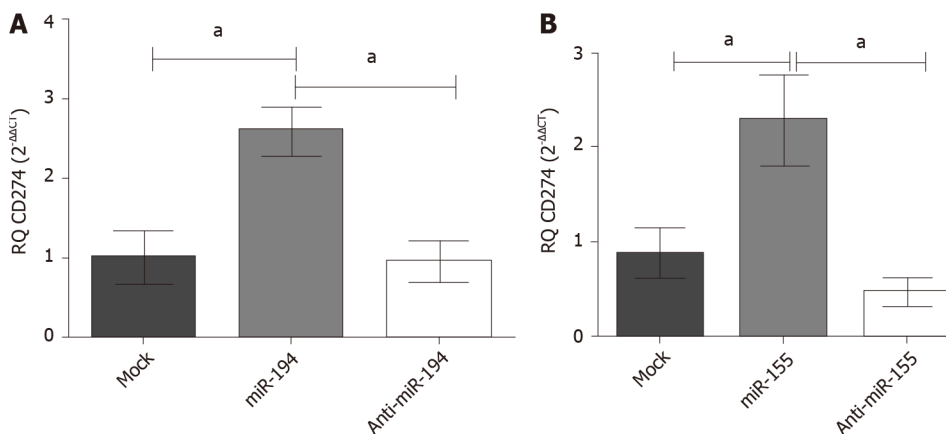


Figure 4 Impact of miR-194-5p and miR-155-5p on programmed death ligand 1 transcript expression in Huh-7 cells. Following ectopic expression manipulation of (A) miR-194-5p and (B) miR-155-5p in Huh-7 cells, programmed death ligand 1 (PD-L1) transcript expression was assessed using quantified real-time polymerase chain reaction and normalized to B2M as an endogenous control. Mimicking of each of the respective miRNAs resulted in significant upregulation of PD-L1 compared to mock untransfected cells ($P = 0.0219$) and ($P = 0.0209$), respectively. On the contrary, PD-L1 transcript expression was significantly downregulated by antagonizing each of the miRNAs in comparison with the mock untransfected cells. ^a $P < 0.05$.

compared to mock untransfected cells. On the other hand, mimicking of miR-155-5p induced the downregulation of MALAT-1 expression compared to mock untransfected cells ($P = 0.0053$).

Impact of knocking down the lncRNAs MALAT-1, XIST and TSIX on PD-L1 expression in Huh-7 cells

Knockdown of MALAT-1 significantly down regulated PD-L1 expression ($P = 0.001$) compared to mock cells. On the other hand, transfection with siRNAs of TSIX induced the upregulation of PD-L1 expression ($P = 0.0358$) compared to mock cells. Knockdown of XIST resulted in an insignificant change in the PD-L1 expression profile compared to untransfected mock cells (Figure 6).

Net impact of combined ectopic expression of miR-194-5p and miR-155-5p together with siRNAs of lncRNAs XIST, TSIX and MALAT-1 on PD-L1 expression profile.

The expression profile of PD-L1 transcript was studied following co-transfection of Huh-7 cells with different combinations of each miRNA; miR-194-5p and miR-155-5p, respectively, with each of the siRNAs of lncRNAs; MALAT-1, XIST and TSIX. Values were normalized to the endogenous housekeeping gene B2M and compared to mock untransfected cells. Following transfection of miR-194-5p with siRNA of MALAT-1, PD-L1 expression was significantly induced ($P = 0.0074$). However, following knockdown of XIST, miR-194-5p did not have a significant impact on PD-L1

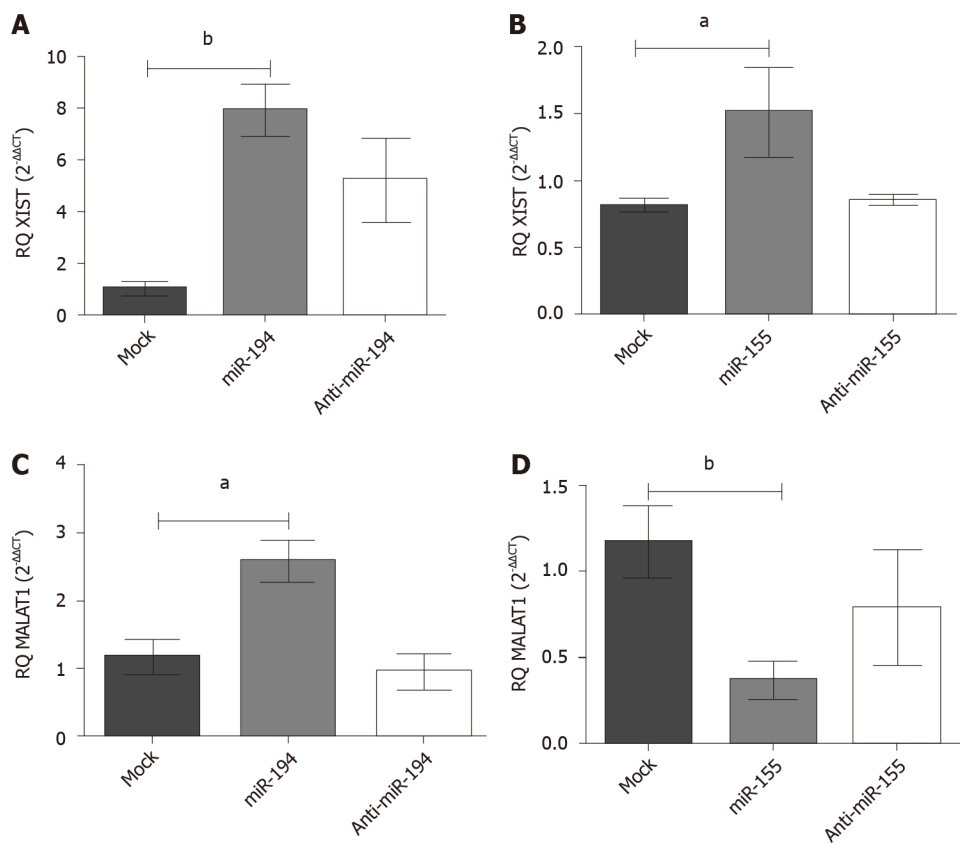


Figure 5 Impact of miR-155-5p and miR-194-5p on lnc-ribonucleic acids X-inactive specific transcript and MALAT-1 expression in Huh-7 cells. Expression levels of lnc-ribonucleic acids X-inactive specific transcript and MALAT-1 were assessed following transfection of miR-194-5p and miR-155-5p oligomirs using quantified real-time polymerase chain reaction and normalized to the endogenous B2M as a housekeeping gene. A: Ectopic expression of both miR-155 and miR-194 resulted in significant upregulation of X-inactive specific transcript expression, ($P = 0.0477$) and ($P = 0.0026$) respectively, compared to mock cells; and B: However, a paradoxical effect of mimicking miR-194-5p and miR-155-5p on MALAT-1 expression profile was observed, as miR-194-5p stimulated the upregulation of MALAT-1 expression ($P = 0.0135$), whereas mimicking miR-155-5p induced downregulation of MALAT-1 expression in comparison to mock cells ($P = 0.0053$). ^a $P < 0.05$ and ^b $P < 0.01$.

expression compared to mock cells. However, co-transfection of miR-194-5p with siRNA TSIX, did have a positive impact on the PD-L1 expression profile compared to mock cells ($P = 0.0067$). Co-transfection of miR-155-5p siRNA MALAT-1 showed a significant upregulation of the PD-L1 transcript expression ($P = 0.0060$). However, miR-155-5P was unable to elevate PD-L1 expression following knockdown of XIST as there was no significant change in PD-L1 expression compared to mock cells. Knockdown of TSIX and co-transfection with miR-155-5P significantly induced the expression of PD-L1 ($P = 0.0188$) (Figure 7).

Impact of oleuropein on the study key players; PD-L1 transcript, miR-194-5p and miR-155-5p and lncRNAs MALAT-1 and XIST

Treatment of Huh-7 cells with pure isolated oleuropein showed surprising results (Figure 8). Oleuropein treatment significantly downregulated the expression of PD-L1 ($P = 0.0011$), XIST ($P = 0.0020$) and miR-155 ($P = 0.0001$); however, MALAT-1 expression profile was not affected following oleuropein treatment. The miR-194-5p expression pattern was upregulated following oleuropein treatment ($P = 0.0022$).

DISCUSSION

The high expression pattern of immune checkpoints is a major cause of inefficient anti-tumor immunity. In this framework, immune checkpoint blockade has been revitalized to unleash the potential of anti-tumor immunity^[38]. Nevertheless, immunotherapeutic approaches have modest responses in HCC. Thus, combinatorial therapeutic strategies including epigenetic modulation through ncRNAs and immunomodulation techniques are implemented to circumvent the limitation of

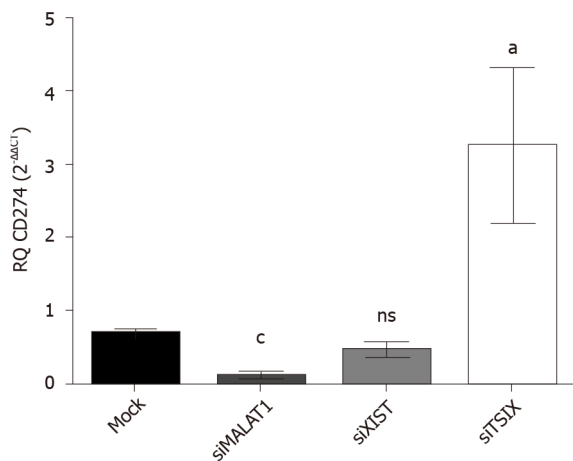


Figure 6 Impact of knockdown of long non-coding ribonucleic acids MALAT-1, X-inactive specific transcript and the negative regulator of X-inactive specific transcript on programmed death ligand 1 expression in Huh-7 cells. Using quantified real-time polymerase chain reaction, the expression profile of programmed death ligand 1 (PD-L1) transcript was determined following transfection of Huh-7 cells with each of the siRNAs for lncRNAs MALAT-1, X-inactive specific transcript and the negative regulator of X-inactive specific transcript. Values were normalized to an endogenous housekeeping gene B2M. PD-L1 expression was induced following knockdown of MALAT-1 ($P = 0.0010$) compared to mock cells. On the contrary, following down regulation of the negative regulator of X-inactive specific transcript, PD-L1 transcript expression was significantly induced ($P = 0.0358$) compared to mock cells. PD-L1 expression level was totally unaffected by knocking down MALAT-1 in comparison with mock cells. TSIX: The negative regulator of X-inactive specific transcript.

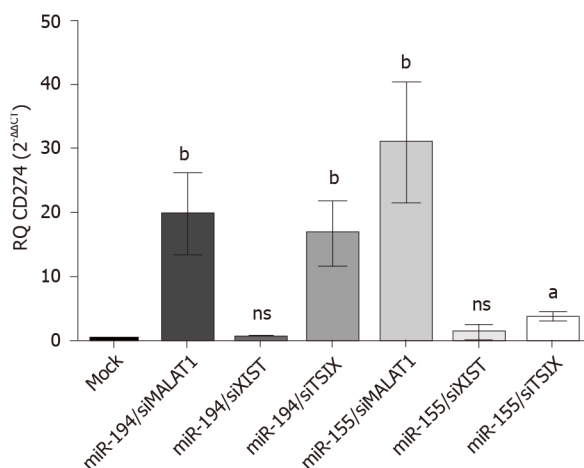


Figure 7 Net impact of ectopic miR-194-5p and miR-155-5p expression on programmed death ligand 1 expression in the presence of siRNA of lnc-ribonucleic acids X-inactive specific transcript, the negative regulator of X-inactive specific transcript and MALAT-1. The impact of both tumour suppressor miR-194-5p and oncogenic miR-155-5p on programmed death ligand 1 (PD-L1) expression in Huh7 cells in the presence of long non-coding ribonucleic acid X-inactive specific transcript (XIST), the negative regulator of X-inactive specific transcript and MALAT-1 siRNA was similar. Both miRNAs induced upregulation of PD-L1 expression when each was accompanied by MALAT-1 siRNA, while knockdown of the long non-coding ribonucleic acid the negative regulator of X-inactive specific transcript, i.e. upregulation of XIST expression, in the presence of miR-155-5p and miR-194-5p mimics down regulated PD-L1 expression. Ectopic expression of each of the miRNAs with knockdown of XIST had no net impact on PD-L1 expression. ^a $P < 0.05$ and ^b $P < 0.01$. TSIX: The negative regulator of X-inactive specific transcript.

immunotherapeutic techniques^[39]. Recently, a novel interaction circuit has been demonstrated in the competing endogenous RNA (ceRNAs) network, composed of three RNAs “lncRNA-miRNA-mRNA”. Here, we showed that PD-L1 in HCC is a member of a ceRNA network orchestrated by miR-155, miR-194 and lncRNA XIST.

Based on in-silico analysis, the oncogenic miR-155-5p and tumour suppressor miR-194-5p were predicted to target PD-L1 mRNA. It has been postulated that miR-155 promotes tumorigenic properties in HCC-derived cell lines and hence is an oncogenic miRNA in HCC pathogenesis^[40-42]. On the other hand, miR-194 has tumour suppressor activity in HCC as it was downregulated in HCC biopsies^[43-45]. Interestingly, a paradoxical function of the tumour suppressor miR-194-5p in HCC was revealed in this study, and was able to elevate the abundance of the oncogenic mediator, PD-L1. Similarly, another study demonstrated the contradictory role of the oncomiR miR-125b in hematological malignancies, in which its oncogenic activity could be overcome in

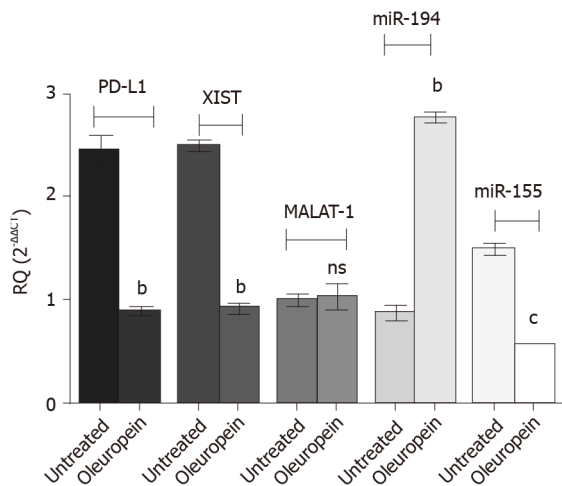


Figure 8 Impact of oleuropein on the expression profile of the study key players. The expression pattern of programmed death ligand 1 (PD-L1), micro ribonucleic acids 194-5p and 155-5p as well as long non-coding ribonucleic acids X-inactive specific transcript (XIST) and MALAT-1 were assessed following oleuropein treatment. Values for micro ribonucleic acids were normalized to RNU6B while that for PD-L1, XIST and MALAT-1 were normalized to B2M. Enhanced expression of miR-194-5p was observed following oleuropein treatment ($P = 0.0022$). However, PD-L1, XIST and miR-155-5p expression pattern were down regulated, ($P = 0.0011$), ($P = 0.0020$) and ($P = 0.0001$), respectively. Oleuropein did not show any significant impact on MALAT-1 expression profile. ^b $P < 0.01$ and ^c $P < 0.001$.

some instances in chronic lymphocytic leukemia to act as a tumour suppressor^[46].

Inspired by the ceRNA regulatory network, we investigated the impact of the key miRNAs players on the proposed lncRNAs. Bioinformatics analysis was adopted to predict the potential lncRNAs targeted by miR-194-5p and miR-155-5p. Based on the literature, two lncRNAs were selected, XIST and MALAT-1. LncRNA XIST is reported to be an oncogenic RNA as it is associated with worsening of survival in HCC patients, in which its oncogenic activity is mediated by AKt signaling pathway activation through the miR-139-5p/PDK1 axis^[47]. Nevertheless, overexpression of miR-194-5p and miR-155-5p induced an elevation in XIST. This finding also confirms the potential paradoxical role of miR-194-5p in HCC pathogenesis.

Several studies have shown upregulation of MALAT-1 in HCC biopsies^[48,49]. However, one study reported that following MALAT-1 knockdown in a hepatoma cell line, no variations in the proliferation pattern, cell cycle progression or nuclear architecture were observed^[50]. Surprisingly, overexpression of miR-194-5p induced the elevation of MALAT-1. In contrast, induced expression of miR-155-5p resulted in downregulation of MALAT-1. Taken together, these findings demonstrate the paradoxical functions of miRNAs in tumours, in which miR-194-5p expression induction elevated the expression of oncogenic members in the Huh-7 cell line. A plausible explanation for this anomaly is the fact that a single miRNA can target tens to hundreds of mRNAs, some of which are tumour suppressors and others are oncogenes. According to the balance in expression of the targeted mRNAs, a net effect of oncogenic or tumour suppressor activity can emerge^[46].

Our study showed that knockdown of MALAT-1 using siMALAT-1 resulted in downregulation of PD-L1 transcript. On the other hand, following knockdown of XIST negative regulator, TSIX, PD-L1 transcript was significantly elevated. These findings are considered to be helpful in clarifying the interesting role of tumour-suppressor miR-194-5p in elevating PD-L1, an activity that could be mediated through XIST and MALAT-1. However, the role of MALAT-1 in PD-L1 transcript elevation in HCC is still questionable, as despite the downregulation of MALAT-1 upon miR-155-5p overexpression, PD-L1 transcript was found to be highly abundant.

In order to have a full understanding of the ceRNA network involved in PD-L1 transcript level modulation in HCC, the combined effect of the respective miRNAs and lncRNAs on PD-L1 transcript abundance was studied. MiR-194-5p elevated PD-L1 transcript abundance even in the absence of MALAT-1. However, when XIST was knocked down, miR-194-5p was unable solely to affect PD-L1 abundance level. Nevertheless, upon XIST upregulation together with mimicking of miR-194-5p, PD-L1 transcript level was restored. These findings provide solid evidence of the pivotal role of XIST in increased PD-L1 transcript abundance. Surprisingly, similar findings were observed following co-transfection of miR-155-5p mimics with each of the siRNAs of the respective lncRNAs, comparable to their co-transfection with miR-194-5p. These

findings provide extra proof of the insignificant role of MALAT-1 in the PD-L1 expression pattern in comparison with XIST and both respective miRNAs.

The *in-vitro* results of our study also demonstrated the dual activity of miR-194-5p. Based on the literature, miR-194-5p has tumour suppressor activity in HCC by exerting a negative impact on cell viability and proliferation^[43]. However, our results indicated that overexpression of miR-194-5p increased the abundance of the two oncogenic HCC members PD-L1 and XIST similar to the impact of oncogenic miR-155-5p. Hence, our next aim was to determine the results *ex-vivo* by screening HCC biopsies for PD-L1, XIST and MALAT-1 expression. An elevated expression of XIST in HCC biopsies was noted which was in accordance with several other studies that have reported the oncogenic role of XIST in HCC^[47,51]. Also, PD-L1 was found to be significantly overexpressed in HCC biopsies compared to normal donor biopsies. This result is similar to that in other studies which reported the elevated expression of PD-L1 in HCC and its mechanistic role in immune evasion^[52]. To our surprise, MALAT-1 was barely detected in HCC biopsies, in contrast to other studies that have reported the oncogenic role of MALAT-1 in HCC^[53]. The interesting finding of downregulated MALAT-1 in HCC biopsies is in accordance with the *in-vitro* finding of the insignificant role of MALAT-1 in PD-L1 expression in HCC cells.

This study highlights the potential therapeutic targets in HCC including the members of the aforementioned upstream regulatory pathways of PD-L1. Nevertheless, the clinical application of ncRNAs as therapeutics is still limited and understudied. Thus, a trend towards using nutraceuticals in cancer therapy has developed due to the feasibility of their clinical application^[54]. Phytochemicals did not only demonstrate epigenetic immunomodulation by targeting lncRNAs and miRNAs, but have also revealed their role in immune checkpoint modulation^[55].

Due to the favorable role of polyphenolic nutraceuticals in epigenetic modulation, the nutraceutical oleuropein was selected for this study in order to determine its impact on the study key players, based on its aforementioned anti-inflammatory and immunomodulatory effects^[33].

At 80 $\mu\text{mol/L}$ ^[37], oleuropein significantly reduced the abundance of PD-L1 in Huh-7 cells. When the abundance of potential upstream regulatory ncRNAs was measured, it was found that XIST expression was significantly down regulated. However, oleuropein did not have a significant impact on MALAT-1 expression. Measurement of the impact of oleuropein on miR-194-5p and miR-155-5p revealed that miR-194-5p expression was markedly upregulated. In contrast, miR-155-5p was significantly downregulated. This finding is in accordance with another study that reported the negative impact of oleuropein on miR-155 in a breast cancer cell line, which manifested anti-proliferative, apoptotic, and anti-metastatic effects in the breast cancer cell line^[56]. Finally, the potential of oleuropein as a therapeutic agent in HCC requires further investigation in order to support these promising findings.

Some limitations must be acknowledged in this study. First, the limited number of patients and subsequently, number of tissue biopsies; however, statistically significant results were obtained. Further studies using a larger number of tissue biopsies should be performed to validate the proposed pathway in a larger cohort of patients. Second, a further robust study design is necessary to analyze the study key players in peripheral blood samples of advanced HCC patients and to investigate the impact of mimicking the miRNAs, miR-155-5p and miR-194-5p, on PD-L1 protein levels in HCC cell lines.

CONCLUSION

In conclusion, this study reported the controversial role of miR-194-5p in HCC as it has the paradoxical function of being both a tumour suppressor and oncogenic activity in HCC, and had the same impact on upregulation of PD-L1 and XIST. Transfection of each of the siRNAs of the respective lncRNAs, showed that XIST and MALAT-1 can have a positive impact on PD-L1 transcript abundance. However, following a series of co-transfections, it was demonstrated that XIST is a cornerstone in PD-L1 expression, while MALAT-1 has no significant impact compared to the respective miRNAs and XIST. Thus, a novel shared upstream regulatory signaling pathway for PD-1/PD-L1 immune checkpoint paradoxically acting on miR-194-5p and miR-155-5p occurs, through XIST expression modulation (Figure 9). Thus, the key regulators of the ceRNA circuit could be employed as therapeutic targets in HCC.

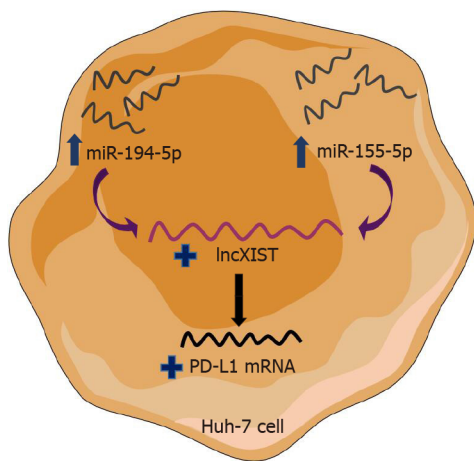


Figure 9 Schematic representation of the shared pathway between miR-155-5p and miR-194-5p. This article highlights the novel shared upstream regulatory signaling pathways for programmed cell-death protein 1/programmed death ligand 1 immune checkpoint between paradoxically acting miR-194-5p and miR-155-5p, through lnc X-inactive specific transcript expression modulation. Mimicking of tumor suppressor miR-194-5p as well as oncomiR-155-5p in the Huh-7 cell line showed the same upregulation pattern of X-inactive specific transcript. X-inactive specific transcript was proposed then to be an intermediate player whose upregulation derived the increase in programmed death ligand 1 transcript abundance.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) develops in an inflammatory milieu containing tumor infiltrating lymphocytes, thus boosting tumor immunogenicity and provides an aspect for developing immunotherapies against HCC. However, immunotherapies have a modest response in HCC, accordingly combinatorial therapies with epigenetic immunomodulation may be a promising modality. Growing scientific evidence has suggested a modulatory role for miRNAs and long non-coding ribonucleic acids (lncRNAs) on programmed cell-death protein 1 (PD-1)/programmed death ligand 1 (PD-L1) immune checkpoint in HCC.

Research motivation

HCC is considered a therapy-resistant disease, and is frequently diagnosed at an advanced stage. Thus, the development of a novel therapeutic modality is essential. It is noteworthy that immune checkpoint blockade therapy in HCC is gaining attention. Additionally, given the wide range of non-coding RNAs (ncRNAs) that orchestrate PD-1/PD-L1 immune checkpoint, we investigated how selected ncRNAs regulate PD-1/PD-L1 immune checkpoint. Hence, the therapeutic potential of combining epigenetic immunomodulation through ncRNAs with immune checkpoint blockade was studied.

Research objectives

This study aimed at exploring potential upstream regulatory ncRNAs of immune checkpoint PD-1/PD-L1. Hence, the potential of combining immune checkpoint blockade with epigenetic immunomodulation was investigated.

Research methods

Based on bioinformatics software and the literature, ncRNAs including miR-155-5p and miR-194-5p as well as lncRNAs X-inactive specific transcript (XIST) and MALAT-1 were selected. 23 HCC tissue biopsies and 10 healthy donor tissue biopsies were used to screen the expression of PD-L1 as well as lncRNAs XIST and MALAT-1. To study the interaction between miR-155-5p, miR-194-5p, lncRNAs XIST and MALAT-1, as well as PD-L1 mRNA, a series of transfections and co-transfections of the Huh-7 cell line was carried out. Quantified real-time polymerase chain reaction was then utilized to study the abundance of selected ncRNAs as well as PD-L1 transcripts in Huh-7 cells in the transfections experiments.

Research results

Based on bioinformatics software and the literature, we hypothesized that a potential upstream regulatory pathway to immune checkpoint PD-L1 is present in HCC,

composed of both miRNAs, tumor suppressor miR-194-5p and oncomiR-155-5p, as well as both lncRNAs XIST and MALAT-1. Following the screening of 23 HCC biopsies, PD-L1 and XIST were found to be significantly upregulated compared to healthy controls; however, MALAT-1 was barely detected. Induced expression of miR-194-5p and miR-155-5p in the Huh-7 cell line showed the same pattern of upregulation of both PD-L1 transcript and XIST. However, ectopic expression of the respective miRNAs had a paradoxical impact on MALAT-1 abundance, *i.e.* miR-194-5p induced the upregulation of MALAT-1 while miR-155-5p downregulated the abundance of MALAT-1. Knockdown of XIST had no impact on PD-L1 expression; however, following knockdown of the negative regulator of X-inactive specific transcript (TSIX), PD-L1 expression was elevated, and MALAT-1 activity was abolished. Upon co-transfection of miR-194-5p with siMALAT-1, PD-L1 expression was elevated. On the other hand, co-transfection of miR-194-5p with siXIST did not have an impact on PD-L1 expression. Following co-transfection of miR-194 with siTSIX, PD-L1 expression was upregulated. Interestingly, the same PD-L1 expression pattern was revealed following the oncomiR-155-5p co-transfection series.

Research conclusions

In conclusion, this study reported the controversial role of miR-194-5p in HCC and despite its paradoxical function of a tumour suppressor and having oncogenic activity in HCC, both had the same impact on upregulation of XIST. LncXIST is thought to be an intermediate player whose upregulation increased PD-L1 transcript abundance.

Research perspectives

Although further investigations are needed, this study proposes a novel competing endogenous RNA circuit made up of both miR-155-5p and miR-194-5p as well as lncXIST and PD-L1 mRNA. This circuit could be regarded as a potential therapeutic target in HCC.

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Case Control Study

Validation of genetic variants associated with metabolic dysfunction-associated fatty liver disease in an ethnic Chinese population

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Abstract

BACKGROUND

Genetic factors play an important role in the pathogenesis and development of metabolic dysfunction-associated fatty liver disease (MAFLD).

AIM

To study the association of single nucleotide polymorphisms (SNPs), previously identified in Western populations, with the risk of MAFLD in a Singapore Chinese population and their interactions with environmental and medical risk factors.

METHODS

A retrospective case-control study was conducted with 72 MAFLD cases and 72 controls with no hepatic steatosis on computed tomography, magnetic resonance imaging, or controlled attenuation parameter score. Subjects were recruited from two tertiary hospitals. Genetic alleles such as *NCAN*, *GCKR*, *LYPLAL1*, *PNPLA3*, *PPP1R3B*, *FDFT1*, *COL13A1*, *EFCAB4B*, *PZP*, and *TM6SF2* were genotyped using the TaqMan® Predesigned SNP Genotyping Assay.

Institutional review board

statement: The study was approved by the Institutional Review Board.

Informed consent statement: All patients gave informed consent.

Conflict-of-interest statement: The authors declare that there is no conflict of interest.

Data sharing statement: No additional data 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.

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RESULTS

Weight and body mass index (BMI) were 1.2-times higher in patients (70.6 kg, 95% confidence interval [CI]: 57.1-84.1 *vs* 60.8 kg, 95%CI: 48.5-73.1, $P < 0.001$ and 26.9 kg, 95%CI: 23-40.8 *vs* 23.3 kg, 95%CI: 19-27.6, $P < 0.001$ respectively). The prevalence of diabetes mellitus in patients was 40.3% and 20.8% in controls ($P = 0.011$). Patients had higher mean triglycerides than controls ($P < 0.001$). *PNPLA3* GG was more likely to be associated with MAFLD (43.4% CC *vs* 69.7% GG, $P = 0.017$, and 44.8% CG *vs* 69.7% GG, $P = 0.022$). In multivariable analysis, hypertriglyceridemia (odds ratio [OR]: 2.04 95%CI: 1.3-3.1, $P = 0.001$), BMI (OR: 1.2 95%CI: 1.1-1.4, $P < 0.001$) and *PNPLA3* GG (OR: 3.4 95%CI: 1.3-9.2, $P = 0.014$) were associated with MAFLD (area under the receiver operating characteristic curve of 0.823).

CONCLUSION

Among the Chinese population of Singapore, *PNPLA3* homozygous GG allele is a strong predictor of MAFLD, whereas *LYPLAL1*, *GCKR*, *FDFT1*, *COL13A1*, *PZP*, and *TM6SF2* are not significantly associated. Hypertriglyceridemia, high BMI, and *PNPLA3* GG are independent predictors of MAFLD.

Key Words: Single nucleotide polymorphism; *PNPLA3*; Genotyping; Metabolic dysfunction-associated fatty liver disease; Non-alcoholic steatohepatitis; Hypertriglyceridemia; Body mass index; Waist-hip ratio; Screening; Hepatic steatosis

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Core Tip: A number of genetic variations (known as single nucleotide polymorphism, SNPs) are reportedly associated with metabolic dysfunction-associated fatty liver disease (MAFLD), mostly by studies from Europe and America. This study examined 10 of the most important SNPs in a Chinese population in Singapore, and found that 1 such variation, the *PNPLA3* GG variation, is strongly linked to MAFLD, whereas the rest are not significantly associated. *PNPLA3*, together with high triglyceride and elevated body mass index, are found to be independent, strong predictors of MAFLD.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) is increasingly recognized as a leading cause of liver morbidity and mortality, and has emerged as the most common chronic liver disease^[1]. The increasing prevalence of MAFLD is associated with the epidemic surge in obesity and metabolic syndrome^[2]. The estimated prevalence of MAFLD in Asia is about 27.4%^[1]. MAFLD is a clinicopathological spectrum that consists of hepatic steatosis and non-alcoholic steatohepatitis (NASH), with up to 20% of NASH patients progressing to cirrhosis and end-stage liver complications^[3-5]. This heralds an expectant future epidemic of hepatocellular carcinoma (HCC) caused by NASH, potentially overshadowing the role of viral hepatitis in the development of HCC^[6-8]. Reflecting this trend, NASH is the most rapidly growing indication for liver transplantation in the United States, increasing 4-fold from 2002 to 2012, and is poised to become the leading indication^[9].

The pathogenesis of MAFLD is multifactorial and complex, and both genetic and epigenetic factors appear to play vital roles in its development. These factors interact with environmental, dietary, and metabolic risk factors, which all contribute to the development of MAFLD and the risk of disease progression. Different genes encode proteins involved in the regulation of lipid metabolism in the liver^[10]. Excess of hepatic

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triglycerides (TGs) associated with insulin resistance is a key mechanism in MAFLD pathophysiology. Adipose tissue insulin resistance is linked with lower circulating adipokines and this leads to increased lipolysis with resultant oxidative stress, lipotoxicity and apoptosis, thus inducing NASH^[11,12].

A multitude of studies have demonstrated a heritable component and familial clustering as important factors in MAFLD. The majority of these studies have been published in the western population. First-degree relatives of individuals with MAFLD have conferred a higher risk of the disease compared to the general population^[13]. Twin studies have shown that genetic factors contribute up to 60% of variability in alanine aminotransferase (ALT) levels and hepatic fat content in subjects^[14]. Over the years, multiple genetic single nucleotide polymorphisms (SNPs) associated with MAFLD have been studied. *PNPLA3* is the major gene associated with hepatic TG content, increasing susceptibility to more aggressive forms of MAFLD and HCC^[15-18]. Specifically, the variant allele rs738409 C>G or I148M of *PNPLA3* is deemed an important genetic factor for a predisposition towards progressive MAFLD and increases hepatic TG accumulation^[15]. The genetic association with *PNPLA3* is also evident in several Asian studies and is estimated to be present in 13%-19% of the general population^[19-21]. In a Chinese study, *PNPLA3* had a stronger correlation with hepatic steatosis in non-obese individuals without metabolic syndrome^[20]. Other SNPs associated with progressive MAFLD include *TM6SF2*, *LYPLAL1*, *NCAN*, *APOB*, *MBOAT7*, *LPIN1*, *GCKR*, *ENPP1*^[22]. The effect size and allele frequency of these genetic variants have shown much diversity across different ethnicities leading to racial and ethnic differences in MAFLD prevalence^[23]. To date, these genetic variants have not been evaluated in our local population.

In this study, we evaluated the association of SNPs, previously identified in Western populations, and environmental and medical risk factors with the risk of MAFLD in a Singapore Chinese population.

MATERIALS AND METHODS

The study was conducted using a retrospective case-control design recruiting subjects from the National University Hospital (NUH) and Khoo Teck Puat Hospital in Singapore. All participants gave informed consent and the study was approved by the Institutional Review Board.

Inclusion criteria

Fatty liver was identified based on computed tomography (CT) or magnetic resonance imaging (MRI) or histopathology of liver (> 20% steatosis) or controlled attenuation parameter (CAP) score. CAP is a non-invasive tool for the detection of hepatic steatosis but is limited by body mass index (BMI). CAP value of S2-3 ($\geq 34\%$ steatosis) is considered as positive hepatic steatosis and S0 (< 5% steatosis) as no steatosis. The enrolled subjects in this study were Singapore residents of Chinese ancestry. These subjects also do not have other chronic liver etiology. The control group will either have a CT or MRI scan or CAP score showing the absence of hepatic steatosis (performed for non-liver disease indications) within 1 year of enrolment into the study.

Exclusion criteria

Patients with secondary causes of steatosis including alcohol abuse, total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of drugs known to precipitate hepatic steatosis were excluded. In addition, patients with any of the following diseases such as autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, and primary sclerosing cholangitis were excluded from participation in this study.

Clinical data collection

Subjects underwent an assessment comprising anthropometric measurements and a questionnaire on health-related behaviors such as smoking and alcohol drinking habits. Smokers are defined as subjects who had smoked at least 100 cigarettes during their lifetime and are henceforth classified as smokers *vs* non-smokers. With regards to drinking history, the study was confined to men and women who drank less than 140 g or 70 g of alcohol per week respectively. Body measurements including weight and height were measured in a standardized fashion by a trained examiner. BMI was calculated as follows: Weight (kg) divided by height squared (m^2). Overweight and/or

obesity were defined as having a BMI > 23 kg/m² and > 28 kg/m² respectively. Venous blood samples were drawn for biochemical and genotyping analyses. Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), TG, fasting plasma glucose (FPG), and liver function tests were measured by standard clinical laboratory techniques in NUH Biochemistry Laboratory. Reduced HDL-C, hypertriglyceridemia, and raised FPG were diagnosed according to the International Diabetes Federation consensus worldwide definition of the metabolic syndrome.

SNP genotyping

Blood samples obtained from the subjects were centrifuged at 1500 rpm for 10 min. The buffy coat layer was separated and transferred into a 1.5-mL centrifuge tube. Genomic DNA was extracted from the concentrated lymphocytes of the buffy coat using the QIAamp DNA Mini Kit (Qiagen, Hilden, German). The related genetic alleles such as *NCAN*, *GCKR*, *LYPLAL1*, *PNPLA3*, *PPP1R3B*, *FDFT1*, *COL13A1*, *EFCAB4B*, *PZP*, and *TM6SF2* were genotyped using the TaqMan® Predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA, United States) on a step one real-time PCR instrument (Applied Biosystems). The total reaction volume for each well was 10 µL containing 5 µL universal mastermix (Applied Biosystems), 0.5 µL assay mix, 3.5 µL distilled water, and 1 mL genomic DNA. The plate was set up at 95 °C holding stage for 20 s, 45 cycles of 95 °C denaturation for 3 s and 60 °C annealing for 20 s and run on a fast reaction (40 min for each run). Negative controls were introduced for every run to ensure genotyping quality.

The pathologist, radiologist, and laboratory technologist, who performed the tests for hepatic steatosis and SNPs, were blinded to the patients' participation in this study. The laboratory team performing and interpreting the SNP assay was blinded to the identity and grouping of the patients and samples.

Statistical analysis

The data were analyzed using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, United States) and $P < 0.05$ was considered statistically significant. All values are presented as the mean \pm standard deviation (SD) for continuous data and as frequency and percent for categorical data. For normally distributed variables (age, BMI, waist circumference, ALT, HDL cholesterol, LDL cholesterol, TC, TG, and systolic blood pressure), two independent sample *t*-test was performed to compare between NASH and simple steatosis. Categorical variables were compared between cases and controls using Chi-square or Fisher's exact test, where appropriate. Multivariable logistic regression analysis was performed to investigate the association of risk factors and SNPs with MAFLD outcome. Area under the curve, positive predictive value and negative predictive value were assessed using receiver operating characteristic analysis.

RESULTS

In total, 150 patients were screened initially; however, 6 were lost to follow-up after recruitment and were removed from the analysis; 72 MAFLD subjects and 72 control subjects completed the study. Among the cases, the methods used to diagnose fatty liver disease were CT (54%); biopsy (24%); combination of ultrasound, laboratory results and clinical features (11%); MRI (10%); and CAP score (1%). Majority (99%) of controls were identified based on CT and MRI results. The mean age was 57 years and 61 years for the NAFLD and control group respectively. 59.7% of the patients in each group were male. 11.8% were smokers. None of the patients in both groups drank more than the 140 g (male) or 70 g (female) of alcohol per week.

The subjects who had MAFLD were more likely to have BMI higher than 24.9 kg/m² (27, 95% confidence interval [CI]: 23-31 *vs* 23, 95%CI: 19-28, $P < 0.001$). While waist-hip ratio was similar between the two groups (0.94, 95%CI: 0.9-1.0 *vs* 0.95, 95%CI: 0.8-1.1), lipid profiles were significantly higher in the MAFLD group than in the control group. Mean TC in MAFLD group was 4.4 (95%CI: 2.9-5.8) while in control was 3.4 (95%CI: 1.2-5.7), $P = 0.0032$, and mean TG in MAFLD group was 1.7 (95%CI: 0.8-2.7) while in control was 1.0 (95%CI: 0.1-1.98), $P < 0.0012$, (Table 1). 36% of MAFLD patients were taking lipid-lowering agents while only 24% in the control group were. Similar proportion of each group had hypertension (35% in MAFLD group *vs* 31% in control group, $P = 0.594$) and were on anti-hypertensive medication. There were 40% of patients who had diabetes mellitus in MAFLD group compared to 21% in the

Table 1 Patients' demographics and clinical characteristics

| | NAFLD patient, <i>n</i> = 72 | Control, <i>n</i> = 72 | Total, <i>n</i> = 144 | <i>P</i> value |
|--|------------------------------|--------------------------------|-------------------------------|-----------------------|
| Clinical characteristics | | | | |
| Age | 57 (46-68) | 61 (48-74) | 59 (46-71) | 0.037 ² |
| Male, % | 43 (59.7%) | 43 (59.7%) | 86 (59.7%) | 1.0 ¹ |
| BMI, kg/m ² | 27 (23-31) | 23 (19-28) | 25 (21-30) | < 0.001 ² |
| Waist-hip ratio | 0.94 (0.9-1.0) | 0.95 (0.8-1.1) | 0.9 (0.8-1.0) | 0.587 ² |
| Smoker, % | 5 (6.9%) | 12 (16.7%) | 17 (11.8%) | 0.071 ¹ |
| DM, % | 29 (40.3%) | 15 (20.8%) | 44 (30.6%) | 0.011 ¹ |
| Hypertension, % | 25 (34.7) | 22 (30.6%) | 47 (32.6%) | 0.594 ¹ |
| On anti-diabetic treatment | 26 (36.1) | 14 (19.4) | 40 (27.8) | 0.026 ¹ |
| On lipid-lowering treatment | 26 (36.1) | 17 (23.6) | 43 (29.9) | 0.101 ¹ |
| Laboratory measures | | | | |
| Serum lipid levels and fasting glucose | | | | |
| Total cholesterol, mmol/L | 4.4 (2.9-5.8) | 3.4 (1.2-5.7) | 3.9 (1.9-5.8) | 0.0032 ² |
| LDL-C, mmol/L | 2.5 (1.5-3.5) | 2.1 (0.7-3.5) | 2.3 (1.1-3.6) | 0.0492 ² |
| HDL-C, mmol/L | 1.3 (0.6-1.9) | 1.0 (0.3-1.7) | 1.6 (0.5-1.8) | 0.0292 ² |
| TG, mmol/L | 1.7 (0.8-2.7) | 1.0 (0.1-1.98) | 1.4 (0.4-2.4) | < 0.0012 ² |
| FPG, mmol/L | 6.1 (5.6-6.6) | 5.9 (5.5-6.3) | 6.0 (5.7-6.3) | 0.4592 ² |
| Liver function test | | | | |
| Albumin, g/L | 40.9 (32.1-49.7) | 41.0 (34.6-47.3) | 40.9 (33.3-48.6) | 0.97422 |
| Bilirubin, mmol/L, Median | 13.1 (4.5-21.7), Median 11.0 | 14.7 (-15.6-44.97), Median 9.0 | 13.9 (-8.3-36.1), Median 10.0 | 0.6672 ² |
| AST, U/L | 37.1 (16.7-57.5) | 26.2 (7.4-44.9) | 31.7 (11.4-51.98) | 0.0012 ² |
| ALT, U/L | 46.2 (15.5-76.8) | 22.2 (8.2-36.1) | 34.3 (7.6-60.9) | < 0.0012 ² |
| ALP, U/L | 87.6 (36.2-138.9) | 77.4 (39.95-114.8) | 82.5 (37.4-127.6) | 0.1792 ² |
| INR | 0.9 (0.5-1.2) | 0.8 (0.2-1.3) | 0.8 (0.3-1.3) | 0.3732 ² |
| Platelet as 10 ⁹ /L | 223 (130-315) | 256 (162-350) | 240 (146-334) | 0.0402 ² |

Categorical variables were in number (%) and continuous variables in range (95% confidence interval).

¹Chi-squared test.

²Student's *t*-test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; DM: Diabetes mellitus; HDL-C: High-density lipoprotein cholesterol; FPG: Fasting plasma glucose; INR: International normalized ratio; LDL-C: Low-density lipoprotein cholesterol; NAFLD: Nonalcoholic fatty liver disease; TGs: Triglycerides.

control group (*P* = 0.011). However, there was no difference in fasting glucose between the two groups (6.1, 95%CI: 5.6-6.6 *vs* 5.9, 95%CI: 5.5-6.3) since most patients were treated with anti-diabetic medication. Both aspartate aminotransferase (AST) and ALT levels were observed to be higher in MAFLD patients than in the controls (Table 1).

A total of 9 SNPs were tested and *PNPLA3* G allele is more likely to be associated with MAFLD compared to C allele (GG *vs* CC OR: 3.0 [95%CI: 1.2-7.5] and GG *vs* CG OR: 2.83 [95%CI: 1.1-6.9]). Some homozygous alleles of *NCAN*, *PPP1R3B*, and *EFCAB4B* that were proven significantly associated with MAFLD in the west are found to be absent among the study population. For instance, *NCAN* homozygous TT allele, *PPP1R3B* homozygous GG allele and *EFCAB4B* homozygous TT allele were not found in any of the recruited MAFLD and non-MAFLD participants. Furthermore, there were some alleles in which each was present only among the minority (< 3%) of the study population, particularly for allele *PPP1R3B* AG, *EFCAB4B* CT, *PZP* CT, *LYPLAL1* TT and *TM6SF2* TT. Despite the high presentation among the MAFLD patient group, four SNPs were not significantly associated with the presence of MAFLD. Two of the insignificant SNPs could be due to small sample sizes as mentioned above in *EFCAB4B* CT *vs* CC (odds ratio [OR]: 3.09, 95%CI: 0.31-30.40) and

PPP1R3B AA *vs* AG (OR: 3.09, 95%CI: 0.31-30.4). Another two insignificant alleles were *COL13A1* AA *vs* GG (OR: 2.3, 95%CI: 0.4-13.1) and *PZP* CC *vs* TT (OR: 2.0, 95%CI: 0.9-4.4) (Figure 1).

We performed multivariable analysis of risk factors and SNPs, independent predictors of MAFLD were hypertriglyceridemia (OR: 2.3 95%CI: 1.3-4.1), BMI (OR: 1.2 95%CI: 1.1-1.4) and *PNPLA3* GG (OR: 4.1 95%CI: 1.3-12.9) (Table 2). This model has a cut-off value of 0.47 (based on Youden index) that can predict 73.8% of positive cases with an area under the receiver operating characteristic curve (AUROC) of 0.823 (95%CI: 0.73-0.91, $P < 0.001$) (Figure 2). Without the *PNPLA3*, the model performance in the prediction of MAFLD was lower with the AUROC of 0.789 (95%CI: 0.71-0.86, $P < 0.001$).

DISCUSSION

In this study, *PNPLA3* GG allele was the only SNP found to be associated with progressive MAFLD in the Singaporean Chinese population. The surprising finding is none of the other SNP variants, including *LYPLAL1*, *GCKR*, *FDFT1*, *COL13A1*, *PZP*, and *TM6SF2*, which were identified in the Western population, demonstrate a similar association. On further analysis, the *PNPLA3* GG variant, elevated BMI, and hypertriglyceridemia were independent predictors of MAFLD.

The *PNPLA3* I148M variant is associated with increased severity of MAFLD, with a higher susceptibility to NASH, fibrosis, and HCC^[24]. The I148M mutation confers double the risk for HCC for each variant allele^[17]. *PNPLA3* I148M predisposes to lower hepatic very-low-density lipoprotein (VLDL)^[25] and also lower levels of adiponectin^[26] which result in inflammation leading to the development of NASH^[27]. Furthermore, this association is independent of predisposition to hepatic steatosis, insulin resistance, dyslipidemia or obesity^[15]. A study of Italian and United Kingdom patients who carried the *PNPLA3* GG genotype had a 3.3-fold risk of MAFLD compared to those with the CC genotype. This data is in line with our results of the predisposition of homozygous GG allele to MAFLD. The GG phenotype was linked with higher LDL levels, fasting insulin levels, homeostasis model assessment of insulin resistance (HOMA-IR) score. However, there was no association with type 2 diabetes. These subjects also had significantly higher ALT levels in comparison with those who possessed the CC or CG genotypes^[28]. In Asians, there is a higher prevalence of the entity that is non-obese MAFLD defined as those with a BMI of less than 25 kg/m². There is some evidence indicating a greater influence of the *PNPLA3* variant in the non-obese MAFLD subjects. Wei *et al*^[29] reported a higher proportion of non-obese MAFLD patients with the *PNPLA3* variant (78.4%) compared with the obese MAFLD group (59.8%) in a population-based study from Hong Kong. But the mechanism linking the two is incompletely understood. On the contrary, these results were not replicated in our study.

Polymorphism in the *PNPLA3* gene may also play an important role in the management of MAFLD as there is data suggesting that lifestyle modification may be more beneficial in this group compared to other genotypes. The effect of degree of weight loss on intrahepatic TG content and liver enzyme levels was more pronounced in MAFLD patients with the homozygous GG allele than in subjects with the homozygous CC allele^[30,31]. Hence, lifestyle modification and weight loss can be strongly advocated in our patient cohort with a predominance of the GG genotype which may result in more optimal outcomes.

Variants in the *GCKR* gene (rs1260326 and rs780094) increase MAFLD susceptibility by inducing lipogenesis *via* activation of hepatic glucose uptake^[22,32]. In a study on Italian MAFLD patients, *GCKR* rs780094 C>T was associated with higher serum TG levels and severity of liver fibrosis^[33]. However, there was no observed association between the *GCKR* SNP and our study cohort. The *FDFT1* gene is involved in the regulation of cholesterol biosynthesis and the rs2645424 SNP has been shown to demonstrate a positive correlation with the MAFLD activity score (NAS)^[34]. Several genes with different SNPs including *COL13A1* and *EFCAB4B* were associated with lobular inflammation in NASH whereas *PZP* SNPs had an association with serum AST levels^[34]. A large GWAS study detected variants in *NCAN* (rs2228603) and *LYPLAL1* correlating with histologic lobular inflammation and fibrosis, but not in *PPP1R3B* which was associated with liver steatosis only^[22]. Another major genetic determinant of MAFLD is *TM6SF2* which is associated with reduced hepatic secretion of VLDL and increased risk of myocardial infarction. However, a study from Hong Kong showed a prevalence of only 0.4% of Chinese who had the variant which conferred a higher risk

Table 2 Multivariable analysis of the environmental factors and single nucleotide polymorphisms

| | Odds ratio | 95%CI | | P value |
|------------------------|------------|-------|-------|---------|
| | | Lower | Upper | |
| TG, mmol/L | 2.338 | 1.3 | 4.1 | 0.003 |
| BMI, kg/m ² | 1.243 | 1.1 | 1.4 | 0.003 |
| <i>PNPLA3</i> GG vs CC | 4.146 | 1.3 | 12.9 | 0.014 |

BMI: Body mass index; CI: Confidence interval; TGs: Triglycerides.

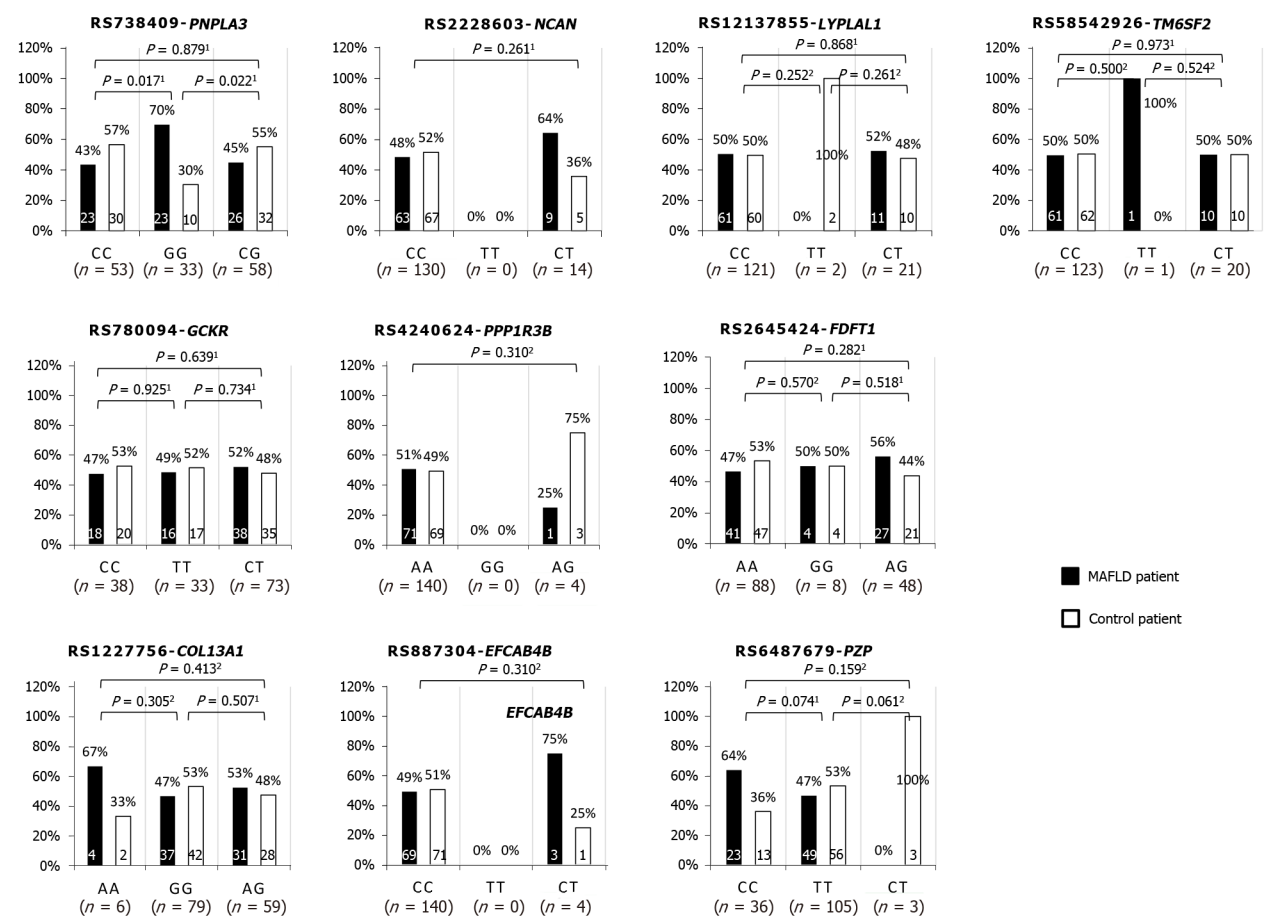


Figure 1 Single nucleotide polymorphisms in patients with or without metabolic dysfunction-associated fatty liver disease. The bar charts represent the percent of each allele comparing metabolic dysfunction-associated fatty liver disease (MAFLD) patients (blue) and controls (green). ¹Chi-squared test; ²Fisher's exact test.

of MAFLD, indicating a potentially limited role of this gene in Asian MAFLD^[35]. Overall, none of these genetic SNPs except for *PNPLA3* were related to MAFLD development in our study population.

This study had limitations. The general limitations of case-control design are well-documented. The sample size may not be large enough to detect the effects of some of the genetic polymorphisms tested, but the effect sizes will likely be too small for clinical application. Despite our best effort to match the control group with the MAFLD subjects, we were not able to completely balance all known risk factors and associated co-morbidities linked to MAFLD. Very few patients with high BMI in our population have completely normal findings for liver steatosis measurement, and also free from other features of metabolic syndrome. As a result, BMI and corresponding risk factors, such as diabetes mellitus and hyperlipidemia are more prevalent in the MAFLD group. The other major limitation is the lack of liver histology in the study population to have the gold standard diagnosis of hepatic steatosis and steatohepatitis. Surrogate markers, including CAP score (on FibroScan), CT, and MRI, are accepted. To

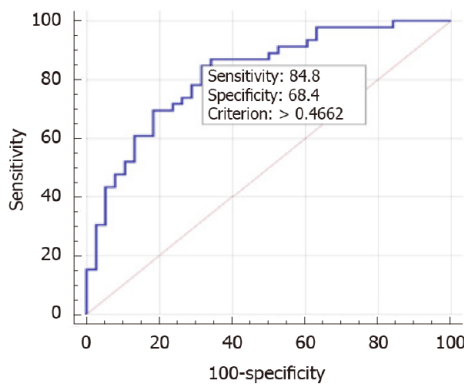


Figure 2 Area under curve of the regression model including *PNPLA3* GG. Area under curve by receiver operating characteristic is 0.823 (95% confidence interval: 0.73-0.91, $P < 0.001$). Regression equation $-7.01 + 0.849$ (triglycerides) $+ 0.218$ (body mass index) $+ 1.422$ (*PNPLA3* GG). Positive predictive value = 72.1%. Negative predictive value = 65.4%.

avoid diagnostic uncertainties, we used more stringent criteria for defining hepatic steatosis. For CAP score, we accepted only subjects with S2 and above into the MAFLD group and only S0 into the control group. For histopathology, we included only those patients with higher liver steatosis of 20% or more into the MAFLD group and $< 5\%$ into the control group.

CONCLUSION

In conclusion, we demonstrated that the genetic variant in *PNPLA3* is associated with an increased risk of MAFLD in the Singaporean Chinese population, but not with the other studied SNPs. While it is possible that with a much larger study population, some of these MAFLD-related SNPs may reach statistical significance, their effect size is likely to be much smaller than *PNPLA3*, and are unlikely to inspire lifestyle changes in affected individuals. Together with the other factors of TG level and BMI, *PNPLA3* can potentially be used as a predictive tool to identify individuals in the community, who are at risk of more progressive forms of MAFLD for targeted close surveillance and early weight loss interventions. The influence of genetic variation can be translated into more precise clinical management, which should be tailored to each individual population in the country.

ARTICLE HIGHLIGHTS

Research background

Metabolic dysfunction-associated fatty liver disease (MAFLD) is increasingly recognized as a leading cause of liver morbidity and mortality and has emerged as the most common chronic liver disease globally. The estimated prevalence of MAFLD in Asia is about 27.4%. Genetic factors play an important role in the pathogenesis and development of MAFLD.

Research motivation

A number of genetic variations (known as single nucleotide polymorphism, SNP) have been reported to be associated with MAFLD, mostly by studies from Europe and America. This study examines 10 of the most important SNPs in the Chinese population in Singapore.

Research objectives

To study the association of SNPs, previously identified in Western populations, with the risk for MAFLD in the Singapore Chinese population and their interactions with environmental and medical risk factors.

Research methods

This is a retrospective case-control study of 72 MAFLD cases and 72 controls with no

hepatic steatosis on imaging or controlled attenuation parameter score. Subjects were recruited from two tertiary hospitals in Singapore.

Research results

PNPLA3 GG was more likely to be associated with MAFLD (43.4% CC *vs* 69.7% GG, $P = 0.017$, and 44.8% CG *vs* 69.7% GG, $P = 0.022$). In multivariable analysis, hypertriglyceridemia (OR: 2.04 95%CI: 1.3-3.1, $P = 0.001$), body mass index (BMI, OR: 1.2 95% confidence interval [CI]: 1.1-1.4, $P < 0.001$) and *PNPLA3* GG (odds ratio [OR]: 3.4 95%CI: 1.3-9.2, $P = 0.014$) were associated with MAFLD (area under the receiver operating characteristic curve of 0.823).

Research conclusions

This study showed that *PNPLA3* GG allele was the only SNP associated with progressive MAFLD in the Singaporean Chinese population. The *PNPLA3* GG variant, elevated BMI, and hypertriglyceridemia were independent predictors of MAFLD.

Research perspectives

PNPLA3, along with triglyceride level and BMI, can potentially be used as a predictive tool to identify and risk-stratify affected individuals in the community for early intervention and targeted surveillance.

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Retrospective Cohort Study

Comparison between hepatocellular carcinoma prognostic scores: A 10-year single-center experience and brief review of the current literature

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Institutional review board statement: The study was conducted retrospectively, without performing any intervention on patients.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) represents the most common primitive liver malignancy. A relevant concern involves the lack of agreement on staging systems, prognostic scores, and treatment allocation algorithms.

AIM

To compare the survival rates among already developed prognostic scores.

METHODS

We retrospectively evaluated 140 patients with HCC diagnosed between February 2006 and November 2017. Patients were categorized according to 15 prognostic scoring systems and estimated median survivals were compared with those available from the current medical literature.

RESULTS

The median overall survival of the cohort of patients was 35 (17; 67) mo, and it was statistically different in relation to treatment choice, ultrasound surveillance, and serum alpha-fetoprotein. The Italian Liver Cancer (ITA.LI.CA) tumor staging system performed best in predicting survival according to stage allocation among all 15 evaluated prognostic scores. Using the ITA.LI.CA prognostic system, 28.6%, 40.7%, 22.1%, and 8.6% of patients fell within stages 0-1, 2-3, 4-5 and > 5 respectively. The median survival was 57.9 mo for stages 0-1, 43 mo for stages 2-3, 21.7 mo for stages 4-5, and 10.4 mo for stage > 5. The 1-, 3-, and 5-year survival

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rates were respectively 95%, 65%, and 20%, for stages 0-1; 94.7%, 43.9% and 26.3% for stages 2-3; 71%, 25.8% and 16.1% for stages 4-5; and 50%, 16.7% and 8.3% for stage > 5. At the same time, although statistically significant in prognostic stratification, the most commonly used Barcelona Clinic Liver Cancer system showed one of the most relevant differences in median survival, especially for stages A and C, when compared to the medical literature. In fact, 10.7%, 59.3%, 27.1%, 1.4%, and 0% of patients were stratified into stages 0, A, B, C, and D respectively. The median survival was > 81.1 mo for stage 0, 44.9 mo for stage A, 21.3 mo for stage B, and 3.1 mo for stage C. The 1-, 3-, and 5-year survival rates were respectively 86.7%, 60%, and 46.7% for stage 0; 91.6%, 50.6%, and 20.5% for stage A; 73.7%, 23.7% and 13.2% for stage B; and 2%, 0% and 0% for stage C.

CONCLUSION

Survival analysis shows excellent prognostic ability of the ITA.LI.CA scoring system compared to other staging systems.

Key Words: Hepatocellular carcinoma; Prognostic score system; Prognostic factors; Survival analysis; Barcelona Clinic Liver Cancer score system; Italian Liver Cancer score system

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Core Tip: Italian Liver Cancer tumor staging system seems a promising prognostic score system with a good applicability and reproducibility for patients with hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma: General aspects

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide, and the third cause of cancer-related mortality^[1]. It is the second most frequent liver malignancy following liver metastasis and the most frequent primitive liver neoplasm, accounting for more than 850000 new diagnoses each year and more than 800000 deaths^[2]. Incidence and death rates are increasing steadily (about 2%-3% per year)^[3,4]. HCC usually arises in patients affected by liver cirrhosis, regardless of the etiology^[5,6]. As chronic liver disease represents the leading risk factor for developing HCC, ultrasound surveillance in this condition is crucial to increase early detection rates and improve the overall survival in treated patients^[7,8]. Current unmet clinical needs involve proper staging, prognosis, and treatment allocation of HCC patients. Both the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver and the European Organization for Research and Treatment of Cancer recommend staging systems that take into account tumor stage, liver function, and physical status in the form of the Barcelona Clinic Liver Cancer (BCLC) staging classification^[9-13]. Also, patients' characteristics, features of the nodules, and liver function drive the choice of treatment, which might be curative (e.g., liver resection, liver transplantation, radiofrequency ablation, microwave ablation, percutaneous ethanol injection) or merely palliative (transarterial chemo-embolization/radioembolization, or specific protein kinases inhibitors such as sorafenib or lenvatinib). However, since the clinical management for HCC can be challenging, treatment should be defined and individualized by a multidisciplinary team composed of hepatologists, hepatobiliary surgeons, interventional radiologists, surgical and medical oncologists.

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HCC staging systems

In the last 30 years, several staging systems have been proposed for the prognosis stratification and treatment choices of HCC. The tumor node metastasis^[14,15] system does not take into account patient characteristics (*e.g.*, liver function tests), thus not allowing for an appropriate prognostic stratification, especially for patients with large tumors^[16,17]; therefore, other systems have been developed. For example, the BCLC is the most widely accepted and used in clinical practice although many others in past times (*i.e.* Okuda Staging System^[18], Cancer of the Liver Italian Program (CLIP) staging system^[19], Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire [GRETCH] staging system^[18,20]) and more recently (*i.e.* Japanese Integrated Staging [JIS] score^[21], Tokyo Scoring System, Hong Kong Liver Cancer [HKLC] classification^[22,23], Model to Estimate Survival in Ambulatory HCC patients [MESIAH] staging score^[24,25], albumin-bilirubin [ALBI] grading system^[26], ALBI-based BCLC staging system, ALBI-T score^[27], model to estimate survival for HCC patients [MESH] scoring system^[28], NIACE score system^[29] and Italian Liver Cancer Group [ITA.LI.CA] score system) allowed physicians allocate all possible presentations of HCC cases. In addition, other scores aimed toward driving treatment procedures have been developed to improve and provide more effective and customized therapy for specific groups of patients; consensus on their use, however, is still to be reached. Meaningful examples are represented by the needle and syringe program (NSP) scoring system^[30], hepatoma arterial-embolization (HAP) scoring system^[31], the Selection for Transarterial chemoembolization Treatment (STATE) scoring system and START strategy^[32] and tumor size and number, baseline alpha-fetoprotein (AFP), Child-Pugh and objective radiological response (SNACOR) staging system^[33]. The main features of the above-mentioned scoring system are reported in **Table 1**.

Proposed in 1999 and updated in 2003, the BCLC staging classification analyzes tumor size, presence of metastasis, portal hypertension, Child-Turcotte-Pugh score, total bilirubin and performance status, stratifying patients into five groups: Stage 0 (very early HCC), stage A (early HCC) which is divided into four subgroups A1-A4; stage B (intermediate HCC); stage C (advanced HCC); stage D (end-stage HCC). The recommended therapy changes according to the stage: Surgical resection is indicated from stage 0 to A2, liver transplant or local ablation procedures from stage A2 to A4, transarterial chemoembolization (TACE) for stage B, sorafenib for stage C, and supportive care for stage D^[34-36]. The median survival for the various stages is over 60 mo for BCLC 0-A, 20 mo for BCLC B, 11 mo for BCLC C and less than 3 mo for BCLC D. Despite its widespread application, the BCLC staging classification has some limitations, especially the strictness in treatment recommendation and the fact that it includes considerably heterogeneous populations in the same stage (principally stage B and C)^[37,38]. Because of the heterogeneity of patients in the intermediate stage (B) of BCLC, several authors have attempted to create subclassifications within this stage to provide more precise prognostic information and allow a more tailored therapeutic approach. In 2012, Bolondi *et al*^[39] proposed a four-class substaging from B1 to B4, based on characteristics such as Child-Turcotte-Pugh score, beyond Milan and up-to-7 criteria, Eastern Cooperative Oncology Group (ECOG) PS and portal vein thrombosis^[35], thus modifying treatment approach according to BCLC scheme^[39]. In 2014, the staging system proposed by Bolondi *et al*^[39] was validated in an Asian population-based study. A year later, the Japanese Society of Transcatheter Hepatic Arterial Embolization (JSTHAE) proposed an alternative subclassification of BCLC stage B, based only on Child-Turcotte-Pugh score and the 4-of-7 cm criterion (total of ≤ 4 tumor nodule, with maximum diameter ≤ 7 cm)^[40-42]. During the same year, researchers from the Kindai University developed other substaging criteria, which appear to perform appropriately; however, external validation is needed^[43]. Another subclassification for intermediate HCC based on the one proposed by Bolondi *et al*^[39] was designed by a Taiwanese group in 2015; however, it has not been validated in Western cohorts of patients. In 2016, a study was conducted to assess whether the ALBI grade could substitute the Child-Turcotte-Pugh score in the BCLC staging system. Concerning the prediction of the clinical outcome of HCC, the ALBI grade performed similarly to the Child-Turcotte-Pugh score when integrated into the BCLC staging system^[44,45]. A few months later, the ITA.LI.CA study group developed and validated its own prognostic system, trying to overcome the shortcomings of previous scores. In particular, 5183 Italian HCC patients (mainly hepatitis C virus infected patients with good performance status and compensated cirrhosis) from the ITA.LI.CA dataset were included in the analysis for internal validation, while other 2651 patients from Taipei (mainly chronic hepatitis B virus infected patients) were recruited for external validation to test the general application of the system. The ITA.LI.CA prognostic system features parameters such as tumor burden (assessed *via* the

Table 1 Description of main features of prognostic score systems for hepatocellular carcinoma

| Score system | Parameters taken into account | Classes/ levels | 1-, 2-, 3-, or 5-yr survival rates/median survival | Ref. |
|-----------------------------------|---|---|---|---|
| BCLC | Tumor size; presence of metastasis; portal hypertension; Child Pugh score; total bilirubin; performance status | Stage 0 (very early HCC); stage A (early HCC, subdivided into A1-A4); stage B (Intermediate HCC); stage C (advanced HCC); stage D (end-stage HCC) | 5-yr survival rates: 50%-70% for BCLC 0-A; 2-yr survival rates: 63% for BCLC B; 1-yr survival rates: 82%, 44% and 11% for BCLC B, C and D respectively | Llovet <i>et al</i> ^[34] , Mazzaferro <i>et al</i> ^[35] , Weinmann <i>et al</i> ^[36] , Barman <i>et al</i> ^[37] , Yopp <i>et al</i> ^[38] |
| Okuda staging system | Tumor size (tumor > 50% of the liver; presence of ascites; albumin < 3 g/dL; bilirubin > 3 mg/dL) | Stage I (0 factors); stage II (1-2 factors); stage 3 (3-4 factors) | 1-yr survival rates: 57% for stage 1, 20% for stage 2 and 3% for stage 3 respectively | Maida <i>et al</i> ^[18] |
| CLIP staging system | Tumor size; tumor morphology (uninodular, < 50%; multinodular, < 50%; massive or > 50%); Child-Turcotte-Pugh score; alpha-fetoprotein levels (< or ≥ 400 ng/mL); presence of portal vein thrombosis | One point each parameter (total score ranging from 0 to 6) | 1-yr survival rates: 86% for CLIP 0, 76% for CLIP 1, 57% for CLIP 2, 38% for CLIP 3, 22% for CLIP 4, 9% for CLIP 5 and 0% for CLIP 6 respectively; 2-yr survival rates: 69% for CLIP 0, 53% for CLIP 1, 25% for CLIP 2, 7% for CLIP 3, 10% for CLIP 4 respectively; 3-yr survival rates: 58% for CLIP 0, 39% for CLIP 1, 15% for CLIP 2, 6% for CLIP 3, 5% for CLIP 4 | [19] |
| GRETCH staging system | Serum bilirubin; alkaline phosphatase; alpha-fetoprotein; evidence of portal obstruction; Karnofsky score | Stage A (low risk); stage B (intermediate risk); stage C (high risk) | 1-yr survival rates are 79%, 31% and 4% for stage A, B and C, respectively | Maida <i>et al</i> ^[18] , Cammà <i>et al</i> ^[20] |
| Japanese integrated staging score | LCSGJ TNM (presence of single mass; dimension < 2 cm absence of vessel invasion); Child-Pugh score | Total JIS score is the sum of LCSGJ TNM (I to IV are assigned 0 to 3 points) and Child Turcotte-Pugh score (A, B or C are assigned 0, 1 or 2 points) | 2-yr survival rates are 94.5%, 88.9%, 78.2%, 52.7%, 30.3% and 15.3% for JIS 0 to JIS 5 | Kudo <i>et al</i> ^[21] |
| Tokyo scoring system | Serum albumin; serum bilirubin; tumor size; number of nodules, each of which is attributed a score | Total Tokyo score is the sum of: 0 points for serum albumin levels > 3.5 g/dL, serum bilirubin levels < 1 mg/dL, tumor size < 2 cm and ≤ 3 tumors; 1 point for serum albumin levels 2.8-3.5 g/dL, serum bilirubin levels 1-2 mg/dL and tumor size 2-5 cm; 2 points for serum albumin levels < 2.8 g/dL, serum bilirubin levels > 2 mg/dL, tumor size > 5 cm and > 3 tumors. | 1-yr survival rates: 100% for score 0, 97.6% for score 1, 94.2% for score 2, 84.6% for score 3, 73.8% for score 4-6; 2-yr survival rates: 98.1% for score 0, 90.5% for score 1, 81.7% for score 2, 70.5% for score 3, 52.4% for score 4-6; 3-yr survival rates: 96.2% for score 0, 90.5% for score 1, 63.5% for score 2, 47.4% for score 3, 33.3% for score 4-6; 5-yr survival rates: 52.8% for score 0, 37.3% for score 1, 27.9% for score 2, 19.2% for score 3, 16.7% for score 4-6 | Tateishi <i>et al</i> ^[54] |
| MESIAH staging score | Tumor size; number of nodules; vascular invasion; extrahepatic metastasis; age; serum albumin; AFP levels; MELD score | Each of the parameters is assigned a specific coefficient. | Along with the score is provided a tailored probability of survival at 1, 3, 6, 12, 24 and 36 mo | Kinoshita <i>et al</i> ^[24] , Choi <i>et al</i> ^[25] |
| ALBI grading system | Serum bilirubin (μmol/L); serum albumin (g/L). | ALBI grade 1 corresponds to a score ≤ -2.60. ALBI grade 2 corresponds to a score > -2.60 and ≤ -1.39. ALBI grade 3 corresponds to a score > -1.39. | In European patients, the median survivals reported in the study were 24.7 mo for ALBI grade 1, 11.4 mo for ALBI grade 2 and 4.9 mo for ALBI grade 3. | Ogasawara <i>et al</i> ^[26] |
| ALBI-based BCLC staging | The procedure to calculate the BCLC stage stays the same, but, instead of Child-Turcotte-Pugh grade A, B and C, ALBI grade 1, 2 and 3 are employed respectively. | An ALBI score 1 can be present in BCLC stage 0, A, B and C; ALBI score 2 can be present in BCLC stage A, B and C; ALBI score 3 is | 1-yr survival rates: 91.3% for ALBI- based BCLC 0, 85.8% for ALBI- based BCLC stage A, 72.6% for ALBI- based BCLC stage B, 32.9% for ALBI- based BCLC Stage C, 26.6% for ALBI- based BCLC stage D. 2-yr survival rates: 79.7% for ALBI- based BCLC 0, | Chan <i>et al</i> ^[45] |

| system | | related to BCLC stage D | 69.2% for ALBI- based BCLC stage A, 46% for ALBI- based BCLC stage B, 14.5% for ALBI- based BCLC stage C, 15.1% for ALBI- based BCLC stage D. 3-yr survival rates: 71.5% for ALBI- based BCLC 0, 69.2% for ALBI- based BCLC stage A, 26.4% for ALBI- based BCLC stage B, 7.2% for ALBI- based BCLC stage C, 15.1% for ALBI- based BCLC stage D. 5-yr survival rates: 50% for ALBI- based BCLC 0, 30.1% for ALBI- based BCLC stage A, 10.2% for ALBI- based BCLC stage B, 2.9% for ALBI- based BCLC stage C, 2% for ALBI- based BCLC stage D. | |
|---|--|---|--|--|
| ALBI-T score | ALBI grade; LCSGJ TNM staging system | The final score, ranging from 0 to 5, is obtained by adding the ALBI grade to the TNM stage and then subtracting 2 | The reported median survival were 137.7 mo for ALBI-T score 0, 83.2 mo for ALBI-T score 1, 53.4 mo for ALBI-T score 2, 27.4 mo for ALBI-T score 3, 5 mo for ALBI-T score 4 and 1.4 mo for ALBI-T score 5 | Hiraoka <i>et al</i> ^[27] |
| MESH scoring system | Tumor burden (within/beyond Milan criteria); vascular invasion; metastasis; Child-Pugh score; Performance Status; serum AFP; ALP | The sum of the points obtained in the various sections leads to the final MESH score (ranging from 0 to 6). | 1-yr survival rates: 89.5% for MESH 0, 82.5% for MESH 1, 74% for MESH 2, 45.2% for MESH 3, 21.4% for MESH 4, 5.7% for MESH 5, 0% for MESH 6; 2-yr survival rates: 72.9% for MESH 0, 52.8% for MESH 1, 74% for MESH 2, 49.4% for MESH 3, 12.8% for MESH 4, 3.7% for MESH 5; 3-yr survival rates: 53.3% for MESH 0, 52.8% for MESH 1, 36% for MESH 2, 14.8% for MESH 3, 8.2% for MESH 4, 1.4% for MESH 5; 5-yr survival rates: 38.6% for MESH 0, 28% for MESH 1, 14.9% for MESH 2, 5.1% for MESH 3, 3.5% for MESH 4, 0% for MESH 5 | Liu <i>et al</i> ^[28] |
| NIACE score system | Number of nodules (N); infiltrative HCC (I); serum AFP levels (A); Child-Turcotte-Pugh grade (C); ECOG PS (E) | The sum of the points obtained in the various sections leads to the final NIACE score (ranging from 0 to 7). | The reported median survivals are 44 mo for NIACE 0, 22 mo for NIACE 1, 20 mo for NIACE 1.5, 14 mo for NIACE 2.5, 9 mo for NIACE 3, 7 mo for NIACE 4, 4 mo for NIACE 4.5, 4 mo for NIACE 5.5, 3 mo for NIACE 6 and 3 mo for NIACE 7 | Adhoue <i>et al</i> ^[29] |
| ITA.LI.CA score system | Tumor burden (assessed <i>via</i> the ITA.LI.CA tumor staging); performance status test; Child-Pugh score; AFP concentration | Each is assigned an amount of points that finally contribute to the total prognostic score (from 0, best prognosis, to 13, worst prognosis) | The median survival was reported to be 61 mo for patients in quartile 1 (ITA.LI.CA score ≤ 1), 38 mo for patients in quartile 2 (ITA.LI.CA score 2-3), 23 mo for patients in quartile 3 (ITA.LI.CA score 4-5) and 8 mo for patients in quartile 4 (ITA.LI.CA score > 5) | Farinati <i>et al</i> ^[46] , Yoo <i>et al</i> ^[47] , Borzio <i>et al</i> ^[48] |
| NSP scoring system | Tumor number (N); tumor size (S); prothrombin time (P) | The sum of the points obtained in the various sections leads to the final NSP score. Using a threshold score of 1 allows to identify 2 subgroups with different prognosis | 1-yr survival rates are 88.4% for NSP ≤ 1 and 62.7% for NSP > 1 ; 3-yr survival rates are 57% for NSP ≤ 1 and 16.9% for NSP > 1 ; 5-yr survival rates are 30.2% for NSP ≤ 1 and 20.4% for NSP > 1 | Zhang <i>et al</i> ^[30] |
| HAP scoring system | Serum levels of albumin; serum AFP; bilirubin; maximum tumor diameter; 1 point is assigned for serum albumin levels < 3.6 g/dL, serum AFP > 400 ng/dL, serum bilirubin > 0.99 mg/dL (17 mmol/L) and for a maximum tumor diameter > 7 cm | HAP A (low risk) for a total score 0, -HAP B (intermediate risk) for a total score 1; HAP C (high risk) for a total score 2; HAP D (very high risk) for a total score > 2 | 1-yr survival rates: 64.7% for HAP A, 50% for HAP B, 38.5% for HAP C, 25% for HAP D; 2-yr survival rates: 17.6% for HAP A, 10.3% for HAP B, 10.3% for HAP C, 10% for HAP D | Kadalayil <i>et al</i> ^[31] |
| STATE scoring system and START strategy | Up-to-7 criteria; serum albumin level; C reactive protein values. A neoplasia within Up-to-7 criteria is assigned 0 points, while a neoplasia beyond the criteria subtracts 12 points. C reactive protein values < 1 mg/dL are attributed 0 points, whereas values ≥ 1 mg/dL subtract 12 points | 2 groups of patients presenting different prognosis were identified: STATE score < 18 and ≥ 18 | Median survival of 20.5 mo for patients with a STATE score ≥ 18 . Median survival of 6.1 mo for patients with a score < 18 | Hucke <i>et al</i> ^[32] |
| SNACOR staging system | Tumor size (S); tumor number (N); baseline AFP (A); Child-Turcotte-Pugh class (C); objective radiological response (OR). No points are assigned for tumors < 5 cm, a number of tumors < 4 , a baseline AFP < 400 ng/mL, a Child-Turcotte-Pugh class A and for complete response or partial response after TACE. 1 point is assigned for tumors ≥ 5 cm and for a Child-Turcotte-Pugh class B; 2 points are assigned for a number of tumors ≥ 4 ; 3 points are assigned for a baseline AFP ≥ 400 ng/ml and for stable disease or progressive disease after TACE | The final SNACOR score is the sum of the points obtained for the previous features and ranges from 0 to 10 | 1-yr survival rates: 80.9% for SNACOR 0-2, 69.4% for SNACOR 3-6, 40% for SNACOR 7-10; 2-yr survival rates: 55.3% for SNACOR 0-2, 38.9% for SNACOR 3-6, 20% for SNACOR 7-10; 3-yr survival rates: 42.6% for SNACOR 0-2, 26.4% for SNACOR 3-6, 6.7% for SNACOR 7-10; 5-yr survival rates: 24.5% for SNACOR 0-2, 16% for SNACOR 3-6, 3.3% for SNACOR 7-10 | Mähringer-Kunz <i>et al</i> ^[33] |

AFP: Alpha-fetoprotein; ALBI: Albumin-bilirubin; BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; ECOG: Eastern Cooperative Oncology Group; GRETCH: Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire; HAP: Hepatoma arterial-embolization; HCC: Hepatocellular carcinoma; HKLC: Hong Kong Liver Cancer; ITA.LI.CA: Italian Liver Cancer; JIS: Japanese Integrated Staging; LSCGJ: Liver Cancer Study Group of Japan; MELD: Model for End-Stage Liver Disease; MESH: Model to estimate survival for hepatocellular carcinoma patients; MESIAH: Model to Estimate Survival in Ambulatory hepatocellular carcinoma patients; NSP: Needle and syringe programmes; SNACOR: Tumour size and number, baseline alpha-fetoprotein, Child-Pugh and objective radiological response; STATE: Selection for Transarterial chemoembolization Treatment; TACE: Transarterial chemoembolization; TNM: Tumor-node-metastasis.

ITA.LI.CA tumor staging), performance status test, Child-Turcotte-Pugh score and AFP concentration, and each is assigned a number of points that finally contribute to the total prognostic score (from 0, best prognosis, to 13, worst prognosis). The ITA.LI.CA tumor staging system, taking into account features such as the diameter of the largest nodule, the number of nodules, vascular invasion or metastasis, classifies patients in stages: 0 (very early), A (early), B (intermediate, divided into B1, B2, and B3) and C (advanced). The median survival was reported to be 61 mo for patients with ITA.LI.CA score ≤ 1 , 38 mo for patients with ITA.LI.CA scores 2-3, 23 mo for patients with scores 4-5 and 8 mo for patients with more than 5 points. In the validation cohorts, the ITA.LI.CA score proved to have the best discriminatory ability among other staging systems such as BCLC, CLIP, JIS, HKLC, and MESIAH^[46]. Compared to the BCLC classification, the ITA.LI.CA prognostic system allows a more thorough analysis of tumor burden, subclassifying intermediate patients into three groups (B1, B2, B3) rather than grouping them as stage B. Furthermore, the ITA.LI.CA prognostic system differentiates patients with intrahepatic or extrahepatic metastasis, who studies proved to have different prognosis^[47]. Finally, external and independent validation studies proved ITA.LI.CA to offer the best predictive ability in terms of calibration, discriminatory ability, and monotonicity of gradients in both treated and untreated patients^[13,48].

MATERIALS AND METHODS

A total of 140 patients diagnosed with HCC and treated at our Liver Clinic (University Hospital of Trieste) between February 2006 and November 2017, were retrospectively enrolled. Follow-up was censored on June 30, 2018. The following variables were analyzed before the first active treatment: Gender, age, etiology of liver disease, presence of portal vein thrombosis and ascites, Child-Turcotte-Pugh classification, Model for End-Stage Liver Disease score, Karnofsky score, and ECOG PS score. Laboratory tests conducted featured serum levels of albumin, total and direct bilirubin, aspartate aminotransferase and alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total proteins, creatinine, hemoglobin, sodium, potassium, white blood cells, red blood cells, platelets, international normalized ratio and activated partial thromboplastin time. The diagnosis of HCC was based on typical imaging features of HCC in computed tomography or magnetic

resonance imaging. Liver biopsy was the technique of choice for diagnosing in case previous imaging studies did not allow diagnostic certainty. Imaging was further employed to obtain information on the number of lesions, tumor diameter, presence of metastasis, and Milan and up-to-7 criteria fulfillment. Depending on their characteristics, patients underwent different therapeutic procedures: Surgical resection, radiofrequency ablation, transarterial chemoembolization (TACE), systemic therapy (sorafenib), or supportive care. Patients were then classified according to different prognostic systems, namely ITA.LI.CA, BCLC (and its subclassifications), CLIP, JIS, HKLC, Tokyo score, Okuda, GRETCH, NIACE, MESH, ALBI (and scores derived from it), HAP, STATE, SNACOR, NSP.

Continuous variables were reported as median (interquartile range) according to the results of the Shapiro-Wilk test. Discrete variables were reported as number (percentage). Between-group comparisons of discrete variables were performed using Pearson's Chi-square test and those of continuous variables using Mann-Whitney *U* test. The overall survival was defined as the time from the date of diagnosis of HCC to the date of death or data censoring (June 30, 2018). Kaplan-Meier survival curves were employed to estimate the median overall survival, and the log-rank test was used to compare differences in survival. All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY, United States).

RESULTS

Patients' clinical, laboratory, radiological characteristics and treatment choice are summarized in [Table 2](#). The median overall survival was 35 (17; 67) mo, and it was statistically different in relation to treatment choice, ultrasound surveillance and serum AFP ([Table 2](#)).

Using the ITA.LI.CA prognostic system, 28.6%, 40.7%, 22.1% and 8.6% of patients fell within stages 0-1, 2-3, 4-5 and > 5 respectively. The median survival was 57.9 mo for stages 0-1, 43 mo for stages 2-3, 21.7 mo for stages 4-5 and 10, 4 mo for stage > 5. 1-, 3-, and 5-year survival rates were 95%, 65% and 20% for stage 0-1, 94.7%, 43.9% and 26.3% for stage 2-3, 71%, 25.8% and 16.1% for stage 4-5 and 50%, 16.7% and 8.3% for stage >5. The Kaplan-Meier curves are shown in [Figure 1](#).

Using the BCLC staging system 10.7%, 59.3%, 27.1%, 1.4% and 0% of patients fell within stages 0, A, B, C and D respectively. The median survival was > 81, 1 mo for stage 0, 44, 9 mo for stage A, 21, 3 mo for stage B and 3, 1 mo for stage C. 1-, 3-, and 5-year survival rates were 86.7%, 60% and 46.7% for stage 0, 91.6%, 50.6% and 20.5% for stage A, 73.7%, 23.7% and 13.2% for stage B and 2%, 0% and 0% for stage C. The Kaplan-Meier curves are shown in [Figure 2](#).

With BCLC stage A substaging 29 (35%), 25 (30.1%), 5 (6%) and 24 (28.9%) patients fell within stages A1, A2, A3, and A4 respectively. The median survival, 1-, 3-, and 5-year survival rates are shown in [Table 3](#), while Kaplan-Meier curves are shown in [Figure 3A](#). With Bolondi's intermediate BCLC subclassification, 13 (34.2%), 19 (50%), 3 (7.9%), and 3 (7.9%) patients fell within stages B1, B2, B3, and B4 respectively. The median survival 1-, 3-, and 5-year survival rates are listed in [Table 3](#), while the Kaplan-Meier curves are shown in [Figure 3B](#).

Median survivals within different stages and 1-, 3- or 5-year survivals for CLIP scoring system, JIS scoring system, HKLC scoring system, Okuda classification, GRETCH scoring system, NIACE scoring system, MESH scoring system, ALBI score, STATE scoring system, SNACOR staging system, NSP staging system are listed in [Table 3](#). The best prognostic performance was achieved by the ITA.LI.CA score ($P < 0.001$), followed by HKLC, GRETCH, BCLC and CLIP ($P = 0.001$); the other showed less accuracy, with STATE and SNACOR staging systems showing no intergroup differences ($P = 0.322$ and $P = 0.09$ respectively). Also, the comparison between the median survival expected from the original studies and median survival in the study population according to the different scores is also shown in [Table 3](#).

DISCUSSION

The main aim of this study was to assess the prognostic efficacy of different staging systems in the local patient population. Fifteen staging systems were analyzed and subsequently compared to data available from the current literature, showing considerably heterogeneous performances ranging from significant prognostic stratification and comparable median survivals to statistical insignificance and

Table 2 Demographic and biochemical factors, liver function, features of hepatocellular carcinoma nodules, treatments and prognosis of our study cohort

| Feature of interest | Study population, <i>n</i> = 140 | Intergroup statistical significance |
|---|----------------------------------|-------------------------------------|
| Gender | | |
| Male | 109 (77.9%) | |
| Female | 31 (22.1%) | |
| Age at diagnosis, yr | 71.6 (65.6; 75.6) | |
| Liver disease etiology | | |
| Viral | 36 (25.7%) | |
| Alcoholic | 30 (21.4%) | |
| Metabolic | 19 (13.6%) | |
| Mixed | 55 (39.3%) | |
| Laboratory parameters at diagnosis | | |
| Albumin, g/dL | 1.12 (0.94-2.23) | |
| INR | 1.12 (0.94-2.23) | |
| Total bilirubin, mg/dL | 1.06 (0.37-14.47) | |
| AST, UI/L | 41 (11-511) | |
| ALT, UI/L | 32 (7-336) | |
| ALP, UI/L | 99 (40-529) | |
| GGT, UI/L | 69 (11-473) | |
| Total serum proteins, g/dL | 7.3 (5.1-8.9) | |
| AFP, ng/mL | 9.3 (5-110) | |
| Creatinine, mg/dL | 0.89 (0.5-2.99) | |
| White blood cells, × 10 ³ cells/μL | 5.04 (1.51-12.18) | |
| Red blood cells, × 10 ⁶ cells/μL | 4.34 (2.85-6.78) | |
| Hemoglobin, g/dL | 13.5 (8.7-17.8) | |
| Platelets, × 10 ³ platelets/μL | 113 (29-346) | |
| Sodium, mmol/L | 139 (128-145) | |
| Potassium, mmol/L | 4.24 (3.40-6.15) | |
| Clinical characteristics at diagnosis | | |
| Ascites | 11 (7.9%) | |
| Portal hypertension | 64 (45.7%) | |
| Hepatic encephalopathy | 10 (7.1%) | |
| Portal vein thrombosis | 10 (7.1%) | |
| Metastasis | 2 (2.4%) | |
| Child-Turcotte-Pugh | | |
| Class A | 116 (82.9%) | |
| Class B | 22 (15.7%) | |
| Class C | 2 (1.4%) | |
| MELD score | 9 (6-25) | |
| Karnofsky score | | |
| 100 | 136 (97.1%) | |
| 90 | 3 (2.1%) | |

| | |
|---|--------------------|
| 80 | 0 (0%) |
| 70 | 1 (0.7%) |
| < 70 | 0 (0%) |
| ECOG PS | |
| 0 | 137 (97.9%) |
| 1 | 3 (2.1%) |
| > 1 | 0 (0%) |
| Number of nodules at diagnosis | |
| 1 | 91 (65%) |
| 2 | 31 (22.1%) |
| 3 | 7 (5%) |
| 4 | 5 (3.6%) |
| 5 | 6 (4.3%) |
| Nodule dimensions | |
| Nodule diameter, mm | 30 (20; 40) |
| Total tumor volume, cm ³ | 14.13 (5.45-36.43) |
| Milan criteria | |
| Within | 99 (71.2%) |
| Beyond | 40 (28.8%) |
| Up-to-7 criteria | |
| Within | 113 (81.3%) |
| Beyond | 26 (18.7%) |
| Treatment | |
| Type | |
| Surgical resection | 28 (20%) |
| Local ablation | 49 (35%) |
| TACE | 54 (38.6%) |
| Sorafenib | 2 (1.4%) |
| Support | 7 (5%) |
| Number | |
| < 2 | 63 (45%) |
| ≥ 2 | 77 (55%) |
| Response at 1 mo after treatment | |
| Complete response | 72 (51.4%) |
| Of whom treated with curative treatment | 56 (77.7%) |
| Partial response | 40 (28.6%) |
| Of whom treated with curative treatment | 17 (42.5%) |
| Stable disease | 14 (10%) |
| Of whom treated with curative treatment | 1 (7.1%) |
| Disease progression | 10 (10%) |
| Of whom treated with curative treatment | 1 (10%) |
| Ultrasound surveillance every 6 mo | |
| Adhesion to ultrasound surveillance | |

| | | |
|---|-------------|-------------|
| Under surveillance | 81 (57.9%) | |
| Not under surveillance | 59 (42.1%) | |
| Nodule diameter, mm | | $P < 0.001$ |
| Under surveillance | 25 (20; 35) | |
| Not under surveillance | 34 (25; 45) | |
| Number of nodules at diagnosis | | $P < 0.001$ |
| Under surveillance, < 2 nodules | 69 (85.2%) | |
| Not under surveillance, < 2 nodules | 22 (37.3%) | |
| Choice of curative treatment | | $P = 0.037$ |
| Under surveillance | 54 (66.6%) | |
| Not under surveillance | 29 (49.2%) | |
| Survival time, mo | | |
| Overall survival | 35 (17;67) | |
| Survival related to gender | | NS |
| Male | 34 (20; 80) | |
| Female | 35 (16; 64) | |
| Survival related to etiology | | NS |
| Viral | 32 (15; 65) | |
| Non-viral | 41 (19; 67) | |
| Survival related to treatment choice | | $P = 0.013$ |
| Curative (surgery/ablation) | 48 (18; 68) | |
| Non-curative (TACE/sorafenib/support) | 23 (14; 34) | |
| Survival related to ultrasound surveillance | | $P = 0.002$ |
| Under surveillance | 48 (20; 75) | |
| Not under surveillance | 30 (12; 49) | |
| Survival related to AFP | | $P < 0.001$ |
| AFP ≤ 200 ng/mL | 55 (34; 75) | |
| AFP > 200 ng/mL | 22 (12; 54) | |

AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ECGO: Eastern Cooperative Oncology Group; GGT: Gamma glutaryl transferase; INR: International normalized ratio; MELD: Model for End-Stage Liver Disease; TACE: Transarterial chemoembolization.

differences in overall survival. The most relevant differences were found for the BCLC, CLIP, JIS, HKLC, Okuda, and GRETCH staging systems and for the ALBI grade, as reported in [Table 3](#).

Despite the unequivocal statistical significance in prognostic stratification of the CLIP and GRETCH staging systems in the study population, the original studies reported substantially shorter survival for almost every stage, although they were validated in European cohorts. However, the reason behind this difference might be related to the advances in treatment for HCC that took place over time since the 1992 and 1994, when the studies were censored. Despite being statistically significant in the study population, the original studies for the Okuda, JIS, and HKLC staging systems reported notably different median overall survival rates. In this case, although the JIS staging system was proven effective by some studies also for Western patients, the explanation is likely to be found in the patient population recruited for the analysis, since validation was performed using only Eastern cohorts along with other factors such as prevalent etiology and different treatment protocols. Moreover, the worse median survival from the original study for the Okuda staging system can be justified by the higher efficacy that therapeutic procedures have reached since 1984. The shorter median survival of patients from the ALBI original study can be explained by the

Table 3 Patients' allocation and their median survival according to prognostic scores taken into account

| Score | Number of patients | Percentage | Median survival in mo | Statistical significance for prognostic stratification | Median survival in the original study in mo |
|------------------|--------------------|------------|-----------------------|--|---|
| ITA.LI.CA | | | | $P < 0.001$ | |
| 0 | 7 | 5% | 93.5 | | |
| 1 | 33 | 23.6% | 57.9 | | |
| 2 | 19 | 13.6% | 63.1 | | |
| 3 | 38 | 27.1% | 40.6 | | |
| 4 | 20 | 14.3% | 25.2 | | |
| 5 | 11 | 7.8% | 21.1 | | |
| 6 | 5 | 3.6% | 20.8 | | |
| 7 | 4 | 2.9% | 10.3 | | |
| 8 | 3 | 2.1% | 4.3 | | |
| > 8 | 0 | 0% | | | |
| ITA.LI.CA | | | | | |
| 0-1 | 40 | 28.6% | 57.9 | | 57-61 |
| 2-3 | 57 | 40.7% | 43 | | 43-48 |
| 4-5 | 31 | 22.1% | 21.7 | | 23 |
| > 5 | 12 | 8.6% | 10.4 | | 9-8 |
| BCLC | | | | $P = 0.001$ | |
| 0 | 15 | 10.7% | > 81.1 | | > 60 |
| A | 83 | 59.3% | 44.9 | | > 60 |
| B | 38 | 27.1% | 21.3 | | 20 |
| C | 2 | 1.4% | 3.1 | | 11 |
| D | 0 | 0% | | | < 3 |
| BCLC A | | | | $P = 0.022$ | |
| A1 | 29 | 20.7% | 61.9 | | 43.4 |
| A2 | 25 | 17.6% | 44.3 | | 28.9 |
| A3 | 5 | 3.5% | 10.7 | | 25.4 |
| A4 | 24 | 17.1% | 34.4 | | 22.3 |
| BCLC B (Bolondi) | | | | $P = 0.007$ | |
| B1 | 13 | 9.3% | 34.7 | | 31.9 |
| B2 | 19 | 15.7% | 25.2 | | 26.9 |
| B3 | 3 | 0.7% | 10.4 | | 13.5 |
| B4 | 3 | 1.4% | 7.8 | | 10.9 |
| CLIP | | | | $P = 0.001$ | |
| 0 | 59 | 42.1% | 50.7 | | 27 |
| 1 | 47 | 33.6% | 53.3 | | 15 |
| 2 | 19 | 13.6% | 20.5 | | 9 |
| 3 | 12 | 8.6% | 17.8 | | 7 |
| 4 | 3 | 2.1% | 3.1 | | 5 |
| > 4 | 0 | 0% | | | 3 |

| | | | | | |
|---------|-----|--------|------|-------------|----------|
| JIS | | | | $P = 0.049$ | |
| 0 | 27 | 19.3% | 70.8 | | 22.6 |
| 1 | 66 | 47.1% | 44.3 | | 22 |
| 2 | 40 | 28.6% | 42 | | 20.6 |
| 3 | 7 | 5% | 10.4 | | 16.9 |
| 4-5 | 0 | 0% | | | 12.1-5.9 |
| HKLC | | | | $P < 0.001$ | |
| 1 | 93 | 67.4% | 47 | | 79.7 |
| 2a | 10 | 7.3% | 19 | | 33.4 |
| 2b | 18 | 13% | 34.7 | | 32.7 |
| 3a | 5 | 3.6% | 10.4 | | 12.5 |
| 3b | 10 | 7.3% | 20.8 | | 5.5 |
| 4a | 1 | 0.7% | 17.8 | | 3.9 |
| 4b | 1 | 0.7% | 3.1 | | 1.9 |
| 5 (a/b) | 0 | 0% | | | 32.5/1.6 |
| Tokyo | | | | $P = 0.002$ | |
| 0 | 10 | 7.1% | 93.5 | | |
| 1 | 48 | 34.3% | 47 | | |
| 2 | 41 | 29.3% | 43.6 | | |
| 3 | 21 | 15% | 30.3 | | |
| 4 | 14 | 10% | 20.8 | | |
| 5 | 4 | 2.9% | 10.4 | | |
| 6 | 1 | 0.7% | 10.3 | | |
| 7 | 0 | 0% | | | |
| 8 | 1 | 0.7% | 0.8 | | |
| Okuda | | | | $P = 0.026$ | |
| 1 | 102 | 72.9% | 45.5 | | 15.8 |
| 2 | 36 | 25.7% | 20.5 | | 3.6 |
| 3 | 2 | 1.4% | 0.8 | | 1.3 |
| GRETCH | | | | $P < 0.001$ | |
| A | 75 | 53.6 % | 57.6 | | 29.3 |
| B | 62 | 44.3 % | 30 | | 7.4 |
| C | 3 | 2.1 % | 7.8 | | 2.1 |
| NIACE | | | | $P = 0.001$ | |
| 0 | 77 | 55 % | 45.7 | | 44 |
| 1 | 10 | 7.1 % | 43 | | 22 |
| 1.5 | 39 | 27.9 % | 21.7 | | 20 |
| 2.5 | 5 | 3.6 % | 10.4 | | 14 |
| 3 | 6 | 4.3 % | 16.5 | | 9 |
| 4 | 3 | 2.1 % | 3.1 | | 7 |
| > 4 | 0 | 0 % | | | 4 |
| MESH | | | | $P < 0.001$ | |
| 0 | 40 | 28.6 % | 57.9 | | 66 |

| | | | | |
|--------------------|--------------------------|--------|--------|--------------------|
| 1 | 46 | 32.9 % | 43 | 37 |
| 2 | 30 | 21.4 % | 19.5 | 21 |
| 3 | 19 | 13.6 % | 20.8 | 10 |
| 4 | 5 | 3.5 % | 10.4 | 5 |
| > 4 | 0 | 0 % | | 4 |
| ALBI | | | | <i>P</i> = 0.008 |
| 1 | 43 | 31.9 % | 79.2 | 24.7 |
| 2 | 87 | 64.4 % | 34.7 | 11.4 |
| 3 | 5 | 3.7 % | 15.7 | 4.9 |
| ALBI | | | | <i>P</i> = 0.008 |
| 2a | 53 | 39.2 % | 44.3 | 14.5 |
| 2b | 34 | 25.2 % | 25.2 | 6.6 |
| BCLC based on ALBI | | | | <i>P</i> = 0.048 |
| 0 | 15 | 10.9 % | > 81.1 | |
| A | 75 | 54.3 % | 44.9 | |
| B | 20 | 14.5 % | 22.2 | |
| C | 1 | 0.7 % | 3.1 | |
| D | 27 | 19.6 % | 21.7 | |
| ALBI-T | | | | <i>P</i> = 0.002 |
| 0 | 12 | 9 % | 93.5 | 137.7 |
| 1 | 42 | 31.6 % | 63.1 | 83.2 |
| 2 | 49 | 36.8 % | 42 | 53.4 |
| 3 | 28 | 21.1 % | 21.3 | 27.4 |
| 4 | 2 | 1.5 % | 0.8 | 5 |
| 5 | 0 | 0 % | | 1.4 |
| HAP | | | | <i>P</i> = 0.004 |
| A | 31 | 22.2 % | 45.7 | 25.5 |
| B | 51 | 36.4 % | 45.7 | 18.1 |
| C | 41 | 29.3 % | 35.7 | 8.9 |
| D | 17 | 12.1 % | 20.6 | 5.9 |
| STATE | | | | <i>P</i> = 0.322 |
| > 37 | 8 | 5.7 % | 25.2 | 20.5 (≥ 18 points) |
| 27-37 | 17 | 12.1 % | 40.6 | |
| 18-27 | 16 | 11.4 % | 44.9 | |
| < 18 | 13 | 9.3 % | 20 | |
| Median STATE score | 29.1 (range: 2.4 - 45.6) | | | 6.1 |
| SNACOR | | | | <i>P</i> = 0.09 |
| 0-2 | 31 | 22.1 % | 25.2 | 31.5 |
| 3-6 | 17 | 12.1 % | 19 | 19.9 |
| 7-10 | 1 | 0.7 % | 10.3 | 9.2 |
| NSP | | | | <i>P</i> = 0.03 |
| 0 | 63 | 45 % | 79.2 | |

| | | | | |
|-----|----|--------|------|-------------|
| 1 | 49 | 35 % | 42 | |
| 2 | 11 | 7.9 % | 14.9 | |
| 3 | 10 | 7.1 % | 20 | |
| 4 | 4 | 2.9 % | 22.2 | |
| 5 | 3 | 2.1 % | 5.6 | |
| NSP | | | | $P = 0.002$ |
| 0-1 | 13 | 9.3 % | 47 | 51.5 |
| > 1 | 25 | 17.9 % | 20.5 | 17.3 |

Comparison with data in original studies which available. NS: Not significant. ALBI: Albumin-bilirubin; BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; GRETCH: Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire; HAP: Hepatoma arterial embolization; HKLC: Hong Kong Liver Cancer; ITA.LI.CA: Italian Liver Cancer; JIS: Japanese Integrated Staging; MESH: Model to estimate survival for hepatocellular carcinoma patients; NSP: Needle and syringe programme; SNACOR: Tumor size and number, baseline alpha-fetoprotein, Child-Pugh and objective radiological response; STATE: Selection for Transarterial chemoembolization treatment.

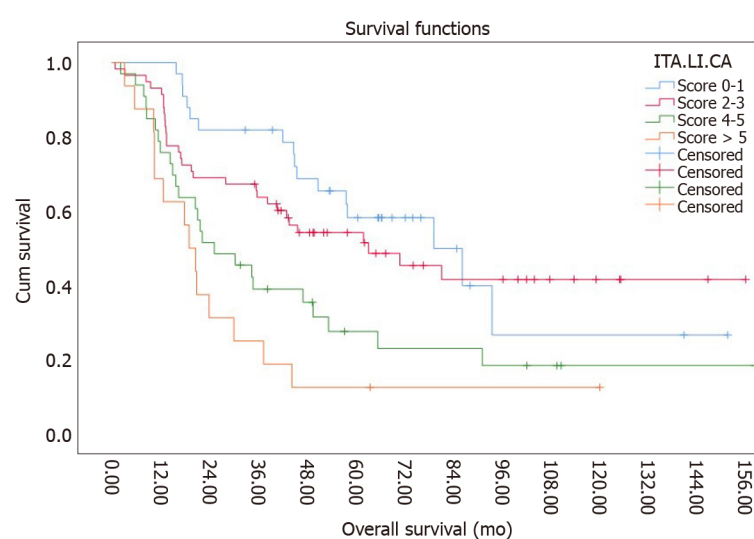


Figure 1 Kaplan-Meier curve for Italian Liver Cancer prognostic score system. $P < 0.001$. ITA.LI.CA: Italian Liver Cancer.

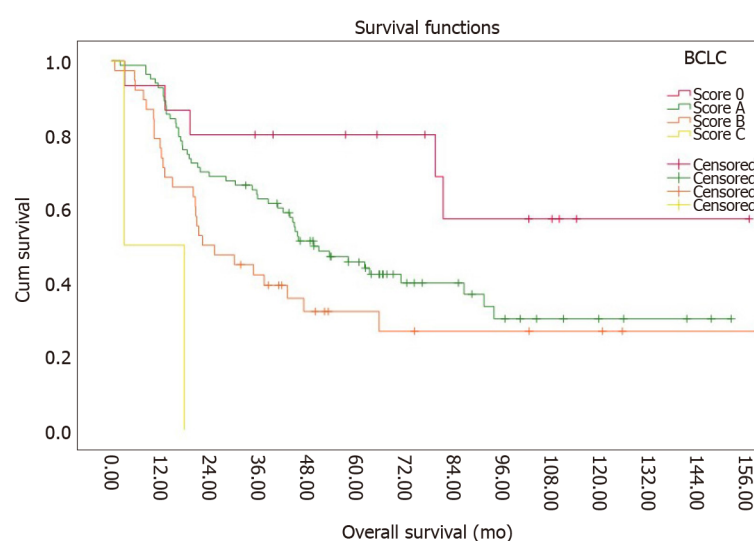


Figure 2 Kaplan-Meier curve for Barcelona Clinic Liver Cancer prognostic score system. $P = 0.001$. BCLC: Barcelona Clinic Liver Cancer.

European population employed as the reference, for all the patients had advanced

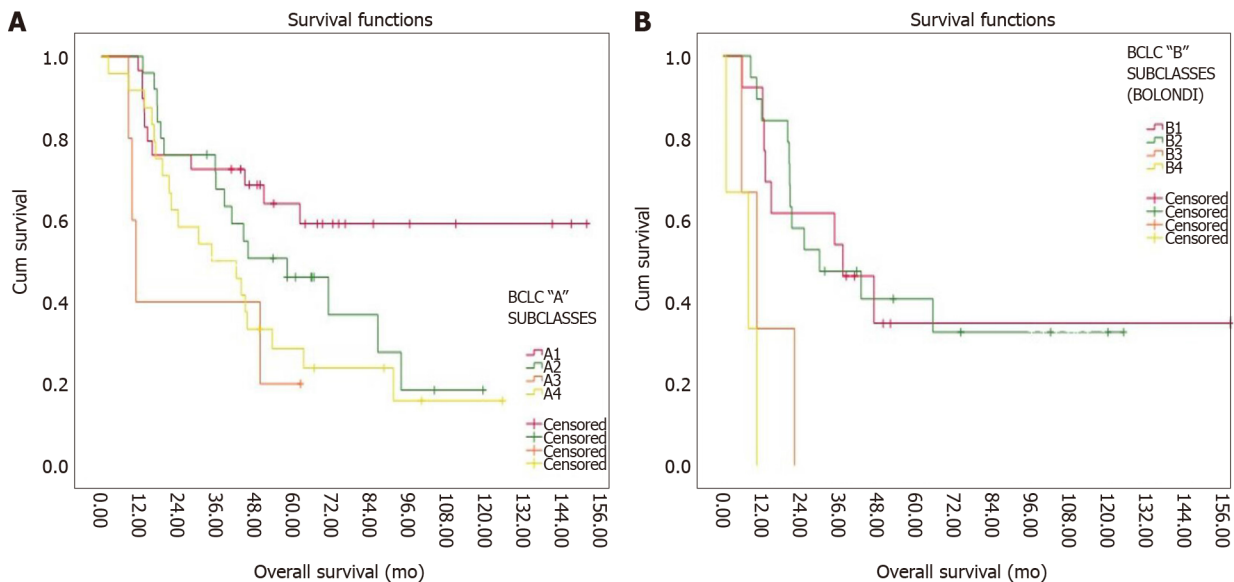


Figure 3 Kaplan-Meier curves. A: Kaplan-Meier curves for Barcelona Clinic Liver Cancer (BCLC) A subclasses. $P = 0.022$; B (right side): Kaplan-Meier curves according to Bolondi's intermediate BCLC subclassification. $P = 0.007$. BCLC: Barcelona Clinic Liver Cancer.

HCC and were treated with sorafenib. Furthermore, if the study population's median survivals are compared with those of the Japanese population of the study, that also included patients who underwent surgical resection, the differences appear much less significant. Despite the difference in survival, however, the ALBI grade showed statistical significance in the study population.

The median survival from the BCLC staging system clearly differs for stages A (and BCLC stage A subclassification) and C in the study population. The difference in survival for stage A might be explained with the heterogeneity in treatment that these patients received in the study population, while the reason for the difference in stage C is to be found in the low number of patients falling within this category in the study population. Nevertheless, BCLC stage B showed similar survivals, and so happened also for the BCLC intermediate subclassification according to Bolondi. The BCLC staging system, BCLC stage A subclassification and Bolondi's BCLC B substaging all resulted statistically significant.

The NIACE staging systems presented median survivals similar to the validation study, and similarly, the MESH staging system presented median survivals comparable to those of the original study, except for stages with lower numbers of patients.

Among all of the staging systems, not only did the ITA.LI.CA show one of the highest statistical significance ($P < 0.001$) for prognostic stratification of the patients, but it also showed almost complete correspondence of median overall survivals for all different stages. Only patients in stage > 5 showed a median survival 2 mo longer than that of the original study (10.4 *vs* 8.9 mo), probably related to the relatively low numerosity of patients in this stage (12 patients, 8.6%). This study further supports the external validation process for the ITA.LI.CA prognostic system in Western patients affected by HCC^[48].

The study also assessed the prognostic performance of scoring systems related to treatment. The median survivals of all three scoring systems (STATE, SNACOR, NSP) in the study population were similar to those of the original studies, but only the NSP system reached inter-group statistical significance.

As could be expected, the median overall survival of patients undergoing ultrasound surveillance every 6 mo was longer than those of patients who were not followed (48 *vs* 30 mo), attributable to an early detection of HCC nodules. In fact, as shown in Table 2, patients undergoing ultrasound surveillance had smaller nodule diameter (25 *vs* 34 mm, $P < 0.001$) and showed lower prevalence of 32 nodules at diagnosis. Also, patients with AFP > 200 ng/mL showed reduced survival if compared to patients with lower AFP levels (22 *vs* 55 mo, $P < 0.001$).

In terms of the treatment regimen, median overall survival was 48 (20; 75) mo for curative (surgery/ablation) treatment and 23 (14; 34) mo for non-curative (TACE/sorafenib/support) treatment. Further analyses were carried out assessing the difference in survival of patients who did and did not receive the treatment

recommended for their stage by the BCLC staging system. For patients treated with surgical resection or TACE, there was no significant difference in survival between the two groups, proving that the BCLC score does not affect the overall survival for the same type of therapy. As could be expected, patients with BCLC stage A who underwent curative treatment (as recommended by the BCLC staging system) presented a significantly better survival compared to those who did not, but at the same time patients with BCLC stage B showed a benefit from curative treatment (not recommended by the BCLC staging system) compared to those who underwent TACE (as recommended), with a median survival of 34.7 mo instead of 22.2 mo. Therefore, the rigorous application of treatment recommendations for each BCLC stage, may shorten patients' survival. In fact, treatment choices based on the sub-classification of the BCLC stage B can furtherly stratify patients and provide the most suitable treatment^[49-53].

CONCLUSION

In conclusion, the study identified the ITA.LI.CA as the most effective staging system in the local population. In addition, the ITA.LI.CA does not propose a treatment algorithm, as opposed to other staging systems such as the BCLC, since numerous variables influence treatment choice, and the use of rigid and categorical flowcharts may not always guarantee the most suitable therapy, as partly shown also in this study. ITA.LI.CA seems a promising prognostic score system with a good applicability and reproducibility for patients with HCC.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma represents the most common primitive liver malignancy.

Research motivation

Currently there is a widespread lack of agreement on staging systems, prognostic scores and treatment allocation algorithms.

Research objectives

Define the prognostic ability of fifteen different prognostic scores.

Research methods

Retrospective study, 10-year enrollment of patients.

Research results

With the Italian Liver Cancer (ITA.LI.CA) prognostic system 28.6%, 40.7%, 22.1% and 8.6% of patients fell within stages 0-1, 2-3, 4-5 and > 5 respectively. The median survival was 57.9 mo for stages 0-1, 43 mo for stages 2-3, 21.7 mo for stages 4-5 and 10.4 mo for stage > 5. 1-, 3-, and 5-year survival rates were 95%, 65% and 20% for stages 0-1, 94.7%, 43.9% and 26.3% for stages 2-3, 71%, 25.8% and 16.1% for stages 4-5 and 50%, 16.7% and 8.3% for stage > 5.

Research conclusions

The median overall survival of the cohort of patients was 35 (17; 67) mo, and it was statistically different in relation to treatment choice, ultrasound surveillance and serum AFP.

Research perspectives

External validation to the ITA.LI.CA staging system.

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Retrospective Study

Effects of proprotein convertase subtilisin/kexin type-9 inhibitors on fatty liver

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Abstract

BACKGROUND

Many studies have investigated the progression of nonalcoholic fatty liver disease (NAFLD) and its predisposing risk factors, but the conclusions from these studies have been conflicting. More challenging is the fact that no effective treatment is currently available for NAFLD.

AIM

To determine the effects of proprotein convertase subtilisin/kexin type-9 (PCSK9) inhibitors on fatty infiltration of the liver.

METHODS

This retrospective, chart review-based study was conducted on patients, 18-year-old and above, who were currently on PCSK9 inhibitor drug therapy. Patients were excluded from the study according to missing pre- or post-treatment imaging or laboratory values, presence of cirrhosis or rhabdomyolysis, or development of acute liver injury during the PCSK9 inhibitor treatment period; the latter being due to false elevation of liver function markers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Radiographic improvement was assessed by a single radiologist, who read both the pre- and post-treatment images to minimize reading bias. Fatty infiltration of the liver was also assessed by changes in ALT and AST, with pre- and post-treatment levels compared by paired *t*-test (alpha criterion: 0.05).

Data sharing statement:

All relevant data has been provided in this article. No additional data is available.

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**RESULTS**

Of the 29 patients included in the study, 8 were male (27.6%) and 21 were female (72.4%). Essential hypertension was present in 25 (86.2%) of the patients, diabetes mellitus in 18 (62.1%) and obesity in 15 (51.7%). In all, patients were on PCSK9 inhibitors for a mean duration of 23.69 ± 11.18 mo until the most recent ALT and AST measures were obtained. Of the 11 patients who received the radiologic diagnosis of hepatic steatosis, 8 (72.73%) achieved complete radiologic resolution upon use of PCSK9 inhibitors (mean duration of 17.6 mo). On average, the ALT level (IU/L) decreased from 21.83 ± 11.89 at pretreatment to 17.69 ± 8.00 at post-treatment (2-tailed $P = 0.042$) and AST level (IU/L) decreased from 22.48 ± 9.00 pretreatment to 20.59 ± 5.47 post-treatment (2-tailed $P = 0.201$).

CONCLUSION

PCSK9 inhibitors can slow down or even completely resolve NAFLD.

Key Words: Proprotein convertase subtilisin/kexin type-9 inhibitor; Fatty liver; Nonalcoholic fatty liver disease; Alanine aminotransferase; Aspartate aminotransferase; Imaging

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Core Tip: This retrospective study evaluated the effects of proprotein convertase subtilisin/kexin type-9 (PCSK9) inhibitors on fatty infiltration of the liver. Among the 29 selected patients, 11 were found to have radiologic diagnosis of hepatic steatosis and 8 of those (72.73%) achieved complete radiologic resolution of the condition upon use of PCSK9 inhibitors for mean duration of 17.6 mo. Both alanine aminotransferase and aspartate aminotransferase levels showed a downward trend after PCSK9 inhibitors for mean duration of 23.69 ± 11.18 mo. These results highlight the potential benefit of PCSK9 inhibitors use for patients with nonalcoholic fatty liver disease.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of fatty infiltration of the liver and is considered the most common chronic liver disease in the world^[1]. Cases range from nonalcoholic fatty liver-a relatively benign condition-to nonalcoholic steatohepatitis (commonly known as NASH), which can eventually lead to the life-threatening condition of cirrhosis^[1-3]. The continually increasing prevalence of NAFLD and numbers of cases progressing to cirrhosis, itself a major co-morbidity and emerging public health concern^[4], have prompted many researchers to investigate the underlying mechanisms of NAFLD progression and the pre-disposing risk factors of such^[3,5-7]. However, the conclusions from these studies have been conflicting. Even more challenging is the fact that there is currently no effective treatment available for NAFLD.

As some recent studies have implicated increased proprotein convertase subtilisin/kexin type-9 (PCSK9) synthesis and release in the pathogenic process of NAFLD^[8,9], we hypothesized that PCSK9 inhibition could lead to improvement in fatty infiltration of the liver. Thus, the aim of this study was to assess improvement in fatty infiltration of the liver among patients on PCSK9 inhibitor drug therapy.

MATERIALS AND METHODS

Study design

This study was designed as a retrospective, chart-based review. After approval from the hospital's Institutional Review Board, the database of the study site was searched for patients who were over 18 years in age and had received PCSK9 inhibitors anytime from January of 2015 until July of 2019.

Exclusion criteria

Patient records were excluded from the study according to: Missing pre- or post-treatment imaging; missing pre- or post-treatment laboratory values; diagnosis of cirrhosis, to avoid confounding by the inherently low alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels; signs of rhabdomyolysis that had developed any time during the PCSK9 inhibitor therapy, to avoid confounding by the inherently elevated ALT and AST; or presence of acute liver injury due to known cause, not thought to be related to PCSK9 inhibitor. For example, 'Female B' was started on PCSK9 inhibitors in February of 2018, and at that time showed ALT of 100 U/L and AST of 90 U/L. She experienced septic shock in May of 2018, due to pneumonia, and subsequent ischemic hepatitis ("shock liver") with ALT of 3000 U/L and AST of 1850 U/L. Her case could not be regarded as "no improvement" or even of "worsening", since a separate injury increased the levels of liver function enzymes.

Outcomes

Radiologic resolution of hepatic steatosis was the primary outcome, and improvement in liver biomarkers was the secondary outcome.

Radiologic resolution: Liver imaging scans [computed tomography (CT) scan, ultrasound (US)] that had been performed before the start of PCSK9 inhibitor therapy were compared with the most recent imaging scans, that had been performed at least 3 mo after the start of the PCSK9 inhibitor therapy. In order to minimize the confounding factors, a single radiologist worked independently to read the scans from before and after treatment. Where possible, attempts were made to compare CT scan with CT scan (or US to US scan), if both had been performed at the pre- and post-treatment times. Hepatic steatosis was defined by the following^[10,11]: (1) ≤ 40 Hounsfield units (HU) on a non-contrast CT scan or during phase when the liver was not contrast-enhanced, such as a chest CT in the very early phase of contrast; (2) < 70 HU during portal venous phase image; and (3) Increased echogenicity on US of the liver parenchyma compared to the kidney, with loss of normal periportal echogenicity.

Liver biomarkers' improvement: ALT and AST were used as the biomarkers to assess improvement of fatty infiltration of the liver, as ALT and AST can be elevated in fatty liver. Pretreatment levels of ALT and AST were compared with those measured post-treatment (at least 3 mo after the start of PCSK9 inhibitor therapy). The most recent ALT and AST measures obtained after the initiation of PCSK9 inhibitors were considered post-treatment values. For example, "Male A" had been started on PCSK9 inhibitors in January of 2015. Liver function tests were conducted during the treatment period (no interruptions in drug therapy), first in June of 2015 and then in January of 2016. For the study, the ALT and AST levels measured in January of 2016 were taken as the post-treatment levels since they were the most recent.

Data analysis

PCSK9 inhibitor use is currently low, due to its few indications as well as its cost. On top of that, selecting patients based on the strict selection criteria (in an attempt to minimize the influence of confounding factors) led to an anticipated small sample size. Radiologic improvement was reported in the form of descriptive data. Improvement in ALT and AST was assessed through paired *t*-test, with alpha criterion of 0.05.

RESULTS

Based on the strict selection criteria (in order to minimize confounders), 29 patients were included in this study.

Patient characteristics

The patient characteristics of the overall study population are summarized in [Table 1](#). The majority of patients ($n = 20$; 68.96%) were started on PCSK9 inhibitors due to statin intolerance and need for lipid control, in particular lowering low-density lipoprotein (LDL) level. Six patients (20.68%) were able to tolerate their statin treatment but never achieved adequate control of their LDL levels, and therefore were started on PCSK9 inhibitors. No specific reason could be found for the initiation of PCSK9 inhibitors for the remaining 3 patients (10.36%).

[Table 2](#) provides a summary of the types of PCSK9 inhibitor used by the study population and the age at which the treatments were initiated.

Primary endpoint

Pretreatment CT scan was compared with post-treatment CT scan for 26 (89.7%) of the patients. Pretreatment US was compared with post-treatment US for 2 patients, and only 1 patient required comparison of pretreatment CT scan with post-treatment US scan. As shown in [Figure 1](#), 11 patients (37.9%) of the total study population had radiologic diagnosis of hepatic steatosis. Among these 11 patients, 8 (72.73%) achieved complete radiologic resolution of the hepatic steatosis after use of PCSK9 inhibitors for a mean duration of 17.6 mo. [Figure 2A](#) and [B](#) provide a comparison of CT images of liver, before and after treatment with PCSK9 inhibitor, from a patient who experienced complete resolution of hepatic steatosis.

Secondary endpoint

Only 2 patients (6.9%) of the total study population had abnormally elevated pretreatment AST levels. One patient had a pretreatment AST level of 41 IU/L, and the other patient had 44 IU/L; the upper limit of normal for AST at the study site is 40 IU/L. Post-treatment, AST level normalized for both patients (27 IU/L and 21 IU/L respectively). Although all other patients in the study had normal pretreatment ALT and AST levels, both markers showed a downward trend after PCSK9 use—showing a statistically significant reduction for ALT, as summarized in [Table 3](#).

DISCUSSION

Though the study population was small, our study showed not only a downward trend in the ALT and AST levels among patients who used PCSK9 inhibitors but also that 8 out of the 11 patients with hepatic steatosis achieved complete radiologic resolution. In general, it appeared that the patient needed to be on PCSK9 inhibitors for approximately 1.5 years to see reasonable radiologic improvement and for about 2 years to see a downward trend in ALT and AST levels.

PCSK9 is an enzyme synthesized mainly by the liver^[12]. It binds to the LDL receptor (LDL-R) on the surface of hepatocytes, leading to the degradation of the LDL-R itself^[13]. When LDL-R receptors are degraded, the result is an increase in plasma LDL-cholesterol levels. Evolocumab and alirocumab, both approved by the United States' Federal Drug Administration (commonly referred to as FDA) in 2015, are fully humanized monoclonal antibodies that bind free plasma PCSK9, promoting degradation of this enzyme. Hence, the result is decreased degradation of LDL-R and increased up-take of the plasma LDL-cholesterol to liver; consequently, the levels of LDL in blood decrease. These PCSK9 inhibitors were initially approved by the FDA only for the treatment of familial hypercholesterolemia^[14-16]. Subsequent approval provided for primary and secondary prevention of cardiovascular events in patients whose LDL-cholesterol was not at target level, despite being on optimal statin therapy, or who were intolerant to statins^[17]. However, the long-term effects and mortality benefits are still unclear^[17,18]. Some recent studies have shown that increased PCSK9 synthesis and release might be involved in NAFLD pathogenesis as well^[8,9], which suggests that inhibition of PCSK9 may actually stop development or progression of NAFLD. Indeed, Theocharidou *et al*^[9] demonstrated such, which prompted our interest in this research project. Despite the small sample size, our results, too, are promising.

Patients with NAFLD are four to five times more likely to develop cirrhosis and three to four times more likely to develop hepatocellular carcinoma, when compared to patients without NAFLD^[19]. However, as of this writing, there is no effective treatment available for NAFLD. Weight loss is considered as the first step in the disease management^[20]. While some pharmacologic therapy options are available, they have shown limited benefit. Vitamin E is recommended for NAFLD patients but only those without diabetes^[21]. For NAFLD patients with type 2 diabetes, in particular,

Table 1 Baseline characteristics of the study population

| Characteristic | Males | Females | Total |
|--|-------|---------|-------|
| Sex | 8 | 21 | 29 |
| Race | | | |
| Caucasian | 7 | 16 | 23 |
| African American | 1 | 4 | 5 |
| Other/unknown | 0 | 1 | 1 |
| Presence of | | | |
| Diabetes | 3 | 15 | 18 |
| Hypertension | 8 | 17 | 25 |
| Obesity | 5 | 10 | 15 |
| Reason for PCSK-9 inhibitor initiation | | | |
| Statin intolerance | 4 | 16 | 20 |
| Inadequate lipid control | 2 | 4 | 6 |
| Not known | 2 | 1 | 3 |

PCSK9: Proprotein convertase subtilisin/kexin type-9.

Table 2 Types of proprotein convertase subtilisin/kexin type-9 inhibitor used by the study population

| | Males | Females | Total |
|--|----------------|----------------|----------------|
| Type of PCSK9 inhibitor | | | |
| Evolocumab | 5 | 17 | 22 |
| Alirocumab | 3 | 4 | 7 |
| Age in year at PCSK9 inhibitor initiation, mean \pm SD | 67.7 \pm 7.6 | 63.6 \pm 9.6 | 64.8 \pm 9.1 |
| Concomitant statin therapy during PCSK-9 therapy | 1 | 5 | 6 |

PCSK9: Proprotein convertase subtilisin/kexin type-9; SD: Standard deviation.

pioglitazone is recommended as an second line anti-diabetic medication, after metformin, due to the slight biochemical and histologic improvements seen with its use^[22]. Since high-dose vitamin E is associated with an increase in all-cause mortality^[23] and pioglitazone can cause both fluid retention and worsening of congestive heart failure^[24], a benefit and risk assessment is necessary before initiation of either of these pharmacologic therapies. Atorvastatin and omega-3 fatty acids have also been studied among patients with NAFLD but remain of uncertain benefit to date.

Since 2010, several new therapies and clinical trials have come forward but their impact has been either limited by a weak degree of improvement or a less favorable side effect profile^[25]. Most notable of all the new agents is obeticholic acid. In a multicenter, randomized, placebo-controlled phase-3 clinical trial of obeticholic acid which lasted from December 9, 2015 until October 26, 2018, the group treated with 25 mg obeticholic acid demonstrated improvement in the liver fibrosis endpoint but the NASH resolution endpoint was not met^[26]. Moreover, more than half of the patients in the 25 mg treatment group experienced pruritis and 14% of patients experienced serious adverse events^[26]. A recently published retrospective study by Zafar *et al*^[27] showed increase in ALT and AST levels with the use of PCSK9 inhibitors, but of only 6.2 mg/dL and 5.8 mg/dL respectively. These are small increases and appear to be clinically insignificant. It is important to note as well that the follow-up time (since start of PCSK9 inhibitors) in that study was only 6 mo. A meta-analysis by Zhang *et al*^[28] evaluated 25 randomized control trials, comprising a total sample of 12200 patients, and found that the PCSK9 inhibitors overall adverse effects profile was not significantly different than placebo. Rather, evolocumab was noted to reduce the rate

Table 3 Effects of proprotein convertase subtilisin/kexin type-9 inhibitor on secondary outcome (alanine aminotransferase and aspartate aminotransferase) and lipid panel

| Parameter | Males | Females | Combined |
|--|-----------------|-----------------|-----------------------------|
| Time elapsed in month from PCSK9 inhibitor initiation until most recent ALT and AST measurements | 18.4 ± 11.2 | 25.71 ± 10.74 | 23.69 ± 11.18 |
| | Pre-treatment | Post-treatment | Sig (2-tailed) ¹ |
| ALT in IU/L | 21.83 ± 11.89 | 17.69 ± 8.00 | 0.042 |
| AST in IU/L | 22.48 ± 9.00 | 20.59 ± 5.47 | 0.201 |
| LDL in mg/dL | 150.43 ± 44.69 | 90.89 ± 35.67 | 0.000 |
| Triglycerides in mg/dL | 220.07 ± 143.36 | 196.34 ± 140.73 | 0.447 |
| HDL in mg/dL | 48.59 ± 12.97 | 48.90 ± 16.27 | 0.884 |

Data are presented as mean ± standard deviation.

¹2-tailed *P* value for paired *t*-test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; PCSK9: Proprotein convertase subtilisin/kexin type-9.

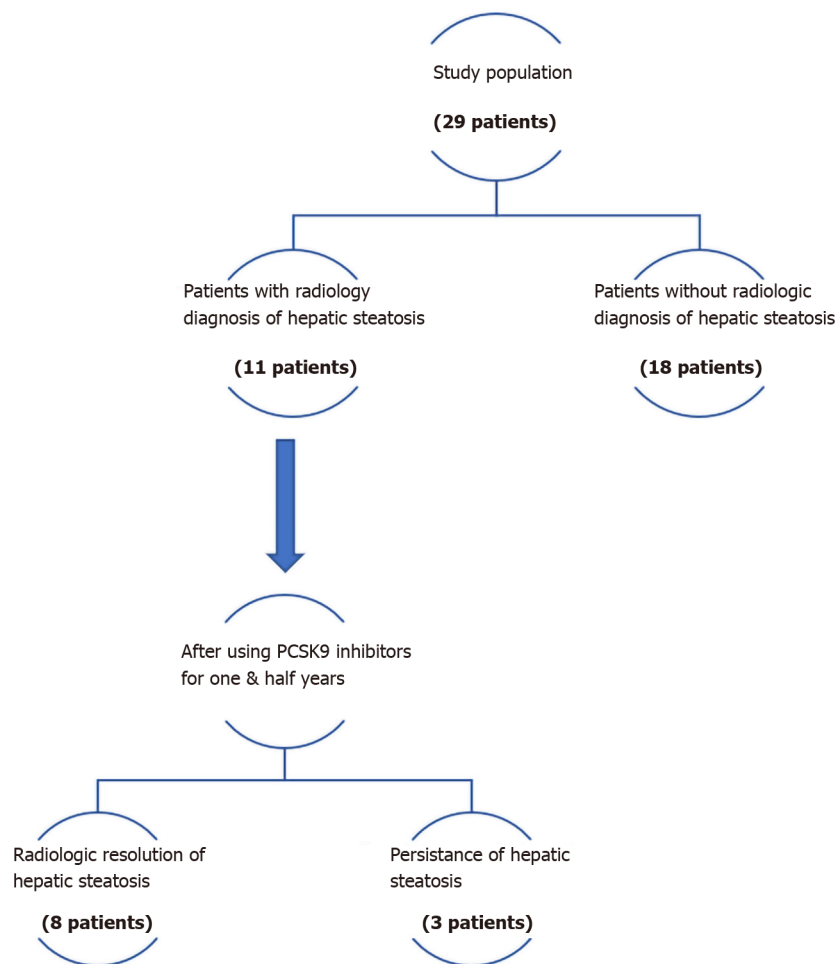


Figure 1 Flow diagram for primary outcome (resolution of hepatic steatosis). PCSK9: Proprotein convertase subtilisin/kexin type-9.

of abnormal liver function, as was noted in our study. Hence, it can be concluded based on the available data that PCSK9 inhibitors are safe to use.

Important limitations of our study include the absence of a control population, a small sample size, the retrospective design, and single-center study design. Each of these limitations may impact the external validity. However, despite these limitations, the potential of PCSK9 inhibitors for NAFLD was supported by our findings of

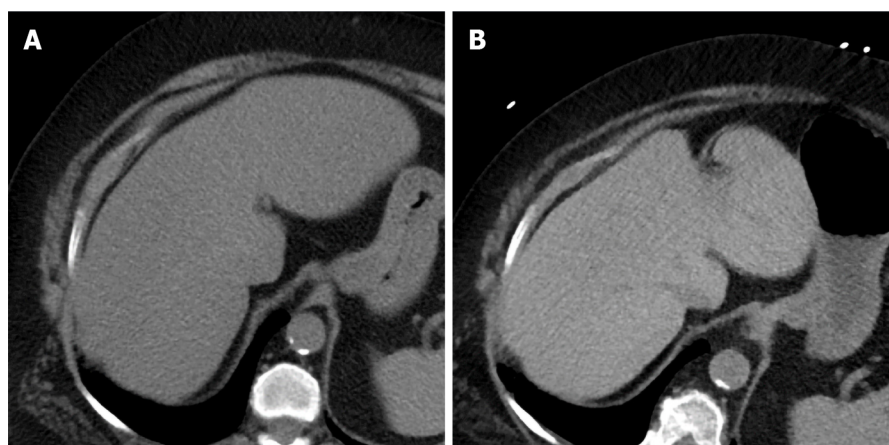


Figure 2 Pre- vs post-treatment computed tomography scans of the liver. A: Liver computed tomography (CT) scan before treatment with proprotein convertase subtilisin/kexin type-9 (PCSK9) inhibitor, showing the liver to be homogeneously hypodense (< 40 HU) and the portal and hepatic veins to have unclear delineation from the surrounding parenchyma; B: Liver CT scan after treatment with PCSK9 inhibitor, showing the liver to have increased density and the portal and hepatic veins to be clear (*i.e.*, linear hypodense structures, in contrast to the more hyperdense liver parenchyma).

radiologic resolution of hepatic steatosis among 8 of 11 patients and of the downward trend of ALT and AST among the entire study population, despite having been within normal range pretreatment. This study, in conjunction with similar studies^[9], can serve as the basis for prospective research to further delineate the potential of PCSK9 inhibitors for NAFLD and NASH.

CONCLUSION

PCSK9 inhibitors can slow down or even result in complete resolution of NAFLD. However, considering the limitations of this study, prospective studies are needed to validate these findings.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is considered the most common chronic liver disease in the world and can be life-threatening, with some cases progressing to end-stage liver disease. Yet, there is no effective treatment available for it.

Research motivation

Proprotein convertase subtilisin/kexin type-9 (PCSK9) inhibitors have produced favorable effects on liver function in some studies. This prompted our interest in determining whether PCSK9 inhibitors can elicit a therapeutic effect on hepatic steatosis.

Research objectives

Radiologic resolution of hepatic steatosis was the primary outcome, and improvement in liver function biomarkers was the secondary outcome.

Research methods

This study was designed as a retrospective chart review and included the medical records of 29 adult patients (18 years and above in age) who had received PCSK9 inhibitors anytime from January 2015 to July 2019.

Research results

Among the total 29 patients, 11 were found to have radiologic diagnosis of hepatic steatosis. Eight of these eleven patients (72.73%) achieved complete radiologic resolution of hepatic steatosis after using PCSK9 inhibitors for a mean duration of 17.6 mo. Levels of both alanine aminotransferase and aspartate aminotransferase levels also

showed a downward trend after use of PCSK9 inhibitors for about 2 years.

Research conclusions

PCSK9 inhibitors can slow down or even result in complete resolution of NAFLD.

Research perspectives

The findings from this study, in conjunction with those from similar studies, can serve as the basis for future prospective research to further explore the effects of PCSK9 inhibitors on hepatic steatosis. PCSK9 inhibitors may represent a significant breakthrough treatment for NAFLD, if prospective studies corroborate the current findings.

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Retrospective Study

Timing of paracentesis and outcomes in hospitalized patients with decompensated cirrhosis

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Institutional review board

statement: The study was reviewed and approved for publication by our Institutional reviewer.

Conflict-of-interest statement: Nothing to disclose.

Data sharing statement: Data set and statistics available from the corresponding author at dr.andreidumitru@gmail.com.

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Abstract

BACKGROUND

Ascites is one of the most common complications of cirrhosis, placing a significant burden on the healthcare system. Data regarding the optimal time of paracentesis and outcomes among patients with cirrhosis and ascites are scarce.

AIM

To assess the outcomes of patients who underwent paracentesis within 12 h after admission compared to patients who underwent paracentesis later than 12 h.

METHODS

The study included 185 patients with cirrhosis and ascites who underwent paracentesis. The early paracentesis group was defined as paracentesis performed < 12 h after admission (65 patients) and the delayed paracentesis group was defined as paracentesis performed > 12 h after admission (120 patients). New-onset complications of cirrhosis, length of hospital stay, weekday or weekend admission, in-hospital mortality rate, and 90-d readmission rates were assessed and compared between the groups.

RESULTS

Significantly more patients in the delayed paracentesis group than in the early paracentesis group developed hepatic encephalopathy (45% vs 21.5%, $P < 0.01$), hepato-renal syndrome (21.6% vs 9.2%, $P = 0.03$) and infections (25% vs 10.7%, $P = 0.02$) during hospitalization. There were no statistically significant differences in the occurrence of spontaneous bacterial peritonitis and upper gastrointestinal bleeding between the two groups. Length of stay was shorter in the early paracentesis group than in the delayed paracentesis group (6.7 d vs 12.2 d) and in-hospital mortality was lower among patients in the early paracentesis group.

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Patients in the delayed paracentesis group had a higher risk of developing complications during hospitalization.

CONCLUSION

Early paracentesis (within 12 h after admission) could be a new inpatient quality metric among patients hospitalized with cirrhosis and ascites as it is associated with fewer complications of cirrhosis, lower in-hospital mortality and shorter length of stay.

Key Words: Cirrhosis; Ascites; Hepatic encephalopathy; Spontaneous bacterial peritonitis; Early paracentesis; Delayed paracentesis

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Core Tip: Data regarding the optimal time of paracentesis and outcomes among patients with cirrhosis and ascites are scarce. We evaluated the outcomes of 185 patients with cirrhosis and ascites who underwent paracentesis within 12 h after admission (65 patients) compared to patients who underwent paracentesis later than 12 h (120 patients) and we found that early paracentesis is associated with fewer complications, lower in-hospital mortality and shorter length of stay.

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INTRODUCTION

Cirrhosis is a leading cause of death worldwide and is also associated with increased healthcare resource use^[1]. Ascites is one of the most common complications of cirrhosis^[2] and a common reason for admission to hospital, placing a significant burden on the healthcare system. Infected ascites leads to spontaneous bacterial peritonitis (SBP) which occurs in 10%-30% of patients with cirrhosis and is associated with high mortality^[2-5].

Paracentesis is a procedure commonly performed in patients with decompensated cirrhosis. Guidelines recommend that diagnostic paracentesis should be performed in all patients who are hospitalized with cirrhosis and new onset grade 2 or 3 ascites, or in those hospitalized due to worsening of ascites or any complication of cirrhosis, to evaluate the presence or absence of SBP^[2,4]. Rapid diagnosis of SBP by early paracentesis is very important for the outcome of patients even in the absence of symptoms given the fact that SBP usually has a subtle presentation or is asymptomatic. Patients with untreated SBP are at high risk of sepsis and early diagnostic paracentesis allows the initiation of rapid specific treatment with potentially better outcomes of the disease. Despite this, the adherence rate in clinical practice may be unsatisfactory^[6,7]. Data regarding the optimal time of paracentesis and outcomes among patients with cirrhosis and ascites are scarce. The aim of this study was to assess the outcomes of patients with cirrhosis and ascites who underwent paracentesis within 12 h after admission compared to patients who underwent paracentesis later than 12 h.

MATERIALS AND METHODS

Data source and study sample

The retrospective study included 307 patients with cirrhosis and ascites admitted to the Department of Gastroenterology, Constanta County Clinical Emergency Hospital, between January 1, 2018 and December 31, 2019.

Information was collected from the digital database of the hospital (each electronic medical file contains demographic data including date of admission, Child-Pugh classification or MELD-Na score, all diagnoses and procedures performed, length of stay (LOS) and data regarding discharge or in-hospital death) and medical files of the patients (to assess the time of paracentesis and to classify ascites according to clinical and ultrasound criteria).

International Classification of Diseases, 10th revision, version 2015^[6] (ICD-10) codes were used to identify patients with primary or secondary diagnoses of cirrhosis (K70.0, K70.2, K70.3, K70.9, K71.0 – K71.9, K74.6), ascites (R18.8) and/or SBP (K65.2). Also, the search engine included the procedure code for paracentesis (30406-00).

Inclusion criteria: Patients with cirrhosis of any etiology and one of the following three conditions: (1) New onset grade 2 or 3 ascites, (2) Ascites which has worsened recently, or (3) Ascites associated with a complication of cirrhosis, who underwent paracentesis during hospitalization.

Exclusion criteria: Patients with cirrhosis but without ascites or with grade 1 ascites (small amount of ascites detectable only on ultrasound studies), patients who did not undergo paracentesis, and patients with other etiologies of ascites (cancer, heart failure, tuberculosis).

Following the electronic search, medical files from the hospital's archive of selected patients were manually checked to assess the time of paracentesis since admission and to assess the occurrence of complications during hospitalization.

Ascites was documented by physical exam and detectable on imaging studies (ultrasound, magnetic resonance imaging, or computed tomography scan). Time to paracentesis after admission was assessed and early paracentesis (EP) was defined as paracentesis performed < 12 h after admission and delayed paracentesis (DP) was defined as paracentesis performed > 12 h after admission.

Of 307 patients with cirrhosis and ascites, after careful application of these criteria, 122 (39.7%) patients were excluded (79 patients who did not undergo paracentesis during hospitalization, 13 patients with grade 1 ascites, and 30 patients with stable ascites and no complications of cirrhosis) and 185 (60.3%) patients met the inclusion criteria. Sixty-five (35.1%) patients were assigned to the EP group and 120 (64.9%) to the DP group. In the case of patients who had multiple hospitalizations during the study period, only the first hospitalization was chosen as the index for analysis.

Variables and outcomes

Demographic data, Child-Pugh classification (A, B, and C) and MELD-Na score were recorded in each group. New-onset complications of cirrhosis (developed after admission, during the index hospitalization) such as SBP, hepatic encephalopathy, hepato-renal syndrome, upper gastrointestinal bleeding, and various infections (urinary, pulmonary, *Clostridium difficile* infections), LOS in days, weekday or weekend admission, the in-hospital mortality rate during the same admission, and 90-d readmission rates were assessed and compared between the groups; the primary reason for readmission was identified using the ICD-10 codes for continuous hospitalization.

Ethics approval

The study was conducted according to good laboratory practice and in concordance with national and international standards. The study protocol was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies of the County Clinical Emergency Hospital of Constanta (approval No. 7/02.03.2020). This was a retrospective study; therefore, a consent form was not required.

Statistical analysis

Statistical analysis was performed using the JASP 0.11.1 statistic software package. Descriptive statistics were used for demographic and baseline data: mean \pm standard deviation for continuous variables, absolute number and frequency for categorical variables. For comparison between variables in the EP and DP groups, a two-sample Student's *t*-test was used for continuous variables and the chi-square test or Fisher exact test were used for categorical variables. The association of each complication developed during hospitalization in each group was analyzed by calculating the odds ratio (OR) together with confidence intervals (CI). Results were considered statistically significant if the *P* value < 0.05.

RESULTS

Demographic and baseline data of the 185 patients enrolled in the study are illustrated in [Table 1](#). Overall, there were no significant differences between the two groups. The mean age was 63.7 ± 10.2 years in the EP group and 62.5 ± 11.1 years in the DP group, and more than half of the patients from both groups were male. The most common etiology of cirrhosis in both groups was alcohol, followed by viral hepatitis B and/or C, mixed etiology (alcohol and viral hepatitis B and/or C) and other etiologies: Non-alcoholic, metabolic associated liver disease (9 patients), primary biliary cholangitis (2 patients), and autoimmune (1 patient). Most of the patients in each group had been classified as Child-Pugh C class. There were no statistically significant differences in the mean Child-Pugh scores and MELD-Na scores between the groups.

With regard to new-onset complications of cirrhosis (after admission, during hospitalization ([Table 2](#)), significantly more patients in the DP group than in the EP group developed hepatic encephalopathy (45% *vs* 21.5%, $P < 0.01$) and hepato-renal syndrome (21.6% *vs* 9.2%, $P = 0.03$). There were no statistically significant differences between the occurrence of SBP and upper gastrointestinal bleeding in the EP and DP group (15.3% *vs* 18.3%, $P = 0.61$, 30.5% *vs* 33.3%, $P = 0.72$, respectively). Hepatocellular carcinoma was present in both groups irrespective of time of paracentesis (18.4% in the EP group, 20.8% in the DP group, $P = 0.70$). Other infections (urinary, pulmonary, and *Clostridium difficile* infections) occurred more frequently in the DP group than in the EP group (25%, *vs* 10.7%, $P = 0.02$).

There was a significantly shorter LOS in the EP group compared to the DP group (6.7 *vs* 12.2 d, $P = 0.01$) ([Table 3](#)).

Regarding the relationship between the time of paracentesis and the time of in-hospital admission, it was observed that more than three-quarters of the patients with early paracentesis (76.9%) were admitted during the weekdays, while most of those with delayed paracentesis (52.5%) were admitted during the weekend ($P < 0.01$).

In-hospital mortality during the same admission was significantly lower among patients in the EP group than in the DP group (6.1% *vs* 17.5%, $P = 0.03$).

Regarding 90-d readmission, from a total of 160 discharged patients (61 patients in the EP, and 99 patients in the DP), 45 (28.1%) patients were readmitted with complications. Among them, significantly more patients from the DP group than from the EP group were readmitted: 34.3% *vs* 18%, $P = 0.03$. The most common cause for readmission within 90-d in the EP group was upper gastrointestinal bleeding (45.4%), followed by ascites (27.3%), hepatic encephalopathy (18.3%), and in the DP group was ascites (47%), followed by hepatic encephalopathy (29.5%), and upper gastrointestinal bleeding (20.5%). Other causes were lower gastrointestinal bleeding in the EP group (1 patient) and hepato-renal syndrome in the DP group (1 patient).

According to the OR calculation ([Table 4](#)), patients in the DP group were more likely to develop hepatic encephalopathy during hospitalization than patients in the EP group (OR = 2.98, 95%CI = 1.49-5.95, $P < 0.01$). The same was true for hepato-renal syndrome (OR = 2.71, 95%CI = 1.05-7.00, $P = 0.03$) and infections (OR = 2.76, 95%CI = 1.13-6.70, $P = 0.02$). Also, weekend admission, LOS ≥ 7 d and in-hospital mortality were more likely to occur in the DP group ([Table 4](#)).

DISCUSSION

In the present study, of 307 patients with cirrhosis and ascites, only 185 (60.3%) of these patients underwent paracentesis. In 43 (14.0%) patients there was no indication for paracentesis as the ascites had been classified as small (grade 1) or stable and without any complications of cirrhosis. However, in 79 (25.7%) patients no paracentesis was performed, although they had an indication for paracentesis, if we comply with the EASL guide of 2018 which states that a “diagnostic paracentesis is recommended in all patients with new-onset grade 2 or 3 ascites, or in those hospitalized for worsening of ascites or any complication of cirrhosis”^[4]. Our findings are similar to previously published data^[6,7,9].

The time of paracentesis is of paramount importance for the outcome of hospitalized patients with cirrhosis and ascites^[6]. In our study, only 35.1% of patients underwent paracentesis within 12 h after admission, and the remaining patients (64.9%) underwent paracentesis later than 12 h after admission. Literature is scarce regarding the optimal time for paracentesis.

Spontaneous bacterial peritonitis occurred in 15.3% of patients in the EP group and in 18.3% in the DP group. This finding is in concordance with the literature^[10,11]. A

Table 1 Patients' demographics

| | EP (n = 65) | DP (n = 120) | P value |
|-----------------------------|-------------|--------------|---------|
| Gender, n (%) | | | 0.43 |
| Male | 34 (52.3) | 70 (58.3) | |
| Female | 31 (47.7) | 50 (41.7) | |
| Age (yr) | | | |
| mean ± SD | 63.7 ± 10.2 | 62.5 ± 11.1 | 0.80 |
| Etiology, n (%) | | | |
| Viral | 20 (30.7) | 45 (37.5) | 0.35 |
| Alcohol | 26 (40.0) | 46 (38.3) | 0.82 |
| Mixed | 12 (18.4) | 24 (20.0) | 0.80 |
| Other | 7 (10.9) | 5 (4.2) | 0.15 |
| Child-Pugh, n (%) | | | |
| A | 0 | 0 | N/A |
| B | 24 (36.9) | 36 (30.0) | 0.41 |
| C | 41 (63.1) | 84 (70.0) | 0.41 |
| Child-Pugh score, mean ± SD | 11.2 ± 2.9 | 11.9 ± 3 | 0.52 |
| MELD-Na score, mean ± SD | 21.4 ± 7.5 | 23.6 ± 8.8 | 0.28 |

EP: Early paracentesis group; DP: Delayed paracentesis group; SD: Standard deviation; N/A: Not applicable.

Table 2 Complications in the early paracentesis group and delayed paracentesis group

| Complications, n (%) | EP (n = 65) | DP (n = 120) | P value |
|----------------------|-------------|--------------|---------------------|
| SBP | 10 (15.3) | 22 (18.3) | 0.61 |
| HE | 14 (21.5) | 54 (45.0) | < 0.01 ^a |
| HRS | 6 (9.2) | 26 (21.6) | 0.03 ^a |
| UGIB | 20 (30.5) | 40 (33.3) | 0.72 |
| Infections | 7 (10.7) | 30 (25.0) | 0.02 ^a |

^aP < 0.05. EP: Early paracentesis group.

DP: Delayed paracentesis group; SBP: Spontaneous bacterial peritonitis; HE: Hepatic encephalopathy; HRS: Hepato-renal syndrome; UGIB: Upper gastrointestinal bleeding.

study conducted by Garcia-Tsao *et al*^[12] found SBP in 12% of patients admitted with cirrhosis and ascites. Interestingly, in our study, SBP was present in both groups irrespective of the timing of paracentesis, and there was no statistically significant difference between the two groups. We assume that in our group of patients, SBP was present on admission to the hospital, so there was no difference in the number of cases diagnosed by EP compared with those diagnosed by DP. However, early diagnosis may be important in the prognosis of the disease, as early diagnosis is followed by early treatment and this may influence the course of the disease.

In addition, significantly more patients in the DP group developed hepatic encephalopathy and hepato-renal syndrome. We can assume that this was due to DP and delayed treatment of SBP as untreated SBP has a worse prognosis and outcome. Also, other infections (urinary, pulmonary, *Clostridium difficile* infections) were more common in the DP group than in the EP group; this also suggests that EP facilitates the diagnosis of SBP and early treatment with antibiotics may also reduce the incidence of some other infections. Overall, patients who received EP had better outcomes and fewer complications in contrast to those who received DP. The relationship between EP and improved outcomes might be due to the rapid initiation of SBP treatment

Table 3 Time of admission, length of stay, in-hospital mortality, and 90-d readmission rate in the early paracentesis group and delayed paracentesis group

| | EP (n = 65) | DP (n = 120) | P value |
|------------------------------|-------------|--------------|---------------------|
| Admission, n (%) | | | |
| Weekday | 50 (76.9) | 57 (47.5) | < 0.01 ^a |
| Weekend | 15 (23.1) | 63 (52.5) | |
| LOS (d) | | | |
| mean ± SD | 6.7 ± 3.8 | 12.2 ± 4.5 | 0.01 ^a |
| In-hospital mortality, n (%) | 4 (6.1) | 21 (17.5) | 0.03 ^a |
| 90-d readmission, n (%) | 11 (18.0) | 34 (34.3) | 0.03 ^a |
| Ascites | 3 (27.3) | 16 (47.0) | |
| UGIB | 5 (45.4) | 7 (20.5) | |
| HE | 2 (18.3) | 10 (29.5) | |
| Other | 1 (9.0) | 1 (3.0) | |

^aP < 0.05. EP: Early paracentesis group; DP: Delayed paracentesis group; LOS: Length of stay; UGIB: Upper gastrointestinal bleeding; HE: Hepatic encephalopathy; SD: Standard deviation.

Table 4 The risk of complications developed during hospitalization in the early paracentesis group and delayed paracentesis group

| Complication | Cases in the EP group, n (%) | Cases in the DP group, n (%) | OR | 95%CI | P value |
|-----------------------|------------------------------|------------------------------|------|-----------|---------------------|
| HE | 14 (21.5) | 54 (45) | 2.98 | 1.49-5.95 | < 0.01 ^a |
| HRS | 6 (9.2) | 26 (21.6) | 2.71 | 1.05-7.00 | 0.03 ^a |
| Infections | 7 (10.7) | 30 (25.0) | 2.76 | 1.13-6.70 | 0.02 ^a |
| LOS ≥ 7 d | 25 (47.6) | 88 (73.3) | 3.01 | 1.60-5.67 | < 0.01 ^a |
| In-hospital mortality | 4 (6.1) | 21 (17.5) | 3.23 | 1.05-9.87 | 0.03 ^a |
| 90 d readmission | 11 (18.0) | 34 (34.3) | 1.94 | 0.90-4.15 | 0.08 |

^aP < 0.05. EP: Early paracentesis group; DP: Delayed paracentesis group; HE: Hepatic encephalopathy; HRS: Hepato-renal syndrome; LOS: Length of stay; OR: Odds ratio; CI: Confidence interval.

based on antimicrobial therapy and albumin. As such, early detection and therapy of SBP are critical for improving favorable outcomes in patients with cirrhosis and ascites. Prior studies showed that paracentesis within one day of admission is associated with lower in-patient mortality and fewer readmissions^[13]. Moreover, higher mortality was noted in patients with cirrhosis and ascites who did not undergo paracentesis compared to patients who did^[6].

Our study found that patients who received EP had a shorter LOS. A reason for this finding could be that rapid diagnosis of SBP or evacuation of tension ascites, and rapid initiation of antimicrobial treatment is effective and prevents the development of other complications leading to shorter hospitalization. On the other hand, patients in the DP group had more complications and this could explain the longer LOS in this group.

Patients were more likely to receive EP if admitted on a weekday compared to being admitted on a weekend and more than half of patients in the DP were admitted on the weekend. This could be explained by the low number of medical staff on call during weekends. Similar findings were noted in another study^[14].

According to the literature^[15], the time of paracentesis is associated with the risk of mortality. In a study conducted by O'Brien *et al*^[16], the majority of patients experienced DP and in-hospital mortality was higher in these patients. Similarly, in our study, patients who received EP had lower in-hospital mortality than patients who received DP (6.1% *vs* 17.5%, *P* < 0.05). Consistent with our findings, a recent study showed that patients who received DP had an increased risk of mortality^[17].

Observing the collected data, we also suppose that DP may also have a negative

impact on healthcare utilization. Given that the patients who received DP developed more complications, and had longer LOS, we can expect increased use of medical resources by these patients.

Cirrhosis is one of the leading causes of morbidity, requires frequent hospitalizations and carries a high risk for readmission^[7]. Studies show 30-d readmission rates between 20%-37%^[18], and a 90-d readmission rate of up to 53%^[19]. In our study, the overall 90-d readmission rate was 28.1% and it was in concordance with the study published by Orman *et al*^[20] and higher than the rate of 12.9% found by Tapper *et al*^[21]. Another study by Volk *et al*^[22] reported a 30-d readmission rate of 37%^[22]. In the literature^[22-24], similar to our study, the most common reasons reported for readmission were complications of cirrhosis. Readmissions frequently occur among patients with advanced liver disease, with increased MELD score being associated with readmission in most studies^[20].

In our study, the majority of patients (75.5%) who were readmitted within 90 d with continuous hospitalization were from the DP group. Only 24.5% of them were from the EP group. Another study conducted by Sobotka *et al*^[25] found that paracentesis was associated with increased 30-d readmission. Despite this finding, paracentesis is recommended by guidelines and it is a quality indicator in cirrhotic patients.

Given that most patients had advanced liver disease, the presence of coagulopathy could be a reason for not performing paracentesis or for performing DP, but there is also strong evidence supporting that paracentesis is a safe procedure even in patients with associated coagulopathy^[26]. Kanwal *et al*^[7] found that the quality of care regarding ascites was better among patients with worse liver disease.

A limitation of our study may be the lack of data regarding discharged patients as we could not assess the survival rates of the patients who were not readmitted to our hospital 90 d after discharge. Another limitation of our study is that it is a retrospective study conducted in a single center, and our results should be confirmed by prospective, multicenter studies on a larger number of patients.

CONCLUSION

In light of the data provided, early paracentesis (within 12 h after admission) could be a new inpatient quality metric among patients hospitalized with cirrhosis and ascites as it is associated with fewer complications of cirrhosis, lower in-hospital mortality and shorter length of stay.

ARTICLE HIGHLIGHTS

Research background

Cirrhosis is a leading cause of death worldwide and ascites is one of the most common complications of cirrhosis. Patients are frequently admitted to hospital, placing significant burden on the healthcare system.

Research motivation

Data regarding the optimal time of paracentesis and outcomes among patients with cirrhosis and ascites are scarce in the literature.

Research objectives

The aim of this study was to assess the outcomes of patients with cirrhosis and ascites who underwent paracentesis within 12 h after admission compared to patients who underwent paracentesis later than 12 h.

Research methods

This was a retrospective study of 185 patients with cirrhosis and ascites who underwent paracentesis. The early paracentesis group was defined as paracentesis performed < 12 h after admission (65 patients) and the delayed paracentesis group was defined as paracentesis performed > 12 h after admission (120 patients). Complications of cirrhosis occurring during hospitalization were assessed and compared between the groups.

Research results

Significantly more patients in the delayed paracentesis group than in the early paracentesis group developed hepatic encephalopathy, hepato-renal syndrome and infections during hospitalization. There were no statistically significant differences in the occurrence of spontaneous bacterial peritonitis and upper gastrointestinal bleeding between the two groups. Length of stay was shorter and in-hospital mortality was lower in the early paracentesis group. Patients from the delayed paracentesis group had a higher risk of developing complications during hospitalization.

Research conclusions

Early paracentesis (within 12 h after admission) is associated with fewer complications of cirrhosis, lower in-hospital mortality and shorter length of stay.

Research perspectives

Early paracentesis could be a new inpatient quality metric among patients hospitalized with cirrhosis and ascites and deserves to be investigated further in larger studies.

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Retrospective Study

Bioelectrical impedance vector analysis evaluates cellularity and hydration in cirrhotic patients

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Abstract

BACKGROUND

Malnutrition in cirrhotic patients is correlated with mortality and a better response to liver transplantation. However, recovery of the nutritional status in these patients is a challenge due to the difficulty in establishing a reliable nutritional diagnosis. The bioelectrical impedance vector analysis (BIVA) method appears as a feasible tool in clinical practice to define the physiological state of cirrhotic patients by assessing hydration and body cellularity.

AIM

To evaluate body composition in cirrhotic patients using BIVA.

METHODS

This retrospective cross-sectional study was carried out by following cirrhotic outpatients at a hospital in Porto Alegre, Brazil. A tetrapolar bioelectrical impedance analysis device was used to evaluate cellularity and hydration and to perform the BIVA. The BIVA graphic was elaborated by software and for statistical analysis a significance level of 5% ($P \leq 0.05$) was considered.

RESULTS

Data sharing statement: No additional data are available.

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One hundred and ninety patients, 61.1% males, with a mean age of 56.6 ± 11.0 years, were evaluated. Of these, 56.3% had Child-Turcotte-Pugh (CTP) A score, and the prevalent etiology was hepatitis C virus (47.4%). The patients were classified according to cellularity and hydration by the quadrants and ellipses of the BIVA method, quadrant 1 (47.9%); quadrant 2 (18.9%); quadrant 3 (14.2%); and quadrant 4 (18.9%). Those classified in quadrant 1 and 2 had a higher phase angle compared to those in quadrants 3 and 4 ($P < 0.001$). Quadrant 2 patients had a lower average age than the other groups. The association with CTP score showed that patients in quadrant 2 had a higher proportion of CTP A, and those in quadrant 4 had a higher proportion of CTP C ($P < 0.052$).

CONCLUSION

The BIVA method allows identification of the cellularity and hydration status of cirrhotic patients, and its association with clinical factors determines the disease severity, age and prognostic index.

Key Words: Body composition; Hydration; Cellularity; Hepatic cirrhosis; Electrical Bioimpedance; Bioelectrical impedance vector analysis

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Core Tip: Using the bioelectrical impedance vector analysis method, it is feasible in clinical practice to identify hydration and cellularity status in patients with liver cirrhosis, regardless of their etiology. This tool allows health professionals to establish an effective treatment for these patients with the objectives of clinical improvement, a better quality of life and better response to orthotopic liver transplantation.

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INTRODUCTION

One of the main clinical complications of liver cirrhosis is protein-calorie malnutrition, which has a prevalence ranging from 10% to 100%, regardless of the stage and etiology of the disease. It has been observed in different studies that the general prognosis of the disease worsens in the presence of malnutrition, contributing negatively to the quality of life of patients^[1-7].

The evaluation of body composition in cirrhotic patients is a challenge, as there is no recognized gold standard. Anthropometric measurements are non-invasive and low-cost methods, but they can be impaired by changes in hydration status (ascites and edema) and have moderate reliability due to interobserver variation^[6,8]. Among the most frequently used methods for assessing body composition, electrical bioimpedance (BIA) is capable of providing information on lean mass and fat mass through the parameters of electrical current of tissues, resistance (R) and reactance (Xc), which help to identify the functionality and integrity of cell membranes^[9].

Using R and Xc, the phase angle (PA), a marker of the nutritional status independent of the device's pre-established formulas can be calculated, where the patient's hydration, for example in cirrhotic patients, could generate estimation errors. In addition, PA values have been shown to be an excellent prognostic index in several clinical conditions^[6,10-15]. The BIA can further provide angular vectors of alterations in body fluid levels and cellularity of the patient, and this method is known as bioelectrical impedance vector analysis (BIVA)^[16-18].

The PA provides us with a large amount of data that, analyzed in a specific way, with specific statistical programs, allows new analyses of body composition to deepen our knowledge. BIVA uses graphic vectors for the analysis of BIA data, where impedance is plotted as a vector by its components R (X axis) and Xc (Y axis) after

standardization by weight^[11,12,18].

The electrical properties of the tissues (R and Xc) must be standardized by sex and race, with their tolerance intervals, in relation to a given population. The resulting graph provides ellipses of tolerance, *i.e.*, 50%, 75% and 95% percentiles (confidence intervals) that are divided into quadrants that represent groups of patients with more or less hydration, more or less cellularity. The advantage of this method is that it allows simultaneous information on changes in body hydration or soft tissue mass, regardless of body weight. Thus, BIVA is able to correctly interpret, even if the patient is extremely heavy, the distribution of water volume in different diseases, and assess the general composition of the body^[19-21].

To date, no studies have analyzed body composition regarding hydration and cellularity in cirrhotic patients using the BIVA method. Therefore, the main objective of this study was to evaluate the results of BIVA regarding hydration and cellularity, and compare them with the PA and other clinical parameters in cirrhotic patients.

MATERIALS AND METHODS

Population

This was a retrospective cross-sectional study with data collected between May 2007 and December 2015, at the Santa Casa de Misericórdia Hospital Complex in Porto Alegre, RS, Brazil.

A total of 224 patients with cirrhosis undergoing outpatient follow-up were included in the data collection. Of these, 34 were excluded due to incomplete data and 190 patients were included in the final analysis. The etiology of cirrhosis was as follows: Hepatitis C virus (HCV), hepatitis B virus, alcohol, autoimmune, non-alcoholic fatty liver disease, cryptogenic or cholestatic disease, and some patients had two concomitant etiologies.

Clinical evaluation

Data on age, gender, socioeconomic status, social history (smoking and alcohol consumption), and chronic diseases were obtained. In addition to the anamnesis, data on the etiology of cirrhosis, staging of the disease, medications used, complementary examinations, laboratory, imaging or anatomopathological data were obtained.

Body mass and height

Body mass measurement was verified by the Filizola® scale, with a scale of 100 g, previously calibrated. The patients were measured wearing light clothing and barefoot. Height was determined with a fixed stadiometer on the wall, with the patient standing erect and barefoot.

Evaluation by BIA

The BIA evaluation was performed in the outpatient department, without previous specific preparation for fasting. The patients were evaluated in a comfortable dorsal decubitus position and relaxed, without shoes, socks and metallic fittings. According to the procedure, the legs were spread apart, hands open and supported on the stretcher. Skintak® electrodes were used as follows: One electrode was placed at the base of the middle toe on the right foot and another electrode slightly above the line of the ankle joint between the medial and lateral malleoli. Another pair of electrodes was distributed at the base of the middle finger of the right hand, and slightly above the line of the right wrist joint, coinciding with the styloid process.

The device used was Biodynamics®, model 450, with an electric current intensity of 800 μ A and frequency of 50 kHz. Nominal voltage was 8.4 V, with a rated capacity of 600 mA/h. The amplitude of R was 200-1500 Ω , with a resolution of 0.1 Ω and accuracy of 0.1%. The amplitude of Xc was 0-300 Ω , the resolution was 0.1 Ω and the precision was 0.2%. The unit also had a 0°-20° PA amplitude, 0.1° resolution and 0°-2° accuracy.

PA

The PA was automatically provided by the equipment from the values of R and Xc. PA was classified according to the cut-off point of 5.4°, based on the reference parameters of the study by Fernandes *et al*^[6] and Selberg *et al*^[22], in which values below this point are considered predictive of a bad prognosis, and the values above are predictive of a good prognosis.

BIVA

In this method, the raw measures of the BIA (R and Xc) are used graphically, standardized by height in meters, and plotted as vector bivariate points, with their confidence and tolerance intervals, which are ellipses in the graphical plane RXc. The method is based on the analysis of the bivariate distribution of vector impedance in a healthy population. Graph RXc can be observed with the tolerance intervals of 50%, 75% and 95% of the impedance value (*i.e.*, the ellipses containing the vector values and the probabilities of 50%, 75% and 95%)^[19-21].

The upward or downward displacement of vectors in the direction of the largest axis (h) of the ellipse indicates progressive change in tissue hydration (dehydration towards the upper pole, hyperhydration with apparent edema toward the lower pole). Vectors migrating towards the lower axis (c) above, to the left, indicate more body cell mass and below, to the right, less body cell mass^[19].

BIVA measurement points were determined for each quadrant, considering areas between the h and c axes, according to body conditions (hydration and cellularity), being classified as: Quadrant 1 (Q1): More cellularity, more hydration; quadrant 2 (Q2): More cellularity, less hydration; quadrant 3 (Q3): Less cellularity, less hydration; and quadrant 4 (Q4): Less cellularity, more hydration (Figure 1).

Statistical analysis

Quantitative variables were described by the mean \pm SD and categorical variables by absolute and relative frequencies. One-way ANOVA was used to compare the means, complemented by the Tukey's test. In the comparison of proportions, the Chi-square test was applied along with the analysis of the adjusted residuals. For control of confounding factors, the Poisson regression analysis was applied to the factors that presented a $P < 0.10$ in the bivariate analysis.

The significance level adopted was 5% ($P \leq 0.05$) and the analyses were performed using the SPSS program version 21.0.

RESULTS

A total of 190 patients with a mean age of 56.6 ± 11.0 years were evaluated. Sixty-one percent of the patients were male. Of these, 56.3% had Child-Turcotte-Pugh (CTP) A score, and the most prevalent etiology was HCV (47.4%). The characteristics of the studied population are presented in Table 1.

A significant association ($P = 0.025$) between etiology and CTP score was observed, with CTP A more prevalent in patients with an etiology related to alcohol. Also, patients with HCV + alcohol etiology had a higher prevalence of CTP C (Figure 2).

The patient sample was plotted on the RXc chart and classified by BIVA quadrants, according to hydration and cellularity (Figure 3).

The patients were evaluated by the BIVA method in relation to age, sex, disease staging (CTP score), etiology and PA (Table 2). The Q2 patients had a lower mean age than those in the other quadrants ($P < 0.001$). Patients classified in Q1 and Q2 had higher PA than those in Q3 and Q4 ($P < 0.001$).

There was an association between the BIVA quadrants and CTP classification. The patients classified in Q2 had a significantly higher proportion of CTP A than the other quadrants. In addition, Q4 patients had a significantly higher CTP ratio than those in the other quadrants.

The association between staging and the CTP score according to the different BIVA quadrants is plotted on the BIVA graph (Figure 4).

Graphical representation of the BIVA shows the distribution of the patients evaluated according to staging of the disease. According to the CTP score, more severe disease was observed in Q4, using the prognosis by the PA values; the opposite was observed in Q2 ($P < 0.002$).

The sample showed an association between the BIVA quadrants and prognosis, using the PA values. There was a statistically significant association between the BIVA and PA classifications ($P < 0.002$), with the values of PA corresponding to a better prognosis in Q1 and Q2 ($P < 0.007$).

The population was evaluated according to age group in relation to PA, BIVA quadrants, gender, disease staging (CTP score) and etiology (Table 3). It was observed that PA was significantly lower in patients aged 50 years or older when compared to those younger than 40 years. Patients younger than 40 years also showed an association with BIVA Q2, and these same patients had a higher prevalence of other etiologies (Table 3).

Table 1 Characteristics of the patients included

| Variables ¹ | Total sample (n = 190) |
|------------------------|------------------------|
| Age (yr) | 56.6 ± 11.0 |
| Gender | |
| Male | 116 (61.1) |
| Female | 74 (38.9) |
| Child-Turcotte-Pugh | |
| A | 107 (56.3) |
| B | 48 (25.3) |
| C | 35 (18.4) |
| Etiology | |
| HCV | 90 (47.4) |
| Alcohol | 51 (26.8) |
| HCV + alcohol | 21 (11.1) |
| Other ² | 28 (14.7) |
| Phase angle | 6.06 ± 2.20 |

¹Described by mean ± SD or n (%).

²Indicates hepatitis B virus, autoimmune, cholestatic disease, non-alcoholic fatty liver disease, and cryptogenic. HCV: Hepatitis C virus.

When adjusted for age, patients with the etiology related to HCV + alcohol had a significantly higher prevalence of being classified as CTP C [hazard rate (HR) = 2.28, 95%CI: 1.12-4.67, $P = 0.024$] when compared to patients with HCV only. Also, patients with alcohol-related etiology had a 31% higher prevalence of CTP A when compared to those with HCV (HR = 1.31, 95%CI: 1.01-1.71, $P = 0.044$). When adjusted for age, CTP C patients had a 17% higher prevalence of being in Q4 by BIVA (HR = 1.17, 95%CI: 1.04-1.33, $P = 0.012$) when compared to Child A patients. The prevalence of bad prognosis by PA was approximately 5 times higher in patients classified in quadrants 3 (HR = 4.47, 95%CI: 2.70-7.40, $P < 0.001$) and 4 (HR = 5.64, 95%CI: 3.54-8.97, $P < 0.001$) when compared to patients in quadrants 1 and 2. When adjusted for age and CTP, the effect measures did not change in quadrants 3 (HR = 4.18, 95%CI: 2.51-6.97, $P < 0.001$) and 4 (HR = 5.01, 95%CI: 3.10-8.10, $P < 0.001$).

There was a statistically significant association between the classification of PA and CTP score ($P < 0.001$). Patients with CTP A were associated with a good prognosis and were classified in quadrants 1 and 2, and patients with CTP C had a bad prognosis and were classified in quadrants 3 and 4 (Table 4).

DISCUSSION

The evaluation of body composition in cirrhotic patients presents some difficulties in measurement due to its peculiarities, and relevant studies suggest that there is no gold standard for diagnosing clinical conditions, such as malnutrition, in these patients.

In the present study of adult cirrhotic patients, a higher proportion of males was observed, which was in accordance with previous studies^[23]. With regard to the classification of CTP, there is a stepwise progression of CTP A, B and C, and because these were outpatients, there was a greater number of CTP A and B than CTP C patients. The etiology of cirrhosis in this study was predominantly due to HCV, alcohol, and HCV associated with alcohol, and was a regional peculiarity, and may differ from other geographical locations^[6].

According to the BIVA, it was possible to differentiate patients according to the disease stage. Younger patients in the sample (50.3 ± 14.3 years) were less hydrated and had more cellularity (Q2), according to the BIVA, and with a greater number of patients classified as CTP A presenting higher values of PA, reflecting a better prognosis. On the other hand, patients classified as more hydrated (water retention)

Table 2 Characteristics of the patients according to the bioelectrical impedance vector analysis method, related to age, sex, staging (Child-Turcotte-Pugh score), etiology and phase angle

| Variables ¹ | Total sample (n = 190) | Classification quadrants - BIVA | | | | |
|------------------------|------------------------|---------------------------------|--------------------------|--------------------------|--------------------------|---------|
| | | Q1 (n = 91; 47.9%) | Q2 (n = 36; 18.9%) | Q3 (n = 27; 14.2%) | Q4 (n = 36; 18.9%) | P value |
| Age (yr) | 56.6 ± 11.0 | 56.8 ± 9.6 ⁴ | 50.3 ± 14.3 ³ | 57.7 ± 9.2 ⁴ | 61.6 ± 8.9 ⁴ | < 0.001 |
| Gender | | | | | | 0.642 |
| Male | 116 (61.1) | 54 (59.3) | 20 (55.6) | 17 (63.0) | 25 (69.4) | |
| Female | 74 (38.9) | 37 (40.7) | 16 (44.4) | 10 (37.0) | 11 (30.6) | |
| Child-Turcotte-Pugh | | | | | | 0.052 |
| A | 107 (56.3) | 52 (57.1) | 27 (75.0) | 13 (48.1) | 15 (41.7) | |
| B | 48 (25.3) | 25 (27.5) | 5 (13.9) | 9 (33.3) | 9 (25.0) | |
| C | 35 (18.4) | 14 (15.4) | 4 (11.1) | 5 (18.5) | 12 (33.3) | |
| Etiology | | | | | | 0.380 |
| HCV | 90 (47.4) | 45 (49.5) | 16 (44.4) | 13 (48.1) | 16 (44.4) | |
| Alcohol | 51 (26.8) | 29 (31.9) | 7 (19.4) | 7 (25.9) | 8 (22.2) | |
| HCV + alcohol | 21 (11.1) | 6 (6.6) | 4 (11.1) | 4 (14.8) | 7 (19.4) | |
| Other ² | 28 (14.7) | 11 (12.1) | 9 (25.0) | 3 (11.1) | 5 (13.9) | |
| Phase angle | 6.06 ± 2.20 | 6.49 ± 2.44 ⁴ | 7.30 ± 2.12 ⁴ | 5.07 ± 0.67 ³ | 4.46 ± 0.70 ³ | < 0.001 |

¹Described by mean ± SD or n (%).²Indicates hepatitis B virus, autoimmune, cholestatic disease, non-alcoholic fatty liver disease, and cryptogenic.³Numbers with superscript 3 do not differ statistically at 5% significance by Tukey's test.⁴Numbers with superscript 4 do not differ statistically at 5% significance by Tukey's test. BIVA: Bioelectrical impedance vector analysis; HCV: Hepatitis C virus; Q1: Quadrant 1; Q2: Quadrant 2; Q3: Quadrant 3; Q4: Quadrant 4.

and with lower cellularity (Q4) were older (61.6 ± 8.9 years), mostly with CTP C and with lower PA, and a possible association between greater severity and bad prognosis.

The BIVA has been studied in several clinical situations in an attempt to understand the human body composition in relation to hydration and cellularity alterations, such as heart failure, compensated cirrhosis, hemodialysis, chronic obstructive pulmonary disease and cancer^[12,17,24-26]. Norman *et al*^[9] established that the BIVA method reflects the actual state of hydration and composition of the cell mass, recognizing its importance in the evaluation and monitoring of possible modifications of body composition. This method has become an important tool in the management of cirrhotic patients.

The use of the BIVA method to determine the status of body fluids is well established in the literature and has been gaining prominence in the management of several diseases^[26]. A classic example where the BIVA method can be used systematically is in the assessment of body fluids of patients in the intensive care unit (ICU), mainly with the diagnosis of acute kidney injury (AKI), which is associated with increased mortality due to the disturbance in water balance^[27]. In the study by Hise *et al*^[28] which evaluated critically ill patients with AKI using the BIVA method, it was observed that the survivors presented vectors of longer and steeper groups, characterized by higher values of R and Xc ($P < 0.05$).

When the patients were plotted according to the clinical classification of CTP, it was possible to conclude that CTP A patients had higher cellularity and lower hydration, with PA values indicating a good prognosis. It was also possible to identify that patients with CTP C were more prevalent in Q4 and had a bad prognostic value assessed by PA ($P < 0.001$). These data corroborate with the findings of Fernandes *et al*^[6], in which the PA was associated with staging of the disease *via* the CTP score.

Guglielmi *et al*^[29] evaluated 810 cirrhotic patients with different etiologies using the BIVA method and compared them with a control group of 208 healthy individuals, and showed differences in hydration between the two groups. Similar findings were observed in the present study, where higher hydration status was associated with patients who had decompensated cirrhosis. On the other hand, the above controlled study did not evaluate the participants' cellularity.

The mean PA in this sample was 6.06 ± 2.20, similar to the findings of Fernandes

Table 3 Distribution of the sample by age group related to phase angle classification, bioelectrical impedance vector analysis quadrants, sex, Child-Turcotte-Pugh score and etiology

| Variables ¹ | Age (yr) | | | | P value |
|------------------------------|--------------------------|----------------------------|--------------------------|--------------------------|---------|
| | 20-39 (n = 11; 5.8%) | 40-49 (n = 32; 16.8%) | 50-59 (n = 66; 34.7%) | ≥ 60 (n = 81; 42.6%) | |
| PA | 7.67 ± 2.50 ⁴ | 6.68 ± 2.63 ^{3,4} | 5.79 ± 1.61 ³ | 5.83 ± 2.29 ³ | 0.014 |
| PA classification | | | | | 0.007 |
| Good prognosis | 11 (100) ⁵ | 24 (75.0) | 43 (65.2) | 43 (53.1) | |
| Bad prognosis | 0 (0.0) | 8 (25.0) | 23 (34.8) | 38 (46.9) ⁵ | |
| BIVA Quadrant classification | | | | | 0.002 |
| Q1 | 4 (36.4) | 15 (46.9) | 36 (54.5) | 36 (44.4) | |
| Q2 | 7 (63.6) ⁵ | 8 (25.0) | 10 (15.2) | 11 (13.6) | |
| Q3 | 0 (0.0) | 6 (18.8) | 5 (7.6) | 16 (19.8) | |
| Q4 | 0 (0.0) | 3 (9.4) | 15 (22.7) | 18 (22.2) | |
| Gender | | | | | 0.162 |
| Male | 9 (81.8) | 22 (68.8) | 42 (63.6) | 43 (53.1) | |
| Female | 2 (18.2) | 10 (31.3) | 24 (36.4) | 38 (46.9) | |
| Child-Turcotte-Pugh | | | | | 0.194 |
| A | 9 (81.8) | 18 (56.3) | 31 (47.0) | 49 (60.5) | |
| B | 2 (18.2) | 8 (25.0) | 17 (25.8) | 21 (25.9) | |
| C | 0 (0.0) | 6 (18.8) | 18 (27.3) | 11 (13.6) | |
| Etiology | | | | | 0.030 |
| HCV | 1 (9.1) | 14 (43.8) | 34 (51.5) | 41 (50.6) | |
| Alcohol | 3 (27.3) | 9 (28.1) | 16 (24.2) | 23 (28.4) | |
| HCV + alcohol | 1 (9.1) | 4 (12.5) | 6 (9.1) | 10 (12.3) | |
| Other ² | 6 (54.5) ⁵ | 5 (15.6) | 10 (15.2) | 7 (8.6) | |

¹Described by mean ± SD or n (%).²Indicates hepatitis B virus, autoimmune, cholestatic disease, non-alcoholic fatty liver disease, and cryptogenic.³Numbers with superscript 3 do not differ statistically at 5% significance by Tukey's test.⁴Numbers with superscript 4 do not differ statistically at 5% significance by Tukey's test.⁵Indicates statistically significant association by the test of the residuals adjusted to 5% of significance. PA: Phase angle; BIVA: Bioelectrical impedance vector analysis; Q1: Quadrant 1; Q2: Quadrant 2; Q3: Quadrant 3; Q4: Quadrant 4; HCV: Hepatitis C virus.

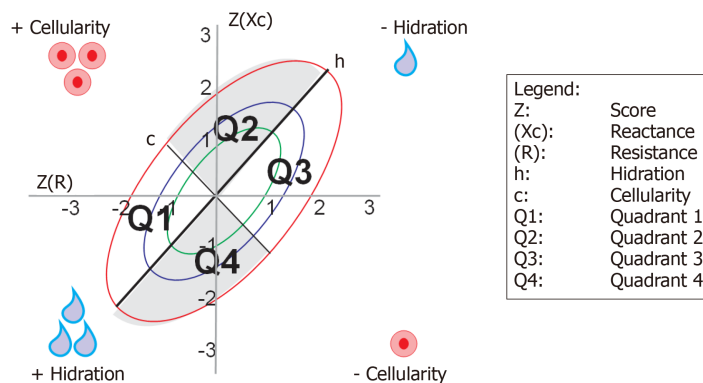
et al^[6] and Selberg *et al*^[22], where values below 5.4° were characterized as having a poor prognosis. When we analyzed the patients divided into two groups according to the PA cut-off point of 5.4°, differences were observed between the two groups, with younger patients, males and those with CTP A having a good prognosis, which may be indicative of the absence of sarcopenia in this population. Sarcopenia is characterized by progressive loss of skeletal muscle mass and strength, negatively influencing body homeostasis associated with functional limitations and morbidity and mortality^[30,31].

Studies have shown that PA is a good prognostic indicator in severe clinical situations^[6,10-14]. Gupta *et al*^[10] demonstrated that PA was a more potent indicator of survival than traditional nutritional assessment parameters, such as albumin, prealbumin and transferrin in patients with advanced pancreatic cancer, and showed that the cut-off point for PA was 5.0°. In a similar study of patients with advanced lung cancer, the patients were stratified using a cut-off point for PA of 4.5°^[11]. Alves *et al*^[12], in a study of chronic heart failure, identified that the BIVA method associated with PA was capable of identifying significant changes in the hydration state during the acute decompensation phase of the disease.

Stapel *et al*^[32] when assessing 196 patients in the ICU showed that patients with higher PA had a lower 90-day mortality rate than those patients with a low PA (5.0° ±

Table 4 Association of the classification of phase angle prognosis related to age, sex, staging (Child-Turcotte-Pugh) and etiology

| Variables ¹ | Classification PA – 5.4° | | P value |
|------------------------|----------------------------------|--------------------------------|---------|
| | Good prognosis (> 5.4°; n = 121) | Bad prognosis (< 5.4°; n = 69) | |
| Age (yr) | 54.2 ± 11.3 | 60.9 ± 9.0 | < 0.001 |
| Gender | | | 0.040 |
| Male | 81 (66.9) | 35 (50.7) | |
| Female | 40 (33.1) | 34 (49.3) | |
| Child-Turcotte-Pugh | | | < 0.001 |
| A | 81 (66.9) ³ | 26 (37.7) | |
| B | 26 (21.5) | 22 (31.9) | |
| C | 14 (11.6) | 21 (30.4) ³ | |
| Etiology | | | 0.060 |
| HCV | 51 (42.1) | 39 (56.5) | |
| Alcohol | 39 (32.2) | 12 (17.4) | |
| HCV + alcohol | 11 (9.1) | 10 (14.5) | |
| Other ² | 20 (16.5) | 8 (11.6) | |

¹Described by mean ± SD or n (%).²Indicates hepatitis B virus, autoimmune, cholestatic disease, non-alcoholic fatty liver disease, and cryptogenic.³Indicates association statistically significant by the test of the residuals adjusted to 5% of significance. PA: Phase angle; HCV: Hepatitis C virus.**Figure 1 Graphic representation of bioelectrical impedance vector analysis by quadrants and ellipses, according to body conditions.**

1.3° vs 4.1° ± 1.2°, $P < 0.001$). It is important to highlight that BIA was performed within 24 h of the patient's admission to the ICU, clearly showing that PA reflects the patient's physiological status (catabolism) and can be classified as a biological marker, as described by Marroni *et al*^[15].

Ruiz-Margáin *et al*^[17], in a pilot study of patients with compensated cirrhosis, the cut-off point for PA of 4.9° was established, indicating this bad prognostic factor is an independent risk factor of mortality. Belarmino *et al*^[33] obtained similar findings in a study of cirrhotic patients using the PA cut-off of ≤ 4.9° established by Ruiz-Margáin *et al*^[17], and observed that PA is an independent prognostic factor associated with mortality, and identified associations with poorer metabolic profiles, nutrition and disease progression. However, these two studies did not evaluate cellularity and body fluid in cirrhotic patients; thus, clinical and nutritional behaviors were not assessed early.

There are some limitations in the present study, such as the use of an Italian population as a reference, and the Piccoli Software^[19] to calculate the BIVA, as there are no data available for the Brazilian population. The population in the study region suffered great miscegenation, having a high Italian genetic component and therefore we believe that it does not significantly compromise the results.

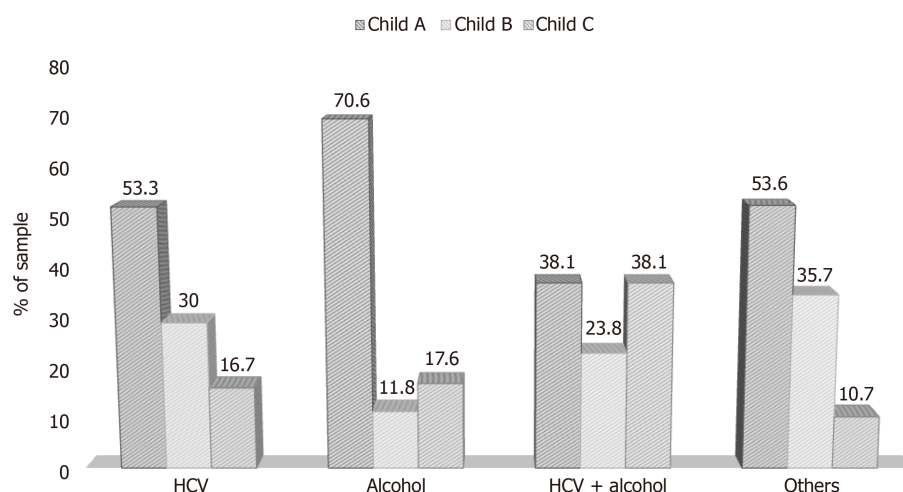


Figure 2 Association between etiology and the Child-Turcotte-Pugh score. Others refer to hepatitis B virus, autoimmune, cholestatic disease, non-alcoholic fatty liver disease, and cryptogenic. HCV: Hepatitis C virus.

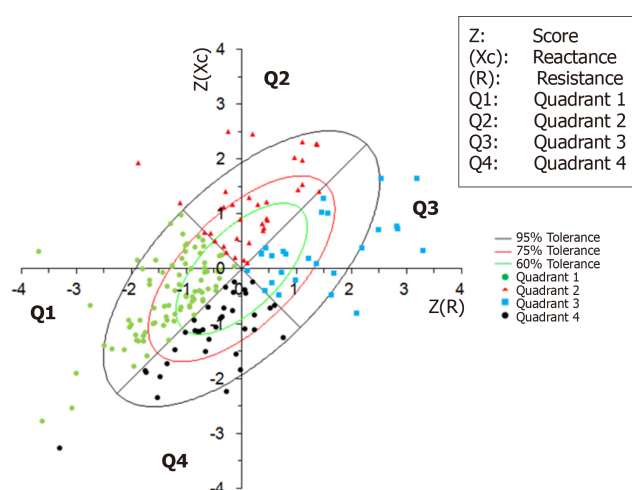


Figure 3 Bioelectrical impedance vector analysis - sample distribution.

BIVA offers advantages over traditional methods in evaluating body composition, due to its non-invasive nature and simplicity. BIVA has a methodological advantage over traditional BIA calculations due to its independence from regression equations. In addition, BIVA can facilitate longitudinal assessment of changes in body composition over time. These properties are useful for assessing nutrition and hydration in cirrhotic patients, who are unable to tolerate more invasive assessment methods. This research demonstrates the potential of using published BIVA data for further analysis, especially in decompensated cirrhotic patients.

The evaluation at different points in the disease trajectory can demonstrate changes in body composition over time. Our data demonstrate that body composition appears to be related to the clinical status of cirrhotic patients.

The main limitation of this study is that nutritional screening tools were not used, which makes it difficult to compare the nutritional basis. Therefore, our ability to assess how BIVA relates to nutritional status is limited.

A small number of studies were evaluated in this analysis, which only included English language studies, and it is possible that studies using BIVA in different cultural contexts have been excluded. There are challenges in using the BIVA method correctly when there is variability in how reference populations are chosen. The BIVA method does not provide quantitative data on body composition variables; therefore, stratification is required, according to clinical variables of BIVA data to determine clinically significant outcomes.

As already mentioned, evaluations of BIA were performed in clinical medical

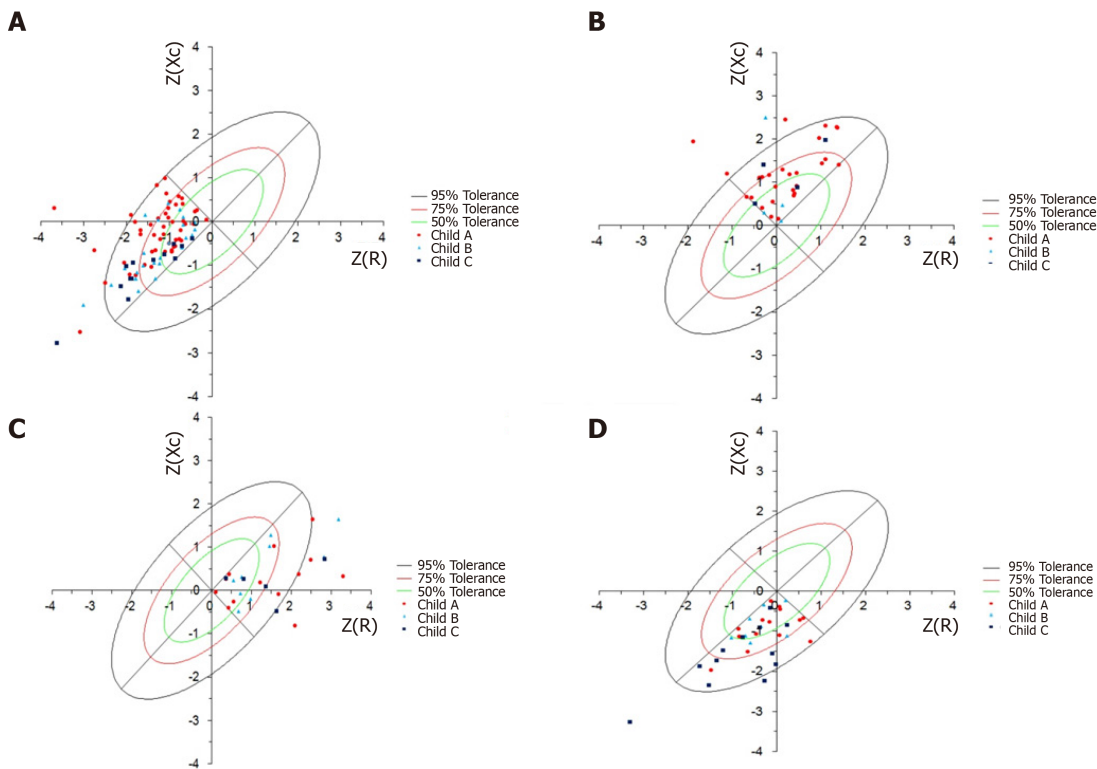


Figure 4 Classification of bioelectrical impedance vector analysis quadrants associated with Child-Turcotte-Pugh classification. A: Bioelectrical impedance vector analysis (BIVA) - patients plotted in quadrant 1; B: BIVA - patients plotted in quadrant 2 - higher proportion of Child-Turcotte-Pugh A; C: BIVA - patients plotted in quadrant 3; D: BIVA - patients plotted in quadrant 4 - greater proportion of Child-Turcotte-Pugh C ($P < 0.002$). Z: Score; (R): Resistance; (Xc): Reactance.

consultations, and not performed with the recommended preparation for the use of BIA. However, as the results of this study do not depend on pre-established formulas of the apparatus, where hydration is a limiting factor, we believe that this did not influence the results.

Implications for clinical and political practice

This study demonstrated the potential of using the BIVA method to perform comparative, multigroup analyses of body composition, to compare differences in cirrhotic patients according to the stage and type of disease. This has the potential to personalize therapeutic, nutritional and hydration interventions according to an individual's physiology.

More studies are needed to recommend the BIVA method for routine clinical use, due to the limited number of studies using this method.

CONCLUSION

In conclusion, the BIVA method allows identification of the cellularity and hydration status of cirrhotic patients associated with clinical factors to determine the severity of the disease, such as age, staging and PA. The BIVA method is a new tool for evaluating the body composition of cirrhotic patients, especially in patients with asymmetry, allowing an early and specific nutritional assessment in each case, which will help to improve the clinical condition of these patients.

ARTICLE HIGHLIGHTS

Research background

One of the main clinical complications of liver cirrhosis is protein-calorie malnutrition, the prevalence of which can vary from 10% to 100%, regardless of the stage and etiology of the disease, but which negatively interferes with the general prognosis of

the disease. Therefore, determining the behavior of body composition (cellularity and hydration) using the bioelectrical impedance vector analysis (BIVA) method, seems to be a promising method for improving the health of patients with liver cirrhosis, expanding their life expectancy and quality of life.

Research motivation

There are few studies on the assessment of body composition and functioning in cirrhotic patients, which directly impacts the overall clinical management of these patients. We believe that with the BIVA method we can gain a new tool for analyzing body homeostasis in this population.

Research objectives

The aim of this study was to evaluate the results of the BIVA regarding hydration and cellularity, and compare them with the phase angle and other clinical parameters in cirrhotic patients.

Research methods

This was a retrospective cross-sectional study with data collected between May 2007 and December 2015, at the Santa Casa de Misericórdia Hospital Complex in Porto Alegre, RS, Brazil. The data obtained were related to the protocol for routine pre- and postoperative care at the service's outpatient clinic. Quantitative variables were described by the mean and standard deviation and the categorical variables by absolute and relative frequencies. One-way ANOVA was used to compare the means, complemented by Tukey's test. In the comparison of proportions, the Chi-square test was applied along with the analysis of adjusted residuals. For control of confounding factors, the Poisson regression analysis was applied to the factors that presented a $P < 0.10$ in the bivariate analysis.

Research results

A total of 190 patients with cirrhosis undergoing outpatient follow-up were included for data collection. The BIVA method showed an association with the staging of cirrhosis, showing worsening of cellularity (integrity and functionality) and worsening of the hydroelectrolytic distribution in patients with greater disease severity.

Research conclusions

The BIVA method makes it possible to identify the cellularity and hydration status of cirrhotic patients, being associated with clinical factors that determine the severity of the disease, such as age, staging and PA.

Research perspectives

The BIVA method is a new tool for evaluating body composition in cirrhotic patients, especially in those with asymmetry, allowing an early and specific nutritional assessment in each case, and helps to improve the clinical condition of these patients.

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Retrospective Study

Incidental biliary dilation in the era of the opiate epidemic: High prevalence of biliary dilation in opiate users evaluated in the Emergency Department

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Author contributions: Barakat MT and Banerjee S were involved in conception and design of the study as well as collection, analysis and interpretation of the data in collaboration with the Stanford University Statistics Consulting Service; Barakat MT and Banerjee S were involved in drafting and critical revision of the article for important intellectual content; Banerjee S granted final approval of the article.

Institutional review board

statement: This study was approved by the Stanford University Institutional Review Board (Protocol No. 41605), with associated HIPPA and Consent Waivers.

Informed consent statement:

Informed consent signature not required from patients for this retrospective cohort study, per terms of Institutional Review Board (IRB) protocol approval.

Conflict-of-interest statement: The authors have no conflicts of interest related to this study.

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Abstract

BACKGROUND

Biliary dilation is frequently related to obstruction; however, non-obstructive factors such as age and previous cholecystectomy have also been reported. In the past two decades there has been a dramatic increase in opiate use/dependence and utilization of cross-sectional abdominal imaging, with increased detection of biliary dilation, particularly in patients who use opiates.

AIM

To evaluate associations between opiate use, age, cholecystectomy status, ethnicity, gender, and body mass index utilizing our institution's integrated informatics platform.

METHODS

One thousand six hundred and eighty-five patients (20% sample) presenting to our Emergency Department for all causes over a 5-year period (2011-2016) who had undergone cross-sectional abdominal imaging and had normal total bilirubin were included and analyzed.

RESULTS

Common bile duct (CBD) diameter was significantly higher in opiate users compared to non-opiate users (8.67 mm *vs* 7.24 mm, $P < 0.001$) and in patients with a history of cholecystectomy compared to those with an intact gallbladder (8.98 *vs* 6.72, $P < 0.001$). For patients with an intact gallbladder who did not use opiates ($n = 432$), increasing age did not predict CBD diameter ($r^2 = 0.159$, $P = 0.873$). Height weakly predicted CBD diameter ($r^2 = 0.561$, $P = 0.018$), but weight,

Data sharing statement: No additional data are available.

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body mass index, ethnicity and gender did not.

CONCLUSION

Opiate use and a history of cholecystectomy are associated with CBD dilation in the absence of an obstructive process. Age alone is not associated with increased CBD diameter. These findings suggest that factors such as opiate use and history of cholecystectomy may underlie the previously-reported association of advancing age with increased CBD diameter. Further prospective study is warranted.

Key Words: Biliary dilation; Opiate; Narcotic; Endoscopic ultrasound; Endoscopic retrograde cholangiopancreatography; Bile duct

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Core Tip: What is current knowledge? Biliary dilation is often related to an obstructing process. Non-obstructive factors such as age and prior cholecystectomy have also been associated with biliary dilation. Rates of opiate use have dramatically increased within the United States over the past two decades. There has also been a dramatic increase in utilization of cross-sectional abdominal imaging over the past two decades. What is new here. Opiate use is associated with biliary dilation in the absence of an obstructive process. Increasing opiate use and increasing utilization of imaging are resulting in increased incidental detection of biliary dilation leading to increased referrals for endoscopic workup. Contrary to conventionally held views, our study indicates that age alone is not associated with increased bile duct diameter. Increasing probability of opiate use and cholecystectomy with advancing age may underlie the previously-reported association of advancing age with increased bile duct diameter. Height is weakly associated with increased bile duct diameter, consistent with an organ scaling effect.

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INTRODUCTION

Bile duct dilation is commonly related to an obstructive process such as a stone, stricture or a mass. However, biliary dilation has also been associated with non-obstructive factors such as advanced age and previous cholecystectomy^[1]. The role of other patient factors such as height, weight, body mass index (BMI), and substance use in modulating biliary dilation have not been well defined.

The opioid epidemic sweeping across the United States, has resulted in a 3-fold increase in opiate prescriptions since 1999. Approximately 255.2 million opioid prescriptions were reported in 2012, corresponding to a staggering 81.3 prescriptions per 100 United States residents^[2,3]. Despite the high prevalence of opiate use in the United States, the impact of opiates on bile duct diameter remains under-studied. Data are limited to only a few case series, some of which suggest that opiate use may be associated with dilatation of the bile duct in the absence of biliary obstruction^[4,5]. However, small sample size, and lack of controls have limited the generalizability of these observations^[4-6].

Additionally, perhaps in association with the ongoing national obesity epidemic, rates of cholecystectomy have increased over time, with over 900000 annual cholecystectomies currently performed in the United States^[7]. Following cholecystectomy, it is widely accepted that the bile duct increases in diameter^[1]. In 1894 Oddi postulated that the bile duct dilates following cholecystectomy so as to serve as a reservoir of bile—the pressure of which must then overcome the biliary sphincter pressure to enable bile to flow into the intestine^[8]. Despite the longstanding

recognition of this phenomenon, systematic evaluations of the impact of cholecystectomy on bile duct diameter have only emerged over the past 5 years^[9]. The extent to which other patient factors may modulate the occurrence and the degree of biliary dilation following cholecystectomy remains to be determined.

Studies of the impact of aging on bile duct diameter in adults are similarly limited. In children, bile duct diameter increases with advancing age in relative proportion to a child's growth curve^[10]. In adults, some studies with limited sample sizes have suggested that common bile duct (CBD) diameter gradually increases with age in healthy adults^[1,11,12]; however other studies have not demonstrated this trend^[13].

In parallel with the progressively aging population, the ongoing opiate and obesity epidemics, and the rising rates of cholecystectomy, utilization of cross-sectional abdominal imaging has more than tripled over the past two decades^[14,15]. An unintended consequence of this escalating utilization of cross sectional imaging is detection of incidental biliary dilation^[14]. At our tertiary care academic endoscopy unit, over the last decade, we have noted a 5-fold increase in referrals for endoscopic evaluation of incidentally detected biliary dilation with normal bilirubin in opiate users. Although the majority of these patients were referred for Endoscopic Retrograde Cholangiopancreatography (ERCP), we opted to perform endoscopic ultrasound (EUS) for these patients, as this is a lower risk procedure. However EUS has not revealed pancreatic or biliary pathology in the vast majority of these patients.

Given the escalating endoscopic burden of this important problem, it would be informative to determine when biliary dilation is within the range of expected variation given the clinical context and characteristics of the patient, and when biliary dilation is more pronounced than would be expected, implying obstructive pathology which warrants further diagnostic evaluation. We therefore undertook a formal, controlled study on over 1500 patients to evaluate factors such as opiate use, age, cholecystectomy status, gender, ethnicity, height, weight and BMI which might predict increased bile duct diameter in patients with normal liver function tests and no visualized obstructive process on cross-sectional imaging.

MATERIALS AND METHODS

We utilized an informatics platform, the Stanford Translational Research Integrated Database Environment (STRIDE) integrated standards-based platform^[16]. This informatics resource consists of integrated components including a clinical data warehouse, which is based on the HL7 Reference Information Model, with clinical information on over 2 million pediatric and adult patients cared for at Stanford University Medical Center since 1995 and an application development framework for building research data management applications and initiating queries on the STRIDE platform^[16].

Utilizing this STRIDE informatics platform and a retrospective cohort study design, we evaluated a 20% sample of patients over 18 years of age presenting to our Emergency Department (ED) for all causes over a 5-year period (2011-2016). We identified patients who had undergone computed tomography (CT) or magnetic resonance imaging (MRI) scans of the abdomen with documentation of CBD diameter, and who had normal bilirubin with no evidence of biliary obstruction on imaging using our institutional informatics platform. Opiate use status is a mandatory question for all patients who are cared for in our ED. We extracted opiate use status responses from the electronic medical record (EMR) for all patients. Gallbladder status, age, gender, height, weight, BMI and ethnicity were also determined from the EMR.

Student's *t*-test was performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA). Reported *p*-values are 2-sided, and comparisons attained statistical significance at $P < 0.05$. Linear regression analysis was conducted using standard techniques and categorical age analysis was performed by comparison of decades. This study was approved by the Stanford University Institutional Review Board (Protocol No. 41605).

RESULTS

This study included 1685 patients, 46% female and 54% male. There were 867 patients in the opiate user cohort and 818 in the non-opiate user cohort (mean age = 54.5 years *vs* 58.6 years, $P = 0.20$). Gender did not predict CBD diameter ($P = 0.12$). Stated ethnicity was only available for 56% of patients. For patients in whom ethnicity data

were available, ethnicity did not predict CBD diameter ($P = 0.09$). Height and weight data were available for 86% of patients in this sample. Height weakly predicted CBD diameter ($r^2 = 0.561$, $P = 0.018$), but weight and body mass index did not ($r^2 = 0.177$, $P = 0.29$, $r^2 = 0.210$, $P = 0.21$, respectively).

The mean CBD diameter was significantly higher in opiate users compared to non-opiate users (8.67 mm *vs* 7.24 mm, $P < 0.001$, **Table 1**). The mean CBD diameter was also significantly higher in patients with a history of cholecystectomy compared to those with an intact gallbladder (8.98 mm *vs* 6.72 mm, $P < 0.001$). The lowest CBD diameter was evident in patients with an intact gallbladder who did not use opiates, with sequentially increasing diameters noted in patients with an intact gallbladder who used opiates, and in those with prior cholecystectomy who did not use opiates, with the largest mean CBD diameter observed in patients with a history of both cholecystectomy and opiate use (**Figure 1**). Gallbladder status appeared to modulate the effect of opiates on bile duct diameter. Among patients with an intact gallbladder, opiate users had a CBD diameter that was 43.5% greater than non-opiate users. In contrast, among patients with a history of cholecystectomy, opiate users had a CBD diameter that was only 6.5% greater than non-opiate users (**Table 1**, **Figure 1**). When 7 mm was used as the threshold for normal bile duct diameter in all patients regardless of age and cholecystectomy status, 72% of opiate using patients had biliary dilation, as compared with only 27% of non-opiate using patients (**Figure 2**).

Importantly, increasing age did not significantly correlate with CBD diameter upon analysis as a continuous variable ($r^2 = 0.159$, $P = 0.873$) or across age group categories ($P = 0.217$, **Figure 3**), for the population of patients with an intact gallbladder who did not use opiates ($n = 432$). Increasing age weakly predicted ($r^2 = 0.439$, $P = 0.027$) increased CBD diameter in patients with a history of opiate use and/or a history of cholecystectomy ($n = 1356$). When all patient cohorts were grouped for analysis, including opiate users and non-users, and patients with and without a history of cholecystectomy ($n = 1685$), advancing age very weakly predicted ($r^2 = 0.306$, $P = 0.038$) increased CBD diameter when analyzed as a continuous variable and across age group categories (**Figure 3**).

DISCUSSION

Prescription and illicit use of opiates has increased dramatically over the last 2 decades, with the emergence and escalation of a nationwide opiate epidemic^[2,3]. The number of cholecystectomies performed annually in the United States has increased by more than 20%^[7], and utilization of abdominal imaging has also increased approximately 3-fold over the same time period^[14]. Age has previously been considered a factor associated with biliary dilation^[1,11,13] and the proportion of the United States population aged over 65 has progressively increased and is projected to continue increasing. We therefore sought to evaluate the impact of each of these parameters on biliary dilation. It has been our impression that these concurrent phenomena have led to the increasing incidental detection of bile duct dilation in patients, which in turn is driving increased utilization of invasive, expensive and potentially risky endoscopic procedures. In our own practice we have noted a 5-fold increase in referrals for EUS and ERCP over the past decade, for patients with biliary dilation and a normal total bilirubin. Over 60% of these referrals in 2018 had concurrent opiate use. A few small previous case series have suggested that opiate use may be associated with biliary dilation; however, small sample size, study populations focused on opiate-dependent patients, confounding variables and lack of controls have limited the generalizability of these observations^[4-6].

Our study, the largest conducted to date evaluating the association between opiate use and bile duct diameter, demonstrates that opiate use is a modulating factor associated with biliary dilation in the setting of a normal bilirubin. Patients who have both undergone cholecystectomy and use opiates have the largest CBD diameters overall. The impact of opiate use on CBD dilation is most striking in patients with an intact gallbladder. The opiate impact is muted in patients who have undergone cholecystectomy, perhaps related to a ceiling effect, given the significant pre-existing dilatory effect of cholecystectomy on the bile duct. We find that height is positively correlated with CBD diameter, consistent with an organ scaling effect. Our data indicate that patient weight, BMI, ethnicity and gender are not correlated with bile duct diameter.

Age has long been held to modulate bile duct diameter – this conventional wisdom is commonly asserted in radiology and gastroenterology textbooks. A standard

Table 1 Common bile duct diameter varies with opiate use and cholecystectomy status

| Cholecystectomy status | Non opiate users, mean (SD) CBD diameter in mm | Opiate users, mean (SD) CBD diameter in mm | P value |
|--------------------------------------|--|--|------------------|
| All patients | 7.24 (2.28), <i>n</i> = 818 | 8.67 (1.89), <i>n</i> = 867 | <i>P</i> < 0.001 |
| Gallbladder intact (<i>n</i> = 814) | 5.58 (1.38), <i>n</i> = 432 | 8.01 (1.83), <i>n</i> = 382 | <i>P</i> < 0.001 |
| Gallbladder absent (<i>n</i> = 871) | 8.72 (1.86), <i>n</i> = 386 | 9.30 (1.72), <i>n</i> = 485 | <i>P</i> < 0.001 |

SD: Standard deviation; CBD: Common bile duct.

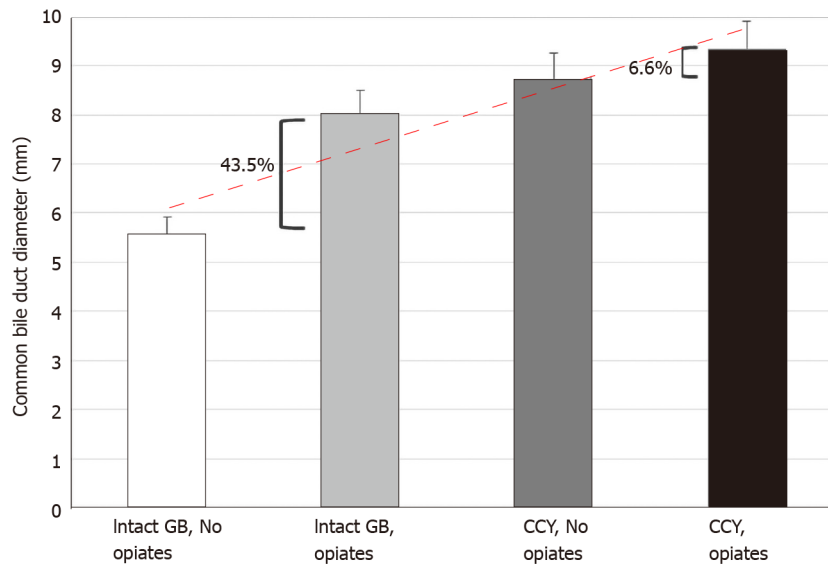


Figure 1 Common bile duct diameter varies with gallbladder and opiate use status. Bar graph depicting mean common bile duct (CBD) diameter for groups categorized by gallbladder status and opiate use. The lowest CBD diameter is seen in patients with an intact gallbladder who did not use opiates, with sequentially increasing diameters noted in patients with an intact gallbladder who used opiates, and in those with prior cholecystectomy who did not use opiates, with the largest mean CBD diameter observed in patients with a history of both cholecystectomy. Trendline (red) depicts this trend, error bars depict standard deviation. Calipers indicate percentage change between the means of indicated categories. GB: Gallbladder; CCY: Cholecystectomy.

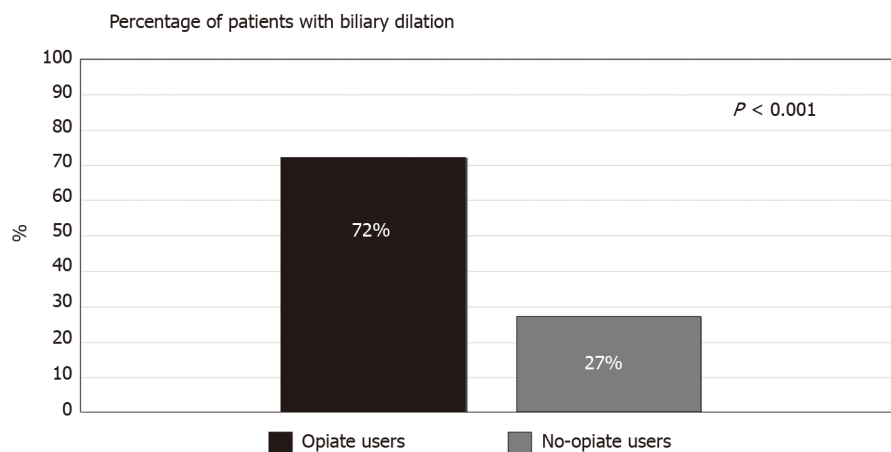


Figure 2 Percentage of patients with biliary dilation. Bar graph depicting proportion of all patients, regardless of age and cholecystectomy status, with biliary dilation when 7 mm was used as the threshold for normal bile duct diameter. With this threshold of normal bile duct diameter, 72% of opiate using patients had biliary dilation, as compared with only 27% of non-opiate using patients.

radiology textbook, for example, indicates that an estimate of normal bile duct diameter at a given age may be roughly derived from considering a 4 mm bile duct diameter normal at age 40, and assuming a 1 mm increase in bile duct diameter for each subsequent decade of life^[17]. The proposed association between age and CBD diameter was supported by a few limited studies conducted over 25 years ago, which concluded that CBD diameter is age-dependent^[18-20]. However, a subsequent small prospective study did not demonstrate this association between age and bile duct diameter^[13]. Our large study has not demonstrated an independent role for age in modulating CBD diameter in the absence of a history of cholecystectomy or opiate use. Our data suggest for the first time that other factors which modulate CBD diameter (cholecystectomy, opiate use) may account for the assertions in previous studies regarding increasing bile duct diameter with age. Further prospective study of this association is warranted.

Workup of incidentally detected biliary dilation in opiate users reflects yet another previously-unrecognized cost of the opiate epidemic. In recent years, cross-sectional imaging of the abdomen has supplanted abdominal radiography as the most frequently reimbursed abdominal imaging study^[15]. Integrated health care systems and Medicare data demonstrate that for every 100 Medicare beneficiaries, over 50 CT scans, 50 abdominal ultrasounds and 15 abdominal MRIs are performed annually^[21,22]. A subset of these patients undergo this imaging for evaluation of non-specific abdominal pain for which they may be prescribed opiates and may potentially then develop associated biliary dilation. Incidental findings from these imaging studies may then result in a cascade of healthcare expenses related to additional studies, diagnostic workup, procedures and ongoing surveillance, each with associated patient anxiety and the potential for adverse events^[14].

In this era of escalating health care costs, our study indicates that the detection on imaging of incidental bile duct dilation without a visualized obstructing process in known opiate users with normal liver function tests may not require expensive and potentially risky endoscopic evaluation. However, the complexity of the problem of incidentally detected biliary dilation must also be acknowledged. The rising rates of Non Alcoholic Fatty Liver Disease (NAFLD) and increased rates of statin utilization in the United States population result in associated liver function tests (LFT) abnormalities in up to 20% of NAFLD patients^[23-25] and around 3% of statin users^[26,27]. LFT abnormalities in these and in similar scenarios will impact the workup of patients referred for workup of incidental biliary dilation.

Additionally, valid concerns of referring and consulting physicians should be acknowledged. Sensitivity for detection of pancreatic adenocarcinoma, ranges from 76%-96% for CT^[28-35] and from 83%-93.5% for MRI^[30-35], with higher sensitivity corresponding with larger masses^[31,33,35]. Additionally, in 5.4%-18.4% of patients with pancreatic malignancy, the lesion is isoattenuating relative to the background pancreas, with smaller lesions more likely to be isoattenuating^[36-39]. Furthermore, cholangiocarcinoma, another concerning potential etiology of biliary obstruction and resultant dilation, often does not present as a mass on cross-sectional imaging and may be only partially occlusive, with incipient obstruction resulting in biliary dilation before significant LFT abnormalities develop^[40-42]. Taken together, these limitations of cross sectional imaging, and the implicit potential for missed malignant lesions, prompt referring physicians to request additional evaluation for patients with incidental biliary dilation and biliary specialists to proceed with additional endoscopic evaluation.

Limitations of this study include its retrospective nature, reliance on data from the electronic medical record and reliance on reports from multiple radiologists for documentation of bile duct diameter. However, the large sample size should neutralize these effects. Due to limitations in opiate type, duration and use pattern details included within the electronic medical record, it was not possible to associate these parameters with CBD diameter. Prospective study of these phenomena would be informative to construct recommendations for when endoscopic evaluation of biliary dilation is most appropriate in the setting of these study limitations.

CONCLUSION

In conclusion, our data indicate that opiate use is associated with bile duct dilation in the absence of an obstructive process. We confirm that a history of prior cholecystectomy is associated with increased CBD diameter. We demonstrate that, in adult populations, height positively correlates with CBD diameter. Finally, we

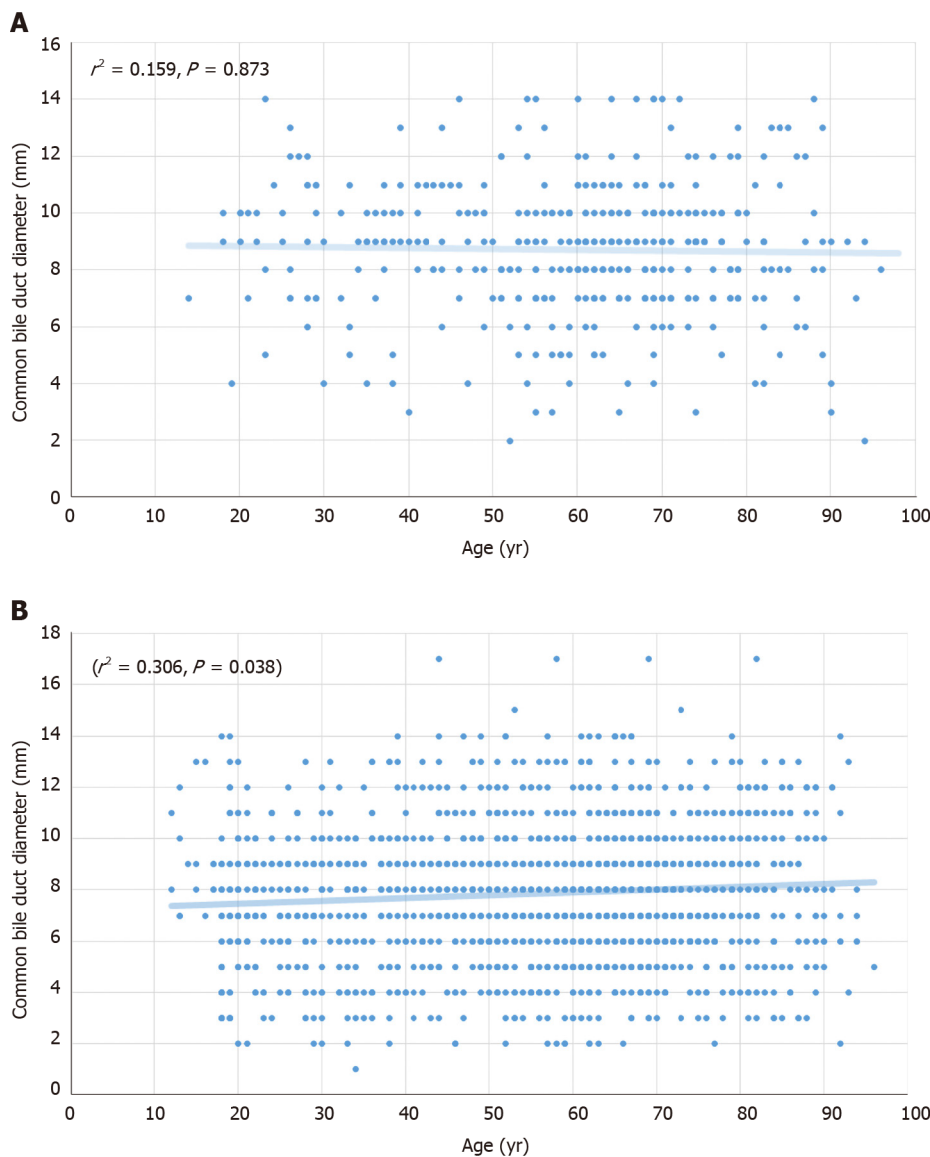


Figure 3 Overall (all groups combined), age weakly predicts common bile duct diameter, suggesting that cholecystectomy status and opiate use may be more common in older individuals and this may be driving previously-described associations between age and biliary dilation. A: Advancing age weakly predicts increased CBD diameter in all patient groups combined; B: Age is not predictive of CBD diameter in patients with an intact gallbladder who do not use opiates.

demonstrate that advancing age does not independently predict a larger CBD diameter in our analysis, and previously-reported associations of advancing age with larger CBD diameter may be attributable instead to other variables such as cholecystectomy and opiate use. Our data suggest that incidentally detected biliary dilation without a visualized obstructive process in the setting of normal bilirubin in known opiate users may not require expensive and potentially risky endoscopic evaluation.

ARTICLE HIGHLIGHTS

Research background

Bile duct dilation is often related to an obstructive process such as a stone, stricture or a mass. The role of other patient factors such as height, weight, body mass index, and substance use in modulating biliary dilation have not been well defined.

Research motivation

In the past two decades, both opiate use/dependence and utilization of cross-sectional

abdominal imaging have sharply increased. We have noted an increase in referrals to our academic tertiary care medical center for incidentally detected biliary dilation, particularly in patients who use opiates.

Research objectives

Our goal was to evaluate associations between opiate use, age, cholecystectomy status, ethnicity, gender, and body mass index to understand how these factors may be related to biliary dilation.

Research methods

We evaluated associations between opiate use, age, cholecystectomy status, ethnicity, gender, and body mass index utilizing our institution's integrated informatics platform. We evaluated 1685 Emergency Department patients (a 20% sample from 2011-2016) who had undergone cross-sectional abdominal imaging and had normal total bilirubin.

Research results

Diameter of the common bile duct was significantly higher in opiate users compared to non-opiate users (8.67 mm *vs* 7.24 mm, $P < 0.001$) and in patients with a history of cholecystectomy compared to those with an intact gallbladder (8.98 *vs* 6.72, $P < 0.001$). For patients with an intact gallbladder who did not use opiates ($n = 432$), increasing age did not predict common bile duct (CBD) diameter ($r^2 = 0.159$, $P = 0.873$).

Research conclusions

A history of cholecystectomy and opiate use are associated with common bile duct dilation in the absence of an obstructive process. Age alone does not appear to be associated with increased common bile duct diameter.

Research perspectives

These findings suggest that factors such as opiate use and history of cholecystectomy may underlie the previously-reported association of advancing age with increased CBD diameter. Future prospective study would be desirable to expand upon these findings.

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Clinical Trials Study

Effect of non-alcoholic beer, diet and exercise on endothelial function, nutrition and quality of life in patients with cirrhosis

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Abstract

BACKGROUND

The implementation of nutritional strategies targeting several variables at once could benefit patients with cirrhosis. Non-alcoholic beer has different compounds that exert antioxidant, anti-inflammatory and nutritional properties.

AIM

To evaluate the effect of diet + exercise and non-alcoholic beer on nutritional status, endothelial function and quality of life in patients with cirrhosis.

METHODS

In this randomized open clinical trial, patients with cirrhosis were randomized into two groups: The intervention (non-alcoholic beer + diet + exercise) and control (water + diet + exercise) group. Treatment consisted of 330 mL non-alcoholic beer/day or the same amount of water, plus an individualized dietary plan and an exercise program with a pedometer-based bracelet to reach at least

Orea-Tejeda A provided endothelial function assessment; Macías-Rodríguez RU, Ruiz-Margáin A, Román-Calleja BM and Flores-García NC completed the supervision; Macías-Rodríguez RU, Ruiz-Margáin A, Román-Calleja BM, Espin-Nasser ME, Flores-García NC, Galicia-Hernández G, Fernández-del-Rivero G and Lozano-Cruz O wrote the original draft; Macías-Rodríguez RU, Ruiz-Margáin A, Galicia-Hernández G and Orea-Tejeda A reviewed and edited the manuscript; all authors have read and agreed to the published version of the manuscript.

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Clinical trial registration statement:

This study is registered at <https://clinicaltrials.gov>. The registration identification number is NCT04041115.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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5000 steps/d and > 2500 above the baseline during 8 wk. Endothelial function (flow-mediated dilation, plethysmography), biochemical and nutritional variables and quality of life (CLDQ) were evaluated.

RESULTS

Forty-three patients were included in the study, 21 in the control group and 22 in the intervention group. The mean age was 53.5 ± 7.8 years, 60% were women, the median MELD score was 8 (7-10) and most patients were Child-Pugh A (88%). Adherence to the interventions was > 90% in both groups, there were no adverse events and all biochemical parameters remained stable in both groups. Endothelial function improved in both groups. All measured nutritional parameters improved in the intervention group, compared to only 2 in the control group and quality of life improved in both groups; however, more domains improved in the intervention group.

CONCLUSION

The intervention consisting of non-alcoholic beer, diet and exercise seems to be safe and well tolerated in patients with cirrhosis, and shows improvement in nutritional status, endothelial function, and quality of life. These results need to be further confirmed.

Key Words: Hops; Portal hypertension; Sarcopenia; Diet; Cirrhosis; Antioxidants

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Core Tip: Malnutrition is a frequent complication in patients with cirrhosis and it is associated with adverse outcomes. Diet and physical exercise are strategies that have shown a beneficial effect on nutritional status. On the other hand, non-alcoholic beer has several nutrients including vitamin B, minerals and flavonoids that make it an attractive "functional" supplement for patients with cirrhosis. The present study evaluated the effect of a multifactorial intervention that included diet, exercise and non-alcoholic beer, showing that it is safe and well tolerated. This intervention showed improvement in endothelial function, quality of life and nutritional status including muscle mass.

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INTRODUCTION

Liver cirrhosis is a frequent disease, leading to poor quality of life and representing the 13th most common cause of death globally^[1,2]. Most of the complications in cirrhosis arise from the development of portal hypertension, including ascites, variceal hemorrhage, hepatorenal syndrome and hepatic encephalopathy^[2-4].

Malnutrition is a frequent complication in patients with cirrhosis, being present in up to 40%-90% of the population during the evolution of the disease^[5]. Malnutrition involves different clinical manifestations, including sarcopenia and cachexia, having higher prevalence in the late stages of cirrhosis^[5-8]. One of the most important facts regarding malnutrition in cirrhosis, is its association with mortality and the development of other complications, such as hepatic encephalopathy^[9,10]. Therefore, any intervention aimed at improving the nutritional status could be helpful in the outcome in patients with cirrhosis and portal hypertension^[11].

Nutritional therapy in patients with cirrhosis includes both the implementation of diets and physical exercise programs^[12-16]. General recommendations of nutritional

established a priori with a legally binding contract to ensure the independence of the research. The non-alcoholic beer used in this study does not belong to or is produced by the Mexican Beer Council (MBC). None of the authors have any actual or past relationship with the company producing the non-alcoholic beer.

Data sharing statement: Participant data that underlie the results reported in this article after deidentification (Text, tables, figures, appendices) and other documents (study protocol, statistical analysis plan and analytic code) are available on request from the corresponding author at ricardomacro@yahoo.com.mx.

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therapy are focused on providing a sufficient and non-restricted energy and protein supply, together with a high amount of fiber^[17,18]. Other useful recommendations in this population include frequent meals and nocturnal supplementation with different nutrients, as well as the use of branched-chain amino acids (BCAAs)^[19-22]. Nutritional supplementation with BCAAs has been extensively studied, and its benefits in patients with cirrhosis are widely recognized, specifically targeting protein metabolism^[17,23-26]. However, implementation of this nutritional strategy is sometimes difficult due to various factors, including availability and tolerability, as well as high cost. The lack of other options in the different clinical settings, considering the factors mentioned above, renders mandatory searching for other options that could be helpful in the nutritional management of these patients.

On the other hand, non-alcoholic beer has several nutrients derived from its ingredients (yeast, xanthohumol and hops), including vitamin B, minerals and flavonoids^[27], rendering it an attractive nutritional supplement in patients with cirrhosis. In addition to these effects, non-alcoholic beer has been shown to beneficially modify gut microbiota diversity^[28], it also improves endothelial function and oxidative stress^[29], and has been used in different clinical settings, including breastfeeding, and post-exercise rehydration among others^[30,31]. Therefore, non-alcoholic beer can be regarded as a "functional" supplement. Physical exercise has proven to be a safe and effective intervention in cirrhosis, providing several benefits, for instance, improvement in nutritional status, quality of life and portal pressure^[16,32,33].

Considering all the previous evidence, the use of a multifactorial intervention, including diet, exercise and a non-alcoholic beer as a supplement could be of benefit in patients with cirrhosis and portal hypertension, influencing the different components of malnutrition in this population. Therefore, the aim of this study was to evaluate the effect of a multifactorial intervention, with diet, exercise and non-alcoholic beer on nutritional parameters, endothelial function, quality of life and safety in patients with cirrhosis and portal hypertension.

MATERIALS AND METHODS

Patients and methods

This was a randomized open-clinical trial, performed at a third level center in Mexico City from March 2015 to September 2018 (Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán). The study protocol was approved by the local Research and Ethics Committee at the institution and registered at ClinicalTrials.gov. It was designed and conducted according to the principles of the Declaration of Helsinki and all patients signed the informed consent before study inclusion.

Inclusion criteria

The inclusion criteria were: (1) Patients with liver cirrhosis established by liver biopsy or the presence of markers of dysfunction in hepatocyte synthesis and portal hypertension (esophageal varices on endoscopy, ascites and other compatible features on ultrasonography); and (2) Patients aged 18-70 years.

Exclusion criteria

Patients with the following characteristics were excluded: (1) Genetic liver disease (hemochromatosis and Wilson's disease); (2) Alcoholic liver disease (including current alcohol intake); (3) History of cancer (including hepatocellular carcinoma); (4) Systemic inflammatory response syndrome; or (5) Decompensation during the past 6 wk.

Intervention

Patients were randomized into 2 groups: (1) Control (diet + exercise + water); and (2) Intervention (diet + exercise + non-alcoholic beer). Randomization was carried out in blocks of 6 patients, using the web-based page randomization.com, and concealment was done with sequentially numbered, opaque, and sealed envelopes.

Randomization, enrollment, and assignment of patients to the groups were performed by different members of the study team.

Diet: Caloric intake was calculated using the Harris-Benedict equation to estimate the resting metabolic rate (RMR) plus 10% of the thermic effect of food and in addition, 20% of calories were added in order to prevent the catabolic effect of exercise. The proportion of macronutrients included was 60% of carbohydrates, 1.3-1.5g of protein/kg body weight/d, and the rest from lipids. Finally, sodium intake was

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restricted (60-90 mEq/d or 1.5-2 g/d of salt) in patients with ascites or edema.

In terms of distribution, 60% of the total caloric intake was obtained from carbohydrates of which 50% were complex carbohydrates, protein was calculated as 1.3-1.5g of protein/kg body weight/d, and the distribution of proteins was 50% vegetable protein and 50% animal protein, and the remaining calories were derived from fat, with less than 10% of saturated fat and 5%-15% of unsaturated fat.

The dietary plan was calculated according to the individual caloric requirement of each patient, with the same macronutrient distribution, as stated in the ESPEN guidelines. The plan was created and prescribed by a trained nutritionist specialized in the field of hepatology and liver transplantation.

The patients received a dietary plan based on exchangeable food sources, where they received the permitted portions of each food group according to the Mexican System of Equivalent Foods (SMAE), the food groups are vegetables, fruits, cereals (with and without fat), legumes, animal-origin foods (with different amounts of fat), milk, fats/oils (with and without protein) and sugars (with and without fat). The dietary plan included a list of all the permitted foods in each food group and the patients were only allowed to eat the food that was listed.

The foods that were excluded from the list were those normally excluded from the diet in patients with chronic liver disease; mainly, canned foods, cold meats such as sausages, ham, salami, *etc.*, high fat meat, high fat dairy, and overly processed drinks.

Exercise: For the physical exercise program, each patient received a physical activity tracker, in the form of a bracelet-based accelerometer, able to measure the number of steps (*i.e.*, functioning as a pedometer, PolarLoop, POLAR, Finland). During the 10-wk period of the study, the patients were allocated to a program that included the following: (1) Educational session: The first intervention that the patients received (both groups), was a 15-min presentation on general information related to cirrhosis and its complications, as well as the benefits, the indications and contraindications of physical exercise and non-alcoholic beer; (2) In the first 2 wk, the participants received a training program in order to learn how the device works and the appropriate use of the activity monitor tracker, as well as the correct record of the baseline physical activity in each patient, before starting the intervention; and (3) In the remaining 8 wk, the patient gradually increased their baseline physical activity, aiming to reach > 2500 steps/d above the average baseline level and a total number of steps of 5000/d. Intensity of exercise was set using the Borg Scale of perceived exertion (scale from 6 to 20), and each patient was trained to reach a target intensity of 10-12 (corresponding to light to moderate intensity and from 3 to 3-6 metabolic equivalents (METs))^[34]. For training, we used a long hallway with a flat surface ([Supplementary Figure 1](#)) allowing the patient to use the monitor, learn how it works and how to reach the intensity of exercise through a chart depicting the required level of effort (Borg scale, [Supplementary Figure 2](#)). There should be no differences in exercise prescription between men and women according to the guidelines^[34,35]. Nutritional requirements were adjusted according to sex.

Non-alcoholic beer nutritional supplementation: Each patient in the intervention group received 1 can (330 mL) of non-alcoholic beer daily (O'Doul's®, St. Louis, MO, United States), which was indicated to be consumed together with food during lunch, for 8 wk. Caloric and nutritional intake derived from the non-alcoholic beer (0.4% alcohol, 90 kcal/350 mL, 0 g fat, 18 g carbohydrates/350 mL, 1.9 g protein/350 mL), was included in the daily caloric intake mentioned previously (detailed information regarding the components and ingredients of the non-alcoholic beer are shown in [Supplementary Material Appendix 1](#)). The control group received a 330 mL bottle of water, in order to control the possible adverse events resulting from the increased fluid intake.

Follow up during the study

Patients were followed for 10 wk (2 wk to determine the baseline physical activity and to adapt to the monitoring device, and 8 wk of intervention), they were evaluated onsite at weeks -2, 0, 4 and 8. In each visit, adverse events, vital signs and nutritional variables were evaluated, as well as adherence to the diet/exercise program and non-alcoholic beer/water consumption by counting the empty cans or water bottles that patients were asked to take back to the center.

At baseline and final evaluations, biochemical tests (liver function tests, serum electrolytes, creatinine, glucose, complete blood count and international normalized ratio (INR)) were performed in every patient, according to the standards of the central laboratory in our institution.

Outcome measurements

Nutritional status: At baseline evaluation, weight and height were measured and later weight at each subsequent visit.

Bioimpedance analysis (BIA) was performed using a monofrequency device at 50 kHz (RJL systems, Quantum IV), at baseline and final evaluations, after an overnight 8-h fasting period, with an empty bladder and removing any metal objects the patient may be carrying. After placing four electrodes, two in the right hand and two in the right foot, the measurement was performed to obtain resistance (R), reactance (Xc) and phase angle (PhA). Malnutrition was considered when PhA was below 4.9° as previously validated and standardized in patients with cirrhosis^[36].

Anthropometry was performed at baseline and final evaluations, including arm circumference, mid-arm circumference (MAC), triceps skinfold thickness, thigh and calf circumferences, and handgrip strength.

Triceps skinfold thickness (TST) was measured on the non-dominant arm to the nearest mm using a Harpenden caliper and mid-arm circumference was measured on the non-dominant arm to the nearest 0.1 cm with a non-stretchable measuring tape. Mid-arm muscle circumference (MAMC), was calculated from the MAC and the TST using the formula $MAMC (mm) = MAC (mm) - (3.14 \text{ TST in mm})$. Subjects were considered malnourished when TST and/or MAMC were below the 5th percentile.

Handgrip strength (HGS) was measured with the patient seated using a Jamar handgrip dynamometer (Patterson Medical, Warrenville, IL, United States). Measurements were made according to the manufacturer's instructions and recorded to the nearest kg.

Quality of life: Quality of life was assessed with the chronic liver disease questionnaire (CLDQ), and the 36-item short form health survey (SF-36) in all the participants before and after completion of the study.

Neurocognitive function: At baseline and final evaluations, both the psychometric hepatic encephalopathy score (PHES) and critical flicker frequency (CFF, Hepatonorm Analyzer R&R Medi-Business Freiburg, Freiburg, Germany), were performed in all the participants.

Endothelial function: Endothelial function was measured using plethysmography. While the patient was sitting, the baseline plethysmography wave was recorded for 30 s, and thereafter ischemia was induced by insufflating an upper arm blood pressure cuff 30 mmHg above the systolic pressure for 5 min. Finally, a new wave was recorded for 120 s after deflating the arm cuff (post-ischemia wave). Every 30 s the plethysmography wave was recorded and contrasted with the baseline wave, and the maximal amplitude time (MAT) and the total time (TT) of the wave were calculated. Endothelial dysfunction was considered when the value of the MAT/TT ratio was < 30, as has been described previously^[37]. The person who performed the study was unaware of the group status of the patient.

Statistical analysis

Sample size was estimated according to a previous study in patients with cirrhosis allocated to a physical exercise program, where nutritional status evaluated through BIA derived-phase angle, showed an improvement after exercise, from a baseline of $5.8^\circ \pm 0.89$ to $6.0^\circ \pm 0.81$ at the end of the study^[33]; this difference was $0.2^\circ \pm 0.89$ (3.4%), and for the present study a total change of 10% was expected ($\Delta = 0.58$). Finally, with α β error of 0.05 and 0.2, and a drop out of 10% the final number was 21 patients per group.

Normality of the data was evaluated with the Shapiro-Wilk test. Data are presented as mean \pm SD, median (P25-P75) or frequencies. Results at baseline and final evaluations in each group (paired data) were analyzed with the Wilcoxon signed-rank test. For comparisons between groups, the Mann-Whitney U or Student's *t*-test was used. Areas under the curve (AUC) were constructed with the repeated values (time 0', 30', 60', 90' and 120'), of the MAT/TT ratio obtained at baseline and final evaluations. In this analysis, only those patients receiving at least 1 d of intervention were included (modified intention to treat, mITT). Finally, to control for baseline differences, ANCOVA was performed. Statistical analysis was carried out with the package software SPSS version 20.0 (Armonk, NY, United States) and figures were created using GraphPad Prism 5.

RESULTS

In total, 44 patients were included in the study (21 in the control group and 23 in the multifactorial intervention) (Figure 1). Only 1 patient in the intervention group was lost during follow-up. The final analysis included 21 patients in the control group, and 22 in the intervention group (mITT).

In the total population, most of the patients were women (60.5%), mean age was 53.5 ± 7.8 years, and the main etiologies of cirrhosis were hepatitis C infection (32.6%), autoimmune hepatitis (20.9%) and non-alcoholic fatty liver disease (NAFLD) (18.6%). All patients were Child-Turcotte-Pugh (CTP) A (88.4%) and B (11.6%), with a median CTP score of 5 (5-6) and model for end-stage liver disease (MELD) score of 8 (7-10). The only baseline difference in biochemical parameters was a higher alkaline phosphatase level in the intervention group ($P = 0.039$) (Table 1). There were no differences between the groups regarding severity of disease, presence of complications or age.

The exercise program was successful in both groups, reaching an increase of almost 3000 steps/d after the intervention ($P < 0.001$ in both groups). In the biochemical parameters, only a mild improvement in transaminases and an increase in the number of platelets were observed in the control and intervention group, respectively. The remaining parameters such as the liver function tests, as well as creatinine, glucose, and serum electrolytes, showed no change compared to their baseline values (Table 2).

Of the subjects with endothelial dysfunction at the beginning of the study, in the control group 8 of 11 (72.7%) patients improved endothelial function at the end of the study, and 5 of 6 patients (83.3%) in the multi-intervention group. This change was significant in both groups ($P < 0.001$) (Figure 2).

Endothelial function was further evaluated by the AUC of the MAT/TT ratio and an improvement was also seen in both groups; however, the behavior of the curves was different among groups (Table 3).

Figure 3 shows the changes in the MAT/TT ratio at baseline and final evaluations. Regarding the baseline evaluation, the behavior of the curve (repeated measurements over time) was similar in both groups, both showing higher MAT/TT ratio at time 120' compared to time 0', although a higher MAT/TT ratio was observed in the control group at time 0'. In terms of the final evaluation (end of interventions) a steadier curve was observed in the group receiving non-alcoholic beer, as well as a return to the time 0 values, showing better endothelial function, which was not observed in the control group.

Hemodynamic variables including heart rate, diastolic, systolic, and mean arterial pressure remained stable throughout the study (Table 3).

One of the most important results in this study was the improvement in nutritional status, where several nutritional markers (thigh circumference, HGS and sit-to-stand test), improved after the multifactorial intervention, compared to an increase only in the sit-to-stand test in the control group (Table 4). Adherence to the diet was evaluated at each visit, and it was found that in both groups adherence to the diet was $> 90\%$ in the first month, and $> 95\%$ in the following weeks. There was no statistical difference in the raw value of MAMC, but when this parameter was analyzed by the diagnosis of malnutrition with percentiles, a significant improvement was observed in the intervention group ($P = 0.045$) (Figure 4). There was a trend showing a more frequent improvement in PhA in the intervention group, where 63.6% of the patients receiving non-alcoholic beer had an improvement, compared to 47.6% of patients that did not receive non-alcoholic beer ($P = 0.290$, for the difference between the groups).

Almost all the individual components, as well as the overall score of the quality of life CLDQ score, improved after the intervention in the group that received non-alcoholic beer, compared to only minor changes in the control group (Table 5). Finally, in the SF-36 there was an improvement in the physical function role in both groups, in the general health role in the control group and in the vitality role in the multifactorial intervention group. Globally, the improvement in quality of life was higher in the group receiving non-alcoholic beer.

In order to adjust for baseline differences among the groups, ANCOVA was also performed, controlling for baseline MAMC, number of steps, AUC for endothelial function, PhA, HGS, sit-to-stand test, and CLDQ (global score). The main results, including PhA, remained significant after adjustment for these baseline variables (Supplementary Material Appendix 2).

Adverse events

There were no reported adverse events or side effects derived from the intervention, this was evidenced by the fact that there were no changes in biochemical tests at the

Table 1 Baseline characteristics of the study population

| | All (n = 43) | Control (n = 21) | Intervention (n = 22) | P value |
|----------------------------------|---------------------|--------------------|-----------------------|---------|
| Sex n (%) F/M | 26 (60.5)/17 (39.5) | 12 (46.2)/9 (52.9) | 14 (53.8)/8 (47.1) | 0.663 |
| Age (yr) | 53.5 ± 7.8 | 53.7 ± 8.2 | 53 ± 7.6 | 0.768 |
| BMI (kg/m ²) | 29.5 ± 4.2 | 29.2 ± 3.7 | 29.8 ± 4.8 | 0.635 |
| Etiology of cirrhosis | | | | |
| HCV n (%) | 14 (32.6) | 6 (28.5) | 8 (36.4) | 0.063 |
| AIH n (%) | 9 (20.9) | 3 (14.3) | 6 (27.3) | |
| NAFLD n (%) | 8 (18.6) | 5 (23.8) | 3 (13.6) | |
| Others ¹ n (%) | 12 (27.9) | 7 (33.4) | 5 (22.7) | |
| History of complications [n (%)] | | | | |
| Variceal bleeding | 2 (4.7) | 2 (9.5) | 0 (0) | 0.233 |
| Encephalopathy | 1 (2.3) | 0 (0) | 1 (4.5) | 0.500 |
| Ascites | 5 (11.6) | 3 (14.3) | 2 (9.1) | 0.664 |
| Child-Pugh stage | | | | |
| A | 38 (88.4) | 20 (95.2) | 18 (81.9) | 0.345 |
| B | 5 (11.6) | 1 (4.8) | 4 (18.1) | |
| Child-Pugh | 5 (5-6) | 5 (5-6) | 5 (5-6) | 0.617 |
| MELD score | 8 (7-10) | 8 (7.5-9.5) | 8.5 (7-10) | 0.524 |
| Biochemical parameters | | | | |
| TB (mg/dL) | 1.19 (0.82-1.95) | 1.15 (0.75-1.44) | 1.28 (0.85-2.11) | 0.284 |
| ALT (U/L) | 43 (26-77) | 43 (30-86.5) | 43.1 (20.75-77) | 0.882 |
| AST (U/L) | 48 (37-93) | 47 (40-80) | 50.5 (32-98) | 0.881 |
| AP (mg/dL) | 142.2 ± 57.7 | 123.7 ± 44.1 | 159.8 ± 64.4 | 0.039 |
| Albumin (g/dL) | 4.0 (3.6-4.2) | 4.1 (3.8-4.3) | 3.9 (3.3-4.1) | 0.170 |
| Leukocytes (K/μL) | 4.2 (3.7-5.3) | 4.0 (3.7-5.15) | 4.75 (3.725-5.925) | 0.233 |
| Platelets (K/μL) | 80 (62-125) | 77 (61.5-125) | 93.5 (63.5-125) | 0.520 |
| Hemoglobin (g/dL) | 14.1 ± 1.94 | 14.2 ± 3.3 | 14.0 ± 2.4 | 0.755 |
| INR | 1.1 (1.1-1.2) | 1.1 (1.1-1.2) | 1.1 (1.1-1.2) | 0.870 |
| Glucose (mg/dL) | 91 (83-103) | 94 (83.5-109) | 91 (81-100.5) | 0.882 |
| Creatinine (mg/dL) | 0.72 (0.63-0.80) | 0.72 (0.60-0.81) | 0.71 (0.64-0.79) | 0.892 |
| Sodium (mmol/L) | 139 (137-141) | 140 (137.5-141) | 138 (137-140.2) | 0.290 |
| Potassium (mmol/L) | 4.08 ± 0.32 | 4.0 ± 0.37 | 4.1 ± 0.25 | 0.198 |
| CO ₂ (mmol/L) | 24 (21-26) | 23.0 (21.5-26) | 24.5 (21-27.25) | 0.518 |

¹Other etiologies included cryptogenic, primary biliary cholangitis and overlap syndrome (HAI/CBP). Data reported as mean ± standard deviation or median (p25-p75), according to the distribution of the data, or absolute frequency (percentage %). BMI: Body mass index; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis; NAFLD: Non-alcoholic fatty liver disease; TB: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AP: Alkaline phosphatase; INR: International normalized ratio.

end of the intervention in either group, or worsening quality of life specifically of abdominal symptoms.

DISCUSSION

Nutritional status in cirrhosis has a central role in the prognosis of the disease,

Table 2 Changes in clinical and biochemical parameters

| | Control (n = 21) | | | Intervention (n = 22) | | |
|----------------------------|------------------|------------------|---------|-----------------------|--------------------|---------|
| | Baseline | Final | P value | Baseline | Final | P value |
| Number of daily steps | 8718 ± 2998 | 11391 ± 4298 | 0.000 | 8533 ± 4072 | 11142 ± 4055 | 0.000 |
| PHES score | -1 ± 2.2 | 0 ± 2.2 | 0.130 | -1 ± 2.1 | 0 ± 2.3 | 0.179 |
| CFF (Hz) | 43.9 ± 7.1 | 45.5 ± 5.8 | 0.238 | 44.5 ± 8.1 | 47.2 ± 6.4 | 0.174 |
| TB (mg/dL) | 1.15 (0.75-1.44) | 1.12 (0.85-1.28) | 0.985 | 1.28 (0.85-2.11) | 1.13 (0.8-1.865) | 0.068 |
| ALT (U/L) | 43 (30-86.5) | 41 (23.5-71) | 0.024 | 43.1 (20.75-77) | 36 (20.25-67.5) | 0.198 |
| AST (U/L) | 47 (40-80) | 45 (29.5-53.5) | 0.015 | 50.5 (32-98) | 44 (28-75.75) | 0.035 |
| Alkaline phosphatase (U/L) | 123.7 ± 44.1 | 105 (79.50-139) | 0.408 | 159.8 ± 64.4 | 159.9 ± 79.3 | 0.988 |
| Albumin (g/dL) | 4.1 (3.8-4.3) | 4 (3.75-4.2) | 0.668 | 3.9 (3.3-4.1) | 3.9 (3.475-4.3) | 0.501 |
| Leukocytes (K/μL) | 4.0 (3.7-5.15) | 3.9 (3-5) | 0.984 | 4.75 (3.725-5.925) | 4.9 (3.62-6.1) | 0.515 |
| Platelets (K/μL) | 77 (61.5-125) | 75 (59-106.5) | 0.278 | 93.5 (63.5-125) | 107 (78.5-150.25) | 0.046 |
| Hemoglobin (g/dL) | 14.2 ± 3.3 | 14.17 ± 1.52 | 0.968 | 14.0 ± 2.4 | 14.25 ± 2 | 0.834 |
| INR | 1.1 (1.1-1.2) | 1.1 (1.1-1.2) | 0.414 | 1.1 (1.1-1.2) | 1.1 (1.0-1.2) | 0.166 |
| Glucose (mg/dL) | 94 (83.5-109) | 88 (82-108) | 0.145 | 91 (81-100.5) | 87.5 (84.75-96.25) | 0.615 |
| Creatinine (mg/dL) | 0.72 (0.60-0.81) | 0.71 (0.6-0.84) | 0.403 | 0.71 (0.64-0.79) | 0.70 (0.62-0.79) | 0.723 |
| Na (mmol/L) | 140 (137.5-141) | 140 (139-141.5) | 0.118 | 138 (137-140.2) | 140 (138.5-141) | 0.061 |
| K (mmol/L) | 4.0 ± 0.37 | 4.16 ± 0.38 | 0.195 | 4.1 ± 0.25 | 4.2 ± 0.37 | 0.078 |
| CO ₂ (mmol/L) | 23.0 (21.5-26) | 24.0 (22-26) | 0.850 | 24.5 (21-27.25) | 24 (22.95-26.15) | 0.304 |

Data reported as mean ± standard deviation, or median (p25-p75), according to the distribution of the data. PHES: Psychometric hepatic encephalopathy score; CFF: Critical flicker frequency; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio.

Table 3 Endothelial function and hemodynamic variables

| | Control (n = 21) | | P value | Intervention (n = 22) | | P value |
|---------------------------|-----------------------|---------------------|---------|-----------------------|---------------------|---------|
| | Baseline | Final | | Baseline | Final | |
| AUC | 103.13 (92.55-107.15) | 93.15 (86.53-99.61) | 0.021 | 98.04 (92.17-102.35) | 93.62 (85.06-99.60) | 0.046 |
| Systolic pressure (mmHg) | 120 (110-130) | 120 (120-129) | 0.418 | 120 (110-120) | 113 (110-120) | 0.513 |
| Diastolic pressure (mmHg) | 80 (60-80) | 80 (60-80) | 0.862 | 70 (67.5-80) | 70 (60-80) | 0.385 |
| Mean arterial pressure | 90 (76.693.3) | 93.3 (78.3-96) | 0.729 | 86.6 (82.5-93.3) | 83.3 (76.6-90.5) | 0.385 |
| Heart rate (bpm) | 62 (60-72.7) | 63 (60.25-80) | 0.079 | 69 (61.5-75.2) | 67 (61.5-71.25) | 0.235 |

Data reported as median (p25-p75). AUC: Area under the curve.

influencing both mortality (*i.e.*, it has been associated with mortality) and the development of cirrhosis-related complications^[38]. Therefore, any intervention able to positively modify malnutrition in patients with cirrhosis deserves special attention. In the present work, we studied the effect of a multi-level nutritional intervention, including diet, exercise, and non-alcoholic beer, in the nutritional status of patients with cirrhosis.

Even though there are studies addressing the effect of exercise and diet in cirrhosis^[16,32], this is the first study showing the effect of adding a cheap, easy to access and consume and rational-based nutritional supplement (*i.e.*, non-alcoholic beer). Although BCAA supplements play an important role in the nutritional management of patients with advanced cirrhosis^[11], their use is limited in some clinical settings due to availability and cost, as well as poor palatability and gastrointestinal symptoms such as bloating, nausea and abdominal pain^[39,40]. On the other hand, non-alcoholic beer has

Table 4 Changes in nutritional parameters at baseline and final evaluation

| | Control (n = 21) | | P value | Intervention (n = 22) | | P value |
|--------------------------|------------------|------------|---------|-----------------------|-------------|---------|
| | Baseline | Final | | Baseline | Final | |
| Phase angle | 6.0 ± 0.7 | 5.9 ± 0.6 | 0.427 | 5.8 ± 0.7 | 5.9 ± 0.7 | 0.198 |
| MAMC (cm ²) | 24.1 ± 3.1 | 23.9 ± 3.5 | 0.462 | 23.5 ± 3.4 | 23.6 ± 3.9 | 0.783 |
| TST (mm) | 25.5 ± 7.9 | 24.7 ± 4.5 | 0.397 | 26.6 ± 6.7 | 26.9 ± 6.3 | 0.782 |
| Thigh circumference (cm) | 53.1 ± 6.5 | 52.9 ± 7.0 | 0.758 | 51.7 ± 6.4 | 53.1 ± 4.6 | 0.033 |
| Handgrip strength (kg) | 19.9 ± 11.5 | 21 ± 11.2 | 0.121 | 18.1 ± 10.3 | 19.9 ± 10.5 | 0.048 |
| Sit-To-Stand test (s) | 24.2 ± 4.4 | 20.9 ± 6.9 | 0.001 | 23.3 ± 3.9 | 21.0 ± 6.2 | 0.045 |

Data reported as mean ± standard deviation. MAMC: Mid-arm muscle circumference; TST: Triceps skinfold thickness.

Table 5 Changes in quality of life

| | Control (n = 21) | | P value | Intervention (n = 22) | | P value |
|---------------------|------------------|--------------|---------|-----------------------|--------------|---------|
| | Baseline | Final | | Baseline | Final | |
| CLDQ questionnaire | | | | | | |
| Abdominal symptoms | 5.4 ± 1.3 | 5.2 ± 0.8 | 0.592 | 5.0 ± 1.5 | 5.1 ± 1.2 | 0.982 |
| Fatigue | 4.7 ± 1.1 | 5.2 ± 0.8 | 0.063 | 4.2 ± 1.2 | 5.1 ± 1.2 | 0.000 |
| Systemic symptoms | 5.6 ± 1.0 | 5.6 ± 0.9 | 0.892 | 4.3 ± 1.2 | 5.0 ± 0.9 | 0.002 |
| Activity | 5.3 ± 1.2 | 5.3 ± 1.3 | 0.892 | 4.8 ± 1.2 | 5.5 ± 1.2 | 0.028 |
| Emotional function | 4.9 ± 0.9 | 5.3 ± 1.1 | 0.056 | 4.3 ± 1.3 | 5.3 ± 1.0 | 0.000 |
| Worry | 5.1 ± 1.1 | 5.3 ± 1.1 | 0.521 | 4.3 ± 1.8 | 5.2 ± 1.8 | 0.002 |
| CLDQ overall score | 5.2 ± 0.8 | 5.4 ± 0.9 | 0.204 | 4.6 ± 1.0 | 5.3 ± 0.8 | 0.001 |
| SF-36 questionnaire | | | | | | |
| Physical function | 80 (70-90) | 95 (80-95) | 0.005 | 72.5 (65-95) | 87.5 (70-95) | 0.007 |
| Physical role | 75 (25-100) | 100 (50-100) | 0.166 | 62.5 (25-100) | 75 (25-100) | 0.468 |
| Body pain | 84 (66-90) | 84 (72-90) | 0.341 | 78 (61-84) | 78 (60-84) | 0.725 |
| General health | 40 (37-47) | 47 (35-52) | 0.044 | 40 (25-55) | 42 (32-52) | 0.375 |
| Vitality | 65 (50-70) | 70 (60-75) | 0.453 | 62.5 (50-75) | 70 (50-80) | 0.037 |
| Social function | 100 (75-100) | 88 (75-100) | 0.875 | 75 (63-100) | 88 (75-100) | 0.080 |
| Emotional role | 67 (33-83) | 67 (33-67) | 0.972 | 67 (33-72) | 67 (33-67) | 0.752 |
| Mental health | 72 (68-84) | 84 (64-88) | 0.868 | 76 (64-84) | 82 (68-92) | 0.073 |

Data reported as mean ± standard deviation, or median (p25-p75), according to the distribution of the data. CLDQ: Chronic liver disease questionnaire; SF-36: 36-item short form health survey.

different compounds that can be useful in the nutritional management of patients with cirrhosis, and it is an attractive way of providing these compounds in those patients who usually have a limited variety of food. These were the main reasons for exploring the use of non-alcoholic beer in this population, together with a personalized diet and exercise, and involving a multidisciplinary group, as recommended in the guidelines^[11].

First of all, the exercise program was created to be easy to follow, specifically by using pedometer-based bracelets to monitor exercise, and it was well understood and well implemented by the patients in both groups, allowing them to successfully reach the aimed number of steps per day (at least > 2500 per day above the baseline level), with an adherence > 90%. This is especially important because in this study, one of the main concerns was to provide a completely outpatient multifactorial approach,

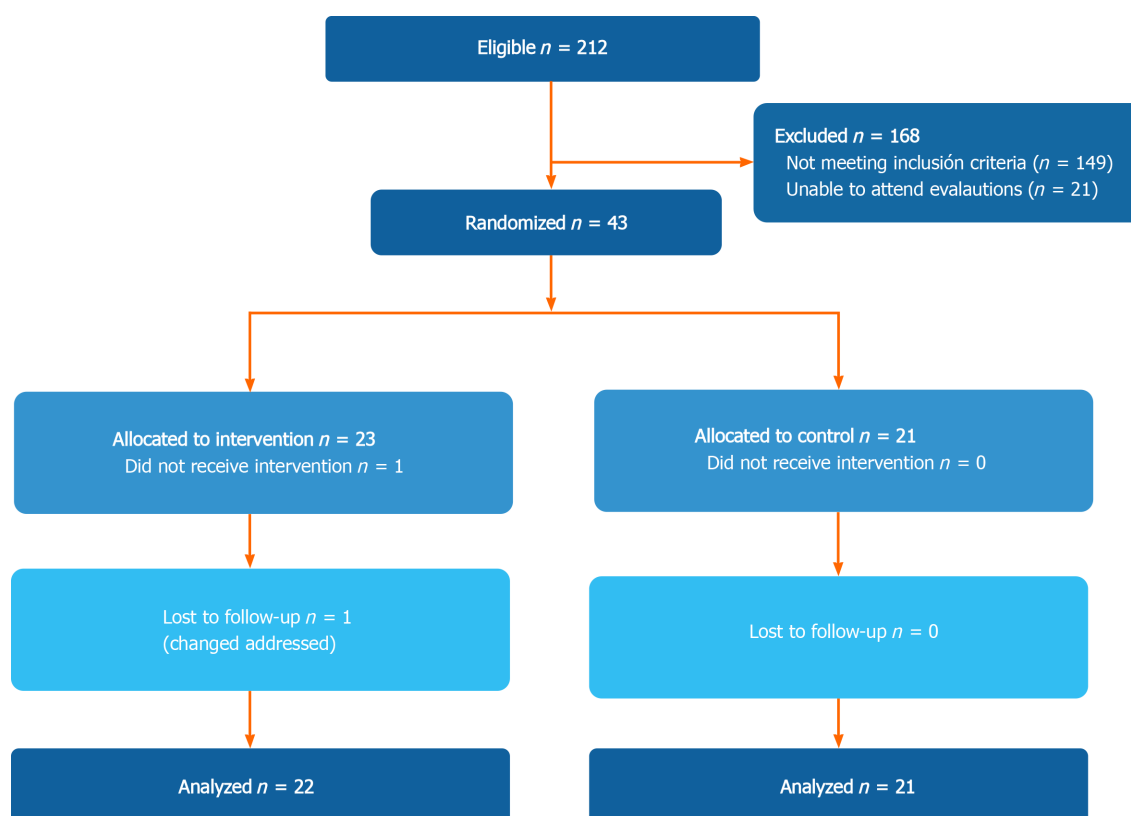


Figure 1 CONSORT enrollment diagram.

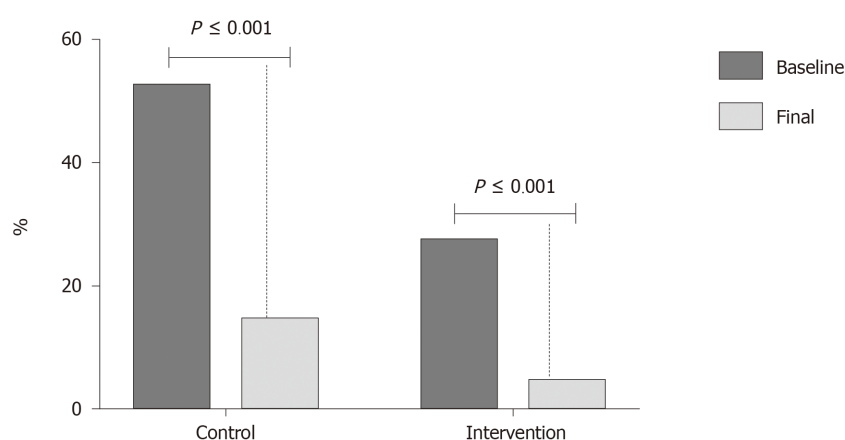


Figure 2 Changes in endothelial dysfunction at baseline and final evaluations. The figure shows the percentage of patients with endothelial dysfunction at baseline and final evaluation.

including an effective exercise program, in order to broaden the use of such an intervention in different clinical settings, and daily life, and therefore not limiting its use only into a very specialized research setting as has been shown in different studies^[16,33].

Endothelial dysfunction has a central role in the pathophysiology of portal hypertension^[41], thus interventions aimed to improve it have been the focus of treatment and research in cirrhosis. In the present study, an improvement in endothelial function was found in both groups, as a consequence of physical exercise. While these effects are expected after physical exercise, the complete picture of the benefits associated with the consumption of non-alcoholic beer, seems to bring additional benefits and enhance exercise performance, including nutritional status and body composition, as well as improvement in quality of life.

Several markers of nutritional status improved, which is the expected change for an effective program with exercise and diet, as has been previously demonstrated in

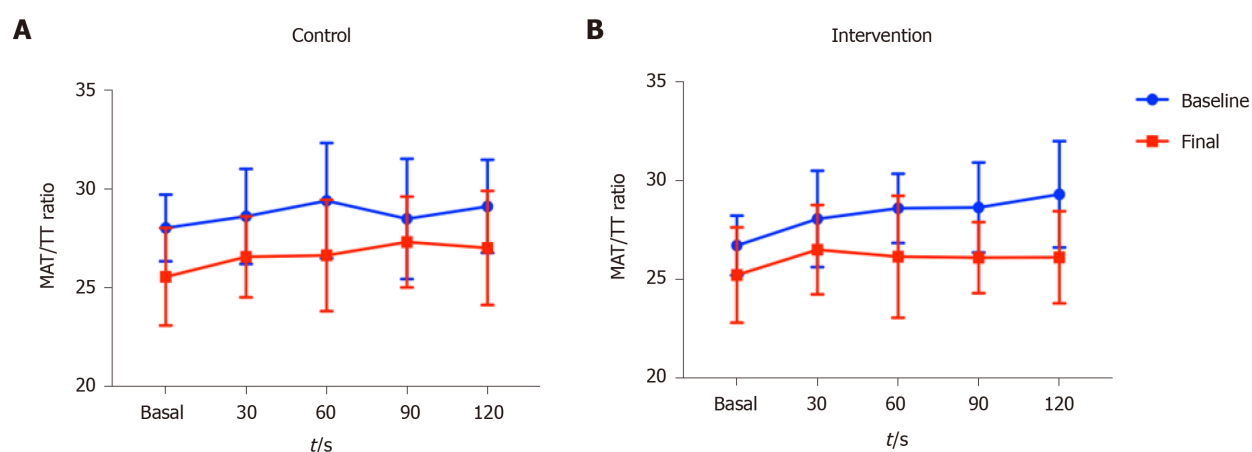


Figure 3 Area under the curve showing the behavior of endothelial function at baseline and final evaluations in the two groups. A: Control; B: Intervention. Data are presented as median (IQR). MAT: Maximal amplitude time; TT: Total time.

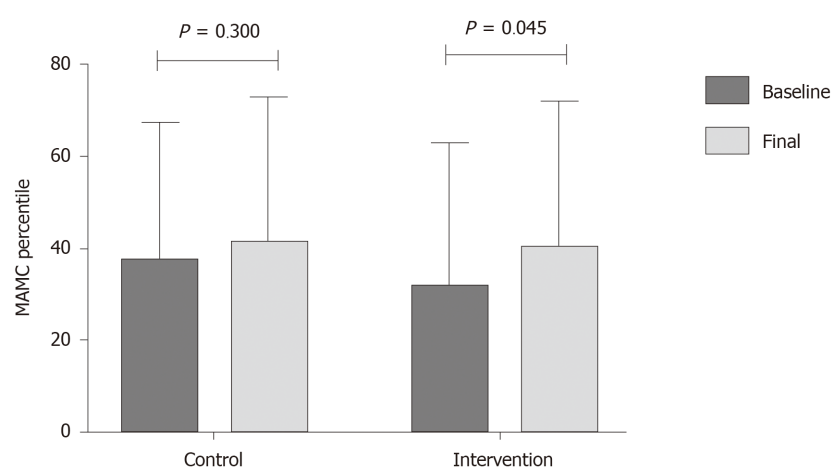


Figure 4 Changes in mid-arm muscle circumference percentile throughout the study. Data are presented as mean percentile \pm SD. MAMC: Mid-arm muscle circumference.

several studies in cirrhotic populations^[16,32]. However, the most prominent findings were observed in the group receiving non-alcoholic beer, where 3 important parameters of nutritional status (thigh circumference, HGS and sit to stand test) improved, compared to only the sit to stand test in the control group. In addition, more patients allocated to the group with non-alcoholic beer had an improvement in the phase angle, although no statistical difference was reached ($P = 0.290$). The overall results for nutritional status suggest a benefit for non-alcoholic beer, and, specifically addressing the results of the improvement in the non-alcoholic beer group, it is important to mention that being able to possibly modify the circumference of the thigh is of great relevance and taken together with the improvement of the two tests evaluating muscle function we can conclude that the intervention targeted the main issues of sarcopenia which is the amount of muscle and its function.

There are various reasons that can explain why non-alcoholic beer had an additive effect in these patients: Improvement in gut microbiota, and palatability of the meals (thus improving dietary intake); Better hydration and higher content of minerals, improving muscle function and therefore facilitating exercise performance; and The amount of micronutrients including vitamin B₁₂, that is a known factor essential for muscle formation and can play a role as an antioxidant, improve exercise tolerance, and neuromuscular function. Given that patients in the control group received the same amount of water, the benefits mentioned above cannot be explained merely by hydration.

On the other hand, there was an improvement in quality of life assessed by the CLDQ and the SF-36 questionnaires, in both groups, but again the most prominent findings were observed in the group receiving non-alcoholic beer. It is noteworthy that all but one domain in the CLDQ improved in the non-alcoholic beer group, with no

worsening of abdominal symptoms as could be expected for the intervention (bloating). There was improvement in the SF-36 only in 2 domains, including physical function, in both groups and in general health and vitality in the control and non-alcoholic beer group, respectively. A clear trend towards an improvement in social function and mental health was observed in the group receiving non-alcoholic beer. This point is extremely important, because usually patients with chronic diseases, including cirrhosis, have a lower quality of life that impedes them from social interaction with other people. Integration of these patients into their normal social environment, by allowing them to participate in activities such as physical exercise, as well as having a “normal” diet supplemented with non-alcoholic beer, can contribute to the benefits observed specifically in this group. Moreover, hops have been shown to improve symptoms of depression, anxiety and stress over a 4-week period partially explaining the results found in QoL^[42].

Regarding the safety of the intervention, there were no reported side effects in any of the groups. In the biochemical tests, no changes in liver function tests, hemoglobin, leukocytes, creatinine or serum electrolytes were observed, and a trend towards a global improvement in those tests was noted after the study. In addition, there was no worsening of abdominal symptoms in the group receiving non-alcoholic beer, as might be expected as a consequence of the beverage.

The most important features in the present study are the design, controlling the amount of additional liquids in both groups, and proposing an easy to follow and reliable exercise protocol together with an affordable and simple nutritional supplement, involving a multidisciplinary group from different specialties (gastroenterology, hepatology, nutrition and cardiology).

There are some limitations in this study; first, the patients included in this study were compensated, therefore the findings are not applicable to patients with decompensated cirrhosis. Another potential limitation is the lack of computed tomography (CT) scan measurements; however, given the monthly evaluations performed in this study, CT scan measurements were not appropriate (due to radiation exposure), therefore it was decided to include other validated markers such as phase angle derived from BIA, that has been validated in cirrhosis against CT scanning with great sensitivity^[43], and HGS, also validated in cirrhosis.

CONCLUSION

In conclusion, a multifactorial program including diet, monitored exercise and non-alcoholic beer is safe, well tolerated and results in improvements in endothelial function, nutritional status, and quality of life. The effects shown in this study should be confirmed with a longer duration of the intervention, and possibly with a larger amount of non-alcoholic beer.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic beer has been shown to positively modify gut microbiota diversity and improve endothelial function and oxidative stress, when used in different clinical settings, including breastfeeding and post-exercise rehydration. Thus, non-alcoholic beer can be regarded as a “functional” supplement. Additionally, physical exercise has proven to be a safe and effective intervention in cirrhosis, providing several benefits, for instance, improvement in nutritional status, quality of life and portal pressure.

Research motivation

Although nutritional therapy (diet + physical exercise) has beneficial effects in patients with cirrhosis, the availability and implementation of other nutritional strategies, such as supplements, is sometimes difficult due to different factors. Non-alcoholic beer has different compounds that exert antioxidant, anti-inflammatory and nutritional properties, and these properties are highly attractive in the treatment of patients with cirrhosis. Therefore, we hypothesize that it could be beneficial as a nutritional supplement in these patients.

Research objectives

The aim of the study was to evaluate the effect of diet + exercise and non-alcoholic beer on nutritional status, endothelial function, and quality of life in patients with cirrhosis.

Research methods

In this randomized open-clinical trial, eligible patients were randomized into two groups: (1) Control: Diet + physical exercise and (2) Intervention: Diet + physical exercise + non-alcoholic beer who were treated for 8 wk. The evaluated outcomes were nutritional status, endothelial function, and quality of life. For the analysis, only those patients receiving at least 1 d of intervention were included (modified intention to treat). Paired data were analyzed using the Wilcoxon signed-rank test. For comparisons between groups, Mann-Whitney U or Student's *t*-test was used. Areas under the curve (AUC) were constructed for repeated measurements of endothelial function.

Research results

Forty-three patients were included in the study, 21 in the control group and 22 in the intervention group. The mean age was 53.5 ± 7.8 years, 60% were women, the median model for end-stage liver disease (MELD) score was 8 (7-10) and most patients were Child-Pugh A (88%). There were no adverse effects related to the consumption of non-alcoholic beer, or to the diet and exercise. Endothelial function improved in both groups. All the measured nutritional parameters improved in the intervention group, compared to only 2 in the control group and quality of life improved in both groups; however, more domains improved in the intervention group.

Research conclusions

A multifactorial program including the standard treatment diet and monitored exercise as well as a non-alcoholic beer is safe, well tolerated and results in improvements in endothelial function, nutritional status, and quality of life.

Research perspectives

Non-alcoholic beer represents a new nutritional strategy that has beneficial effects in patients with cirrhosis. However, the effects shown in this study should be confirmed with a longer duration of the intervention, and possibly with a larger amount of non-alcoholic beer. Our study did not include patients with decompensated cirrhosis, which limits the extrapolation of the results.

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Observational Study

HIPPOCRATES® project: A proof of concept of a collaborative program for hepatitis C virus micro-elimination in a prison setting

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Informed consent was obtained from all individual participants included in the study.

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Abstract

BACKGROUND

In the last few years we have witnessed a revolution in the treatment of hepatitis C virus (HCV) infection. With the introduction of direct-acting antiviral agents (DAAs), sustained virological response (SVR) is achieved in more than 95% of the patients. The focus is now being turned to the global targets set by the World Health Organization, with the aim of achieving HCV elimination by 2030. Prison inmates constitute one of the high-risk groups, and receive treatment less frequently due to several barriers in access to health care.

AIM

To describe the management and follow-up of a cohort of HCV monoinfected patients treated with DAA in the prison setting, where tertiary referral liver center specialists locally provide, on-site assessment and treatment for the prisoners.

METHODS

A prospective observational study was conducted from April 2017 to March 2020, which included all HCV monoinfected prison inmates in the largest Northern Portugal prison. Demographic, clinical, and laboratory data, as well as transient elastography measurements, were collected onsite by the medical team and prospectively recorded. Patients were treated with DAA according to international guidelines. The primary endpoint was SVR at post-treatment week 12.

RESULTS

There were 98 monoinfected HCV male inmates (mean age, 42.7 ± 8.6 years) included in the analysis. Injecting drugs or tattooing were reported in 74.5%, with

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38.8% of the latter being done in prison. Alcohol consumption of more than 30 g/d was referred in 69.4%. The most prevalent genotype was 1a (54.1%), followed by 3 (27.6%), 4 (9.2%) and 1b (6.1%). Pretreatment fibrosis degree was mild-to-moderate (F0-F2) in 77.6% and severe in 22.4% (F3-F4). Treatment regimens chosen were: 45.9% elbasvir/grazoprevir, 29.6% sofosbuvir/velpatasvir, and 12.2% sofosbuvir/ledipasvir and glecaprevir/pibrentasvir. No major adverse events were observed. SVR at post-treatment week 12 was 99%.

CONCLUSION

In a population considered to be both hard-to-access and a cornerstone for HCV elimination, the onsite evaluation and treatment of HCV-infected prisoners, achieved an exceptional highly effective success rate. This type of collaborative program should be considered to be expanded, to support hepatitis C elimination efforts.

Key Words: Hepatitis C infection; Treatment; Prison setting; Direct-acting antiviral agents; Micro-elimination

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Core Tip: Hepatitis C is a curable infectious disease with high cure rates reaching almost 100% with the use of direct-acting antiviral agents. The World Health Organization defined the aim of achieving hepatitis C virus elimination by 2030. In the first phase, patients identified with hepatitis C virus infection were treated. Although, to achieve this ambitious goal, we had to reach difficult to access groups, such as persons who inject drugs (PWID) and prisoners. We developed a strategy where a medical team (2-3 doctors) from the hospital went to prison and was responsible for the outpatient clinics, liver elastography, and giving the medication on-site, increasing access to care by avoiding any need to move the patients outside the prison. This was so successful, reaching 99% of sustained virological response in a difficult to treat cohort, that a national program was created implementing our strategy. Therefore, in Portugal, every prisoner has access to treatment inside the prison.

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INTRODUCTION

Hepatitis C virus (HCV) is recognized as the leading cause of chronic liver disease worldwide, being one of the major global health problems. The prevalence of HCV infection ranges from 0.5% in Western Europe and 1.3% in United States to 2.5% in Southern Europe and 6% in Eastern Europe^[1]. According to the last report from World Health Organization (WHO), about 71 million people are suffering from chronic HCV infection, with almost 400000 deaths due to cirrhosis or liver cancer^[2-5]. Moreover, it is still the most common indication for liver transplantation. Management of HCV infection places a large burden on local health systems due to social and economic charges^[6].

In the last few years, we have witnessed a major revolution in the treatment of HCV infection. The introduction of direct-acting antiviral agents (DAAs) resulted in fewer side effects, shorter regimen treatments, and higher rates of sustained virological response (SVR). Because cure is attainable in almost all patients, the WHO defined ambitious targets to be achieved by 2030, namely HCV elimination, with 65% reduction in liver-related deaths, 90% reduction of new infections, and 90% of patients with viral hepatitis infections being diagnosed^[5].

However, HCV elimination poses several logistic and political challenges: Most health systems are not prepared to deal with the huge task of HCV testing and



treatment, and, many times, it is difficult to convince health authorities to provide human and financial resources so that HCV elimination could be a realistic goal. Recently, the concept of micro-elimination has emerged, where the main focus is tackling specific individual populations, creating an organized plan to overcome the known barriers, and achieving both high levels of diagnosis and treatment, choosing also the best interventions in accordance with that population's needs.

This strategy is going to target well-recognized high-risk populations such as people who injected drugs, homeless people, and prisoners, all of whom are typically not well engaged with specialist care^[6,7].

Prisons are considered a high-risk environment with a population characterized by risky behaviors such as unprotected sexual intercourse, tattooing, and injecting drug use^[3]. At the same time they also provide a unique opportunity to scale up hepatitis C treatment^[8]. It is estimated that the prevalence of HCV infection in prisons is 10 to 20 times higher than that of the average population. The reported prevalence of HCV infection in prisoners ranges from 15.4% in Western Europe to 20.7% in Eastern Europe^[9,10]. The correctional system may be considered a public health opportunity to treat these patients^[11] for a variety of reasons: Improved adherence due to daily staff distribution of medication, limited access to drugs and alcohol, and monitoring of possible side effects^[12]. Although treatment of HCV infection in the correctional systems seems necessary to achieve the goal of HCV elimination by 2030, there are few reports about the management of HCV in prison, particularly in the DAA era.

The aim of this study is to describe an innovative program of diagnosing and treating HCV monoinfected patients within the prison setting, the HIPPOCRATES® (Hepatitis C In-PrisonProgram fOr enhancing Cure RATES) Project, and evaluate its performance in curing this infection, assessing SVR at post treatment week 12 (SVR 12).

MATERIALS AND METHODS

HCV is recognized as the leading cause of chronic liver disease worldwide, being one of the major global health problems. The prevalence of HCV infection ranges from 0.5% in Western Europe and 1.3% in United States to 2.5 in Southern Europe and 6% in Eastern Europe^[1]. According to the last report from WHO, about 71 million people are suffering for chronic HCV infection, with almost 400000 deaths due to cirrhosis or liver cancer^[2-5]. Moreover, it is still the most common indication for liver transplantation. Management of HCV infection places a large burden on local health systems due to social and economic charges^[5].

In the last few years, we have witnessed a major revolution in the treatment of HCV infection. The introduction of DAA resulted in fewer side effects, shorter regimen treatments and higher rates of SVR. Because cure is attainable in almost all patients, the WHO defined ambitious targets to be achieved by 2030, namely HCV elimination, with 65% reduction in liver-related deaths, 90% reduction of new infections, and 90% of patients with viral hepatitis infections being diagnosed^[5].

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The aim of this study is to describe an innovative program of diagnosing and treating HCV monoinfected patients within the prison setting, the HIPPOCRATES® Project, and evaluate its performance in curing this infection, assessing SVR 12.

Prison setting

Porto's correctional system is an exclusive male prison located in Porto, the second largest city of Portugal. It is the largest prison in the Northern Portugal, having the capacity for 1200 inmates. The Medical Department has one treatment room, four outpatient clinic rooms, and a ward with the capacity for six patients. Personnel staff comprises one permanent doctor and four nurses.

Screening

All inmates were tested for hepatitis B virus, HCV, and human immunodeficiency virus (HIV) on admission and there was no refusal to be screened. All anti-HCV antibody positive inmates were tested for HCV RNA, even in cases of previous treatment or negative RNA on previous admissions due to the possibility of reinfection in this high-risk population. Viral load and genotype were determined according to international guidelines.

Treatment eligibility

To be eligible for treatment, prisoners had to be ≥ 18 -years-old, have evidence of active chronic hepatitis C with a recent detectable serum HCV RNA, and have an adequate sentence duration to facilitate complete hepatitis C treatment while incarcerated (between 8 and 12 wk, depending on the chosen treatment). However, it was not a requirement that prisoners remained in prison till SVR 12, and those who were released earlier were provided with their remaining medication to complete treatment in the community and had follow-up scheduled in the hospital's Hepatology outpatient clinic.

Following identification, HCV monoinfected patients were referred by the Prison doctor to the Liver specialist team. This medical team, from the Gastroenterology Department, consisted of three Hepatologists from Centro Hospitalar São João, the largest liver center in the north of the country. All co-infected patients were treated by the Infectious Disease team.

Those hospital doctors visited once weekly the prison, where all clinical procedures were performed, in coordination with the prison doctor.

For each patient, we had scheduled three clinical visits, for a total of 302. In the first one, careful medical history and demographics were taken including age, place of birth, weight and height, medication, history of previous blood transfusions, tattooing, drugs (injected or inhaled), alcohol and tobacco consumption. Patients were also asked about the exact time of HCV infection diagnosis, as well as previous treatments. During the first visit, a transient liver elastography was performed onsite. After gathering clinical, laboratory, and virological data together with the fibrosis stage, treatment regimen and duration was decided by the medical team and requested to the central pharmacy.

In the second appointment, when treatment was already available, the expected efficacy and possible side effects were again explained. For those patients who decided to participate in this program, treatment started on the day after. The prison staff was responsible for distributing the DAAs to our patients, and were instructed by the medical team before the beginning of the program. They were already previously in charge of distributing the opioid substitution therapy.

The third outpatient visit was performed 12 wk after the end of treatment to inform the patient about SVR. All patients received extensive counseling and education about harmful practices potentially leading to reinfection such as tattooing, sharing needles or unprotected sexual intercourse, and they were instructed to change their own usual hygienic habits from the beginning of treatment. We established a viral hepatitis education program for prisoners and prison staff, including drug service providers and correctional officers, to support clinical service. Prisoners with hepatitis C and cirrhosis were enrolled in surveillance programs for hepatocellular carcinoma and esophageal varices, as recommended by consensus guidelines^[13].

All the procedures were performed by the same three hepatologists and any problems concerning these patients were seen by the prison's doctor and reported to the hospital team.

Laboratory data

Blood samples were all taken in the prison setting by a special team of nurses. Every patient had laboratory data at three-time points: (1) Before starting the treatment; (2) At the end of treatment; and (3) 12 wk after the end of treatment to assess SVR 12. At all time points, full blood count, aspartate aminotransferase, alanine aminotransferase (ALT), γ -glutamyl transpeptidase (GGT), alkaline phosphatase, total and direct bilirubin, albumin, urea, and creatinine were requested. In addition, first and last blood samples were analyzed for iron, transferrin and ferritin, total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol, alpha-fetoprotein, international normalized ratio, hepatitis B surface (HBs) antigen, HBs antibody, hepatitis B core antibody, and HIV.

Fibrosis assessment

The degree of fibrosis was evaluated using the portable Fibroscan 430 mini model from Echosens[®] (owned by our department), performed in the prison by the gastroenterology medical team, and classified according to Metavir classification^[14].

Only procedures with at least 10 valid measurements, an interquartile range/median ratio of less than 30%, and a success rate of at least 60% were included. The median value of liver stiffness was recorded in kPa. All procedures were performed by well-experienced and well-trained investigators.

Treatment

The treatment for each patient was discussed within the medical team based on the laboratory data and liver fibrosis stage, and decided according to international guidelines^[15].

SVR 12 was defined as having an undetectable HCV RNA test at least 12 wk after the end of treatment.

Statistical analysis

Continuous variables are expressed as median (range). Categorical variables are reported as absolute (*n*) or relative frequencies (%). Analysis of variance was used to compare the differences in variable between groups. $P < 0.05$ was considered statistically significant. Data were analyzed using SPSS 21.0 (IBM Corp, Armonk, NY, United States).

Ethical considerations

This study was conducted according to the Declaration of Helsinki. The ethical approval for this study was obtained from the Ethics Committee of Centro Hospitalar São João and Porto's correctional system. Informed consent was obtained from each patient before each treatment.

RESULTS

Clinical features

Screening of 2451 inmates resulted in 276 prisoners (11.3%) positive for anti-HCV antibody and negative for HIV. Among them, 27685 patients were co-infected HCV/HIV patients and 191 were only HCV-antibody positive. From the 191 patients, 108 prisoners tested positive for HCV RNA, resulting in a prevalence of ongoing infection of 4.4%. Two patients refused to participate in the project and eight patients were transferred to another institution before starting the treatment. The selection of cases for treatment is summarized in [Figure 1](#).

The mean age of the final group of 98 patients was 42.7 ± 8.6 years ([Table 1](#)). All patients were Caucasian and had a mean body mass index of 24.7 ± 3.2 .

Regarding risk factors for acquiring HCV infection, 9.2% had a previous history of blood transfusions, 74.5% were injecting drug users, and 74.5% had tattoos (38.8% of them were done in the prison setting). The vast majority (94.9%) also reported previous history of smoked drugs (mostly cocaine and cannabis). Active smoking was established in 90.8%, while 77.6% admitted daily alcohol consumption (more than 30 mg/d in 69.4%). Regular medication consisted of benzodiazepines in 70.4% ($n = 69$), methadone in 26.5% ($n = 26$), and antidepressants in 40.8% ($n = 40$). Clinical characteristics are summarized in [Table 1](#).

For most patients (68.3%), the diagnosis of HCV infection was made in prison, while for a minority, it was established in PWID (18.4%) or during routine laboratory data

Table 1 Clinical characteristics are summarized

| Prisoners characteristics | n (%) |
|---|------------|
| Age | |
| 18-29 yr | 7 (7.1) |
| 30-39 yr | 22 (22.5) |
| 40-49 yr | 47 (47.9) |
| 50+ yr | 22 (22.5) |
| Body mass index, kg/m ² , mean | 24.7 ± 3.2 |
| HCV genotype | |
| 1a | 53 (54.1) |
| 1b | 6 (6.1) |
| 2 | 2 (2.0) |
| 3 | 27 (27.6) |
| 4 | 9 (9.2) |
| 6 | 1 (1.0) |
| Tattooing | |
| No | 25 (25.5) |
| Made in prison | 38 (38.8) |
| Made outside prison | 68 (69.4) |
| Illicit drug use | |
| No | 5 (5.1) |
| Ever smoked heroin/marijuana only | 93 (94.9) |
| Inject drugs | 73 (74.5) |
| Smoker | |
| No | 9 (9.2) |
| Yes, < 10 cigarettes per day | 27 (27.6) |
| Yes, > 10 cigarettes per day and < 20 | 31 (31.6) |
| Yes, > 20 cigarettes per day | 31 (31.6) |
| Alcohol consumption | |
| No | 22 (22.4) |
| Yes, < 30 g day of alcohol | 8 (8.2) |
| Yes, 30 g day of alcohol and < 60 g | 13 (13.3) |
| Yes, 60 g day of alcohol | 55 (56.1) |
| Medication | |
| Opiate substitution therapy | 26 (26.5) |
| Antidepressants | 40 (40.8) |
| Benzodiazepines | 69 (70.4) |

HCV: Hepatitis C virus.

performed outside the prison (13.3%) (Figure 2).

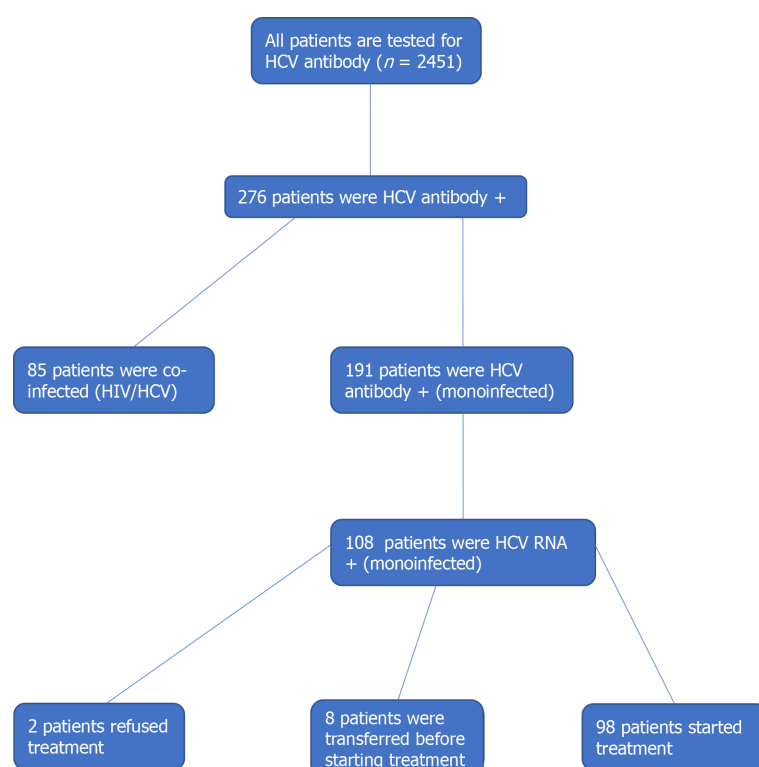
Laboratory data

Laboratory data are depicted in Table 2. Except for slight elevation of ALT and GGT, all laboratory data were within the normal range. There were no patients with positive HBs antigen but there were 41.8% with positive HBc antibody.

Table 2 Laboratory data in the beginning of the treatment

| Laboratory data | Median (IQR) |
|----------------------------------|---------------------|
| Hemoglobin, g/dL | 15.7 (14.9-16.0) |
| Leukocytes, × 10 ⁹ /L | 7.9 (7.0-10.2) |
| Platelets, × 10 ⁹ /L | 204.5 (175.8-238.8) |
| Albumin, g/dL | 58.4 (55.0-61.1) |
| AST, U/L | 36.0 (28.0-47.8) |
| ALT, U/L | 47.0 (31.0-67.0) |
| GGT, U/L | 41.5 (27.0-72.0) |
| Alkaline phosphatase, U/L | 76.5 (65.5-94.5) |
| Total bilirubin, mg/dL | 0.48 (0.32-0.6) |
| Total cholesterol, mg/dL | 162.0 (142.0-200.0) |
| Alpha-fetoprotein, ng/mL | 2.0 (1.4-3.5) |
| Creatinine, mg/dL | 0.8 (0.7-0.9) |
| INR | 1.0 (1.0-1.1) |
| Anti-HBc antibody+ | 41 (41.8%) |
| Anti-HBs antibody+ | 33 (33.7%) |

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transpeptidase; IQR: Interquartile range.

**Figure 1 Selection of cases for treatment.** HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

Virology and fibrosis assessment

The median HCV viral load was 1865000 copies (range 673250-4580000). The most prevalent genotype was genotype 1a (54.1%), followed by genotype 3 (27.6%) genotype 4 (9.2%) and genotype 1b (6.1%) (Figure 3). All liver elastographies performed had more than 10 valid measurements and an interquartile range < 30%.

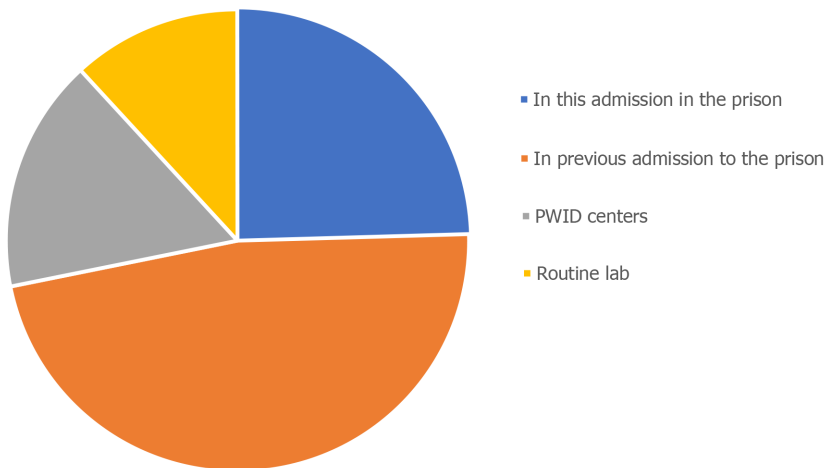


Figure 2 Hepatitis C virus diagnoses. PWID: Persons who inject drugs.

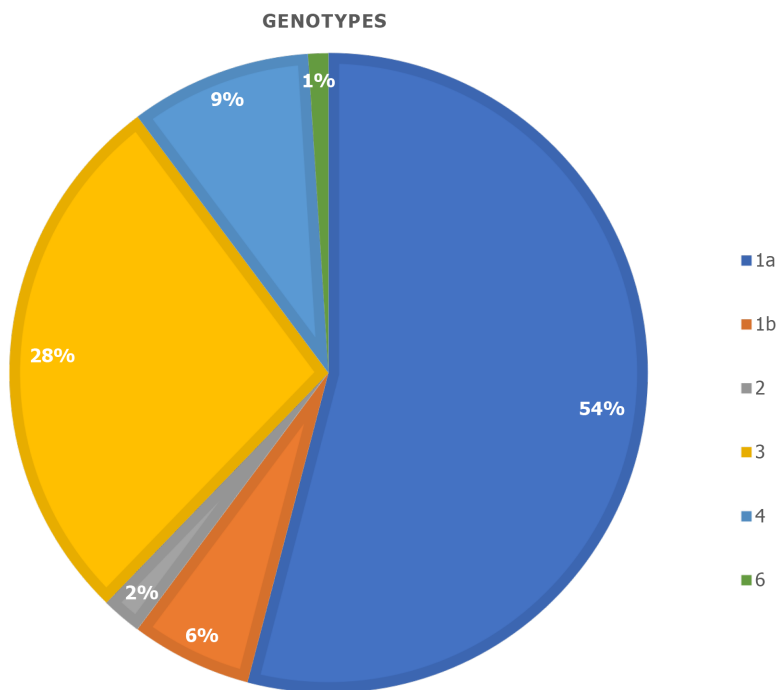


Figure 3 Genotype distribution.

According to the Fibrosan scoring card, 77.6% of the patients were classified as stage F0 to F2 and only 22.4% were classified as stage F3 or F4 (Table 3).

Treatment

All patients completed 12 wk of treatment under daily staff distribution of medication, achieving 100% of adherence. Regarding treatment, regimens were prescribed: 45 patients (45.9%) got elbasvir/grazoprevir, 29 patients (29.6%) took sofosbuvir/velpatasvir and 12 (12.2%) with sofosbuvir/Ledipasvir and 12 (12.2%) with glecaprevir/pibrentasvir, always without ribavirin. There were two patients that refused treatment, and eight patients started treatment in another institution after being moved. During the entire period of treatment, there were no reports of major adverse events and only seven patients complained of mild headache, without the need to take medication for relief.

SVR at week 12 after treatment was 99.0% (97/98). At this time, 52 (53.1%) patients gained weight and 86 (87.8%) referred improved quality of life. There were no cases of hepatitis B virus reactivation during the entire period of follow-up.

Table 3 Liver fibrosis according to liver elastography

| Liver fibrosis according to liver elastography | n (%) |
|--|-----------|
| F0-F1 | 50 (51.0) |
| F2 | 26 (26.6) |
| F3 | 12 (12.2) |
| F4 | 10 (10.2) |

DISCUSSION

This is the first hepatitis C prison management program to be implemented in the country and settled the basis for an innovative national approach to deal with this problem.

This decentralized model of care involving onsite specialist consultation has overcome several of the traditional systemic barriers to provide efficient hepatitis C treatment to prisoners, namely hospital transfer, treatment interruption from transfer between correctional facilities, and low compliance due to non-assisted daily medication for several weeks. With the advent of DAAs, HCV infection treatment has achieved cure rates ranging from 90% to 100%. Therefore, with the aim of achieving HCV elimination in 2030, the focus is being turned into the high-risk groups, considered difficult-to-treat populations, traditionally due to their barriers in being engaged in health care.

The micro-elimination approach relates to target some subpopulations of HCV-infected people, namely high-risk populations, providing tailored services and the best interventions regarding the population's needs in order to reach engagement and high rates of diagnosis and treatment.

One of the main focus of treatment and one of the cornerstones of the global micro-elimination strategy is also to reduce transmission by infected people, potentially contributing to treatment as prevention.

Although prisoners are undoubtedly considered a high-risk population and one of the main targets for micro-elimination, there is no universal policy for the provision of HCV testing/screening in this population. A recent European study showed that in 16 of 25 countries, HCV screening is not performed routinely but only if requested by the inmate^[9]. In this study, where upon all inmates were tested for HCV regardless of the presence of risk factors, we found a prevalence of chronic HCV infection of 4.4%. Therefore, it seems justifiable that systematic screening of all prisoners upon admission to the correctional system should be pursued and recommended as an essential component of every national elimination strategy. In our program, as all the prisoners were always tested at admission in prison, we achieved very good results when compared with other studies^[16-19].

In Portugal, there is unrestricted access to DAA therapy for chronic HCV infection since 2016. Although many patients had already been treated, vulnerable populations such as prisoners, only have access to therapy through general hospitals, with the need for specialist consultation implying frequent visits to hospital, a costly, time-consuming and distressing transport of prisoners. The prison setting could be, however, an ideal opportunity and an appropriate environment to provide hepatitis C treatment, breaking all the usual barriers in the access of treatment that this high-risk population have to face including reasons that lead to treatment interruption or diminished efficacy. In the community setting, these subjects would not normally receive treatment due to substance use related disorders (alcohol/drugs), psychiatric comorbidities, social stigma, lack of access to health care services and poor adherence to treatment. However, in the correctional system, these obstacles can be surpassed, as incarcerated people have limited access to alcohol and drugs and are actively treated for their mental illness, allowing a concentrated and targeted healthcare approach. A DDA-based program started in a correctional facility in Cairns, Australia, resulted in reduction of HCV prevalence from approximately 12% to less than 1% over a 22-mo period, showing that is not only feasible but also a very effective strategy^[12].

As established in this project, hospital doctors moved to prison, allowing all HCV management to be done onsite, avoiding one major hurdle which is the frequent hospital visits these patients would otherwise need. This strategy of relocation of HCV specialists, together with daily staff distribution of medication and the advantages of DAA therapy mentioned above, resulted in a successful 100% of patients' adherence to the treatment plan. Consequently, SVR 12 rate was 99.0% in this difficult-to-treat

population, showing that this strategy is not only feasible but also very effective.

Regarding risk factors to acquire HCV infection, in our study we found that 74.5% of the patients reported a lifetime history of injecting drugs use. Several studies showed higher odds ratio for HCV among prisoners who injected drugs^[9,20]. Tattooing is also an important risk factor, especially if performed in non-licensed settings or in prison^[21]. As drugs injection inside prison is not permitted, one major pathway for transmission of HCV infection is through previously infected material used for tattooing, as inmates perform tattoos with artisanal instruments that are not adequately sterilized. In our study, we found that 74.5% of the patients had tattoos, and 38.8% of whom had performed them inside prison. Interestingly, the majority of inmates did not recognize tattooing as a potential pathway for transmitting infection. Although until now, we have no cases of reinfection, in the Cairns study, six re-infected patients were reported. Thus, it is very important to elucidate the risks of using non-sterile devices to reduce the risk of transmission and reinfection, always bearing in mind that prisoners frequently cycle between incarceration and freedom, creating new networks for hepatitis C transmission. In our program, an important issue was also to prevent re-infection and "*de novo*" infection in the prison setting. Thus, our follow-up strategy was to re-screen all the patients every year (average length of stay in prison was 2 years) and implement educational measures through not only the medical team and the local nurses but also with educational sessions given by the medical team to all the prisoners, where all the risk factors and pathways to acquire HCV infection was explained.

Despite the excellent results reported here, HCV treatment in prison is demanding and there are still challenges to face, as shown from other successful experiences^[17,18,21]. First, treatment of inmates requires careful attention and dedicated training to health care providers in order to create a good doctor-patient relationship, a keystone of care. Prison's nurses played also a major role, not only for therapeutic compliance but also highlighting the measures to prevent reinfection.

Second, to provide dedicated outpatient clinic in the prison setting, it is necessary total availability of the medical team, as it implies several visits to the prison as well as some logistic issues (*e.g.*, availability of a portable Fibroscan equipment). Lastly, the average length of incarceration is very variable, and could only last some weeks. For some patients in whom therapy is initiated in prison, it needs to be completed in the community, making the interaction between the correctional system and the medical team in the community essential to avoid loss to follow-up.

There are also some improvements that can be done in our program. With total availability of pangenotypic drugs that were not available in the beginning of our program, determining genotype is not needed and could be a step forward treatment simplification. In addition, access to new technologies as TRODs GeneXpert and immediate availability of the drugs would reduce the number of outpatients clinics and the time from diagnose and beginning of the treatment.

A major breakthrough achieved with this project was the recognition of its success by the national health authorities who established a strategic plan of a national program in Prisons, aiming to detect and treat all the inmates with HCV infection, under the guidance of specialist doctors moving into prison sites (Diário da República n.º 4/2018, Série II de 2018-01-05, despacho 283/2018).

CONCLUSION

In conclusion, we showed a 4.4% prevalence of HCV infection in a prison setting after universal screening at admission. Although demanding, decentralizing a specialized medical team from the hospital to the prison as well as daily staff distribution of DAA medication, improved the prisoners access to hepatitis C treatment and lead to a very high rate of hepatitis C cure (99.0% rate of SVR 12 in our study).

Micro-elimination plans as the one we have developed should be promoted to benefit both the individual prisoners and the global efforts to the community to achieve hepatitis C elimination.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C is now a curable infectious disease with high rates of cure, reaching almost

100% cure with the use of direct antiviral agents.

Research motivation

The World Health Organization defined the aim of achieving hepatitis C virus (HCV) elimination by 2030. Although, to achieve this ambitious goal, we have to reach difficult to access groups, as persons who inject drugs and prisoners.

Research objectives

The aim of our program was to develop a research program of management and follow-up of a cohort of HCV mono-infected patients treated with direct-acting antiviral agents in the prison setting.

Research methods

We developed a strategy where a medical team (2-3 doctors) from the hospital went to prison and was responsible for outpatient clinics, liver elastography, and give the medication on-site, increasing the access to care by avoiding any need to move the patients outside the prison.

Research results

Screening of 2451 inmates resulted in 276 prisoners (11.3%), of whom 108 prisoners mono-infected for HCV. Two patients refused to participate in the project and eight were transferred to other institution before starting the treatment. All patients completed 12 wk of treatment (100% adherence), achieving a sustained virological response (SVR) at week 12 after treatment of 99.0% (97/98).

Research conclusions

In a particularly difficult to achieve population, we achieved a SVR at week 12 after treatment of 99.0%, with 100% adherence to treatment in patients that accepted the treatment.

Research perspectives

These results show that an innovative and "in-loco" project in this special population could be the pathway to achieve HCV elimination in 2030. After this pilot project, a national program was created implementing our strategy. Therefore, currently in Portugal, every prisoner has access to treatment inside the prison.

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Prospective Study

Subclinical proximal tubulopathy in hepatitis B: The roles of nucleot(s)ide analogue treatment and the hepatitis B virus

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Abstract

BACKGROUND

The recommended monitoring tools for evaluating nucleot(s)ide analogue renal toxicity, such as estimated glomerular filtration rate (eGFR) and phosphatemia, are late markers of proximal tubulopathy. Multiple early markers are available, but no consensus exists on their use.

AIM

To determine the 24 mo prevalence of subclinical proximal tubulopathy (SPT), as defined with early biomarkers, in treated *vs* untreated hepatitis B virus (HBV)-monoinfected patients.

METHODS

A prospective, non-randomized, multicenter study of HBV-monoinfected patients with a low number of renal comorbidities was conducted. The patients were separated into three groups: Naïve, starting entecavir (ETV) treatment, or starting tenofovir disoproxil (TDF) treatment. Data on the early markers of SPT, the eGFR and phosphatemia, were collected quarterly. SPT was defined as a maximal tubular reabsorption of phosphate/eGFR below 0.8 mmol/L and/or uric acid fractional excretion above 10%. The prevalence and cumulative incidence of SPT

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at veronique.loustaud-ratti@unilim.fr. Participants gave informed consent for data sharing.

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at month 24 (M24) were calculated. Quantitative data were analyzed using analyses of variance or Kruskal-Wallis tests, whereas chi-squared or Fisher's exact tests were used to analyze qualitative data. Multivariate analyses were used to adjust for any potential confounding factors.

RESULTS

Of the 196 patients analyzed, 138 (84 naïve, 28 starting ETV, and 26 starting TDF) had no SPT at inclusion. At M24, the prevalence of SPT was not statistically different between naïve and either treated group (21.1% *vs* 30.7%, $P < 0.42$ and 50.0% *vs* 30.7%, $P = 0.32$ for ETV and TDF, respectively); no patient had an eGFR lower than 50 mL/min/1.73 m² or phosphatemia less than 0.48 mmol/L. In the multivariate analysis, no explanatory variables were identified after adjustment. The cumulative incidence of SPT over 24 mo (25.5%, 13.3%, and 52.9% in the naïve, ETV, and TDF groups, respectively) tended to be higher in the TDF group *vs* the naïve group (hazard ratio: 2.283, $P = 0.05$). SPT-free survival at M24 was 57.6%, 68.8%, and 23.5% for the naïve, ETV, and TDF groups, respectively. The median survival time without SPT, evaluated only in the TDF group, was 5.9 mo.

CONCLUSION

The prevalence and incidence of SPT was higher in TDF-treated patients compared to naïve patients. SPT in the naïve population suggests that HBV can induce renal tubular toxicity.

Key Words: Hepatitis B virus; Proximal tubulopathy; Biomarkers; Renal insufficiency; Nucleoside analogues

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Core Tip: The objective of this prospective, multicenter study was to determine the 24 mo prevalence and incidence of subclinical proximal tubulopathy (SPT) in hepatitis B virus (HBV)-monoinfected patients that were either treatment-naïve or treated with nucleot(s)ide analogues. Data on early SPT markers, the estimated glomerular filtration rate and phosphatemia, were collected quarterly from 196 patients. The prevalence and incidence of SPT was higher in tenofovir disoproxil-treated patients compared to naïve patients. The median survival time without SPT (time during which more than 50% of the patients remained SPT-free), evaluated only in the tenofovir disoproxil-treated group, was 5.9 mo. SPT was detected in the naïve population, indicating possible HBV-induced toxicity in renal tubules.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is associated with significant morbidity and mortality due to cirrhosis and hepatocellular carcinoma^[1,2]. Current second-generation antiviral agents are efficient, as they have a high barrier to resistance. They include nucleosidic [*e.g.*, entecavir (ETV)] and nucleotidic [*e.g.*, tenofovir disoproxil (TDF) and tenofovir alafenamide (TAF)] analogues. Nevertheless, the persistence of HBV within hepatocytes in the form of covalently closed circular deoxyribonucleic acid and the

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low probability of hepatitis B surface antigen (HBsAg) clearance necessitates long-term or even life-long treatment. Currently available antiviral agents are eliminated in an active form *via* glomerular filtration and active tubular secretion. Their dosages must be adjusted when the estimated glomerular filtration rate (eGFR) falls under 50 mL/min/1.73 m². Therefore, long-term renal tolerance to antivirals is an important issue.

Although tubular toxicity is well-described in human immunodeficiency virus (HIV) patients treated with TDF^[3-5], less data exist for hepatitis B mono-infection. Registration trials report good tolerance profiles, but real-life studies recount cases of lactic acidosis with ETV treatment, and impaired renal function and rare cases of Fanconi syndrome are reported with TDF^[6-8]. Indeed, these two compounds weakly inhibit host mitochondrial polymerase and may induce tubulopathy^[9]. TDF toxicity may also result from tubular secretion of its active form (tenofovir) and its potential interaction with the metabolism of tubular cells^[3,4,9]. Furthermore, transport proteins may interact with TDF, increasing its intracellular concentration and consequently, its toxicity^[10-12]. Long-term consequences of tubular dysfunction include hypophosphatemia (secondary to hyperphosphaturia), osteomalacia, osteoporosis, and renal failure.

Most studies on nucleot(s)ide analogue (NA) renal toxicity are based on assessments of eGFR and phosphatemia, which are late markers of proximal tubulopathy. Various early markers are available (*e.g.*, non-diabetic glycosuria, hyperaminoaciduria, β_2 -microglobulinuria, and cystinuria), but no consensus exists on their use^[3,4,13,14].

In this study, two early, easy-to-perform, and inexpensive markers were selected: Maximal tubular reabsorption of phosphate per unit volume of eGFR (TmPi/eGFR) and fractional excretion rate of uric acid (FEUA). The objective was to detect and monitor the evolution of subclinical proximal tubulopathy (SPT) over a 2-year period in three populations of HBV-mono-infected patients. The three populations included those who were treatment naïve or those starting treatment with either ETV or TDF.

MATERIALS AND METHODS

Patient selection

A prospective, non-randomized phase IV study involving 20 French centers was conducted. Adult patients with HBV mono-infection and an eGFR above 50 mL/min/1.73 m² were included. They were separated into three populations: Naïve, ETV treatment, or TDF treatment, depending on the investigator's choice. The following exclusion criteria were employed in this study: Patients already receiving the planned treatment; those who have hepatocellular carcinoma; those coinfected with the hepatitis C virus, hepatitis D virus, or HIV; those with serum phosphate levels < 0.48 mmol/L; and pregnant or breast-feeding women.

Data collection

On day 0 (D0), data on the following characteristics were collected: Age, gender, ethnicity, body mass index (BMI), potentially nephrotoxic treatments (*e.g.*, diuretics, non-steroidal anti-inflammatory drugs), prior anti-HBV treatment, viral load, and fibrosis stage. On D0 and then every 3 mo thereafter until month 24 (M24), the eGFR, phosphatemia, 25-hydroxyvitamin D3 [25(OH)D3] vitamin levels, and dipstick test levels were measured. The TmPi/eGFR and FEUA were calculated. Patients with serum 25(OH)D3 vitamin < 30 ng/mL were supplemented systemically.

The monitoring visits were planned according to the patients' usual follow-up appointments. Treatment choices and any modifications made during the study complied with the recommendations made by the European Association for the Study of the Liver in 2012^[15].

TmPi/eGFR and FEUA calculations

The main objective of this study was to determine the prevalence of SPT at M24 in the three groups. SPT was defined as a TmPi/eGFR below 0.8 mmol/L and/or FEUA above 10%.

TmPi/eGFR was estimated according to Bijvoet's diagram and included serum and urine phosphate and creatinine measured from fasting morning blood and urine samples. The eGFR was estimated with a simplified Modification of Diet in Renal Disease formula. FEUA was calculated as follows: [(urine uric acid × serum creatinine)/(serum uric acid × urine creatinine)] × 100%. If data at inclusion (M0) and M24 were missing, M3 and M21 data were used, respectively.

Prevalence and incidence data

The prevalence of SPT resulting from anti-HBV treatment prior to inclusion, if any, was retrospectively described.

At M24, the prevalence of $\text{eGFR} < 50 \text{ mL/min/1.73 m}^2$ or serum phosphate $< 0.48 \text{ mmol/L}$ and the cumulative incidence of SPT were calculated. High urine calcium defined by a urine calcium/blood calcium ratio above 0.5 mmol/mmol was used as a marker of bone involvement at M24.

Ethical considerations

The study was conducted in full compliance with the European and French guidelines of good clinical practices. It was approved by the French Institutional Review Board and the Independent Ethics Committee of Limoges. The study is registered with ClinicalTrials.gov under the number NCT01500265. Eligible patients were given information describing the study in readily understandable language detailing the investigational nature of the study. All patients gave written informed consent for study participation and blood sample conservation.

Statistical methods

Statistical analyses were performed by the Methodological, Epidemiological, and Biostatistical Research Center of the University Hospital of Limoges, using SAS V9.3® software (SAS Institute; Cary, NC, United States). The package *survival* in R v3.2.2 software was used for survival analyses.

Quantitative variables were described using means and standard deviations, medians, and interquartile ranges. Analyses of variance or Kruskal-Wallis tests were used to compare treatment-naïve patients to ETV- and TDF-treated patients.

Qualitative variables were described using the numbers and percentages associated with their 95% confidence intervals (CIs). They were compared using chi-squared or Fisher's exact tests. These tests were also performed to compare the prevalence of SPT at inclusion between previously-treated patients and patients who had not received any antiviral treatment before inclusion, as well as the M24 prevalence of renal insufficiency, hypophosphatemia, or hypercalciuria, depending on the occurrence of SPT during follow-up.

P values less than 0.05 were considered to denote significance except for the main objective and the differences between the naïve group and each treatment group at inclusion, which were deemed significant at $P < 0.025$.

The SPT-free survival curves of the different groups over the 24 mo were plotted using the Kaplan-Meier method. The log-rank test was used to compare survival curves between the groups.

Analyses were adjusted to account for potential confounders. For the main analysis (*i.e.* prevalence of SPT at M24) a multivariate binary logistic regression model was used, whereas a Cox model was used for the cumulative incidence of SPT. The models included variables associated with *P* values of less than 0.2 in the univariate analysis; variables were strained using the step-by-step method.

RESULTS

Study population

Data were obtained from 214 patients between December 2011 and December 2013; 18 were excluded from the analysis (Figure 1). The final dataset was compiled from 196 patients: 116 in the naïve group, 38 in the ETV group, and 42 in the TDF group.

Prevalence of SPT at baseline with or without previous HBV treatment

Of the 196 patients analyzed, 22 (11.2%) had received previous HBV therapy: Adefovir (36%), lamivudine (27.3%), or both (36.7%). At baseline, 40 patients (22.5%) presented with SPT. SPT prevalence did not differ significantly between previously treated and untreated patients (21.5% *vs* 30%, respectively; $P = 0.40$).

SPT prevalence at M24

Forty patients met the criteria of SPT at D0. Eighteen patients with incomplete biological reports, including at D0, were further excluded. The final number of patients with no SPT at D0 was 138: 84 in the naïve group, 28 in the ETV group, and 26 in the TDF group (Figure 1). Clinical and para-clinical characteristics of these 138 patients at inclusion are summarized in Tables 1 and 2.

Table 1 Characteristics of the patients with no subclinical proximal tubulopathy at on day 0

| Variables | Global (<i>n</i> = 138); <i>n</i> (%) ou; median (Q1; Q3); (<i>n</i>) (min-max) | ETV (<i>n</i> = 28); <i>n</i> (%) ou; median (Q1; Q3) (<i>n</i>) (min-max) | Naive (<i>n</i> = 84); <i>n</i> (%) ou; median (Q1; Q3) (<i>n</i>) (min-max) | TDF (<i>n</i> = 26); <i>n</i> (%) ou; median (Q1; Q3) (<i>n</i>) (min-max) | <i>P</i> value; ETV vs naive | <i>P</i> value; TDF vs naive |
|--|---|--|--|--|------------------------------------|------------------------------------|
| Male | 72 (52.2%) | 19 (67.9%) | 39 (46.4%) | 14 (53.8%) | 0.05 ¹ | 0.51 ¹ |
| Age in yr | 37.5 (29; 47); (<i>n</i> = 138); (18-74) | 45.5 (31; 57.5); (<i>n</i> = 28); (18-66) | 36.5 (29; 45); (<i>n</i> = 84); (18-74) | 35.5 (24; 42) (<i>n</i> = 26); (21-56) | 0.08 ² | 0.22 ² |
| BMI in kg/m ² | 24.5 (21.3; 27.8); (<i>n</i> = 115); (16.6-38.8) | 25 (22.2; 29); (<i>n</i> = 24); (16.6-36.3) | 24.8 (21.7; 28.7); (<i>n</i> = 71); (17.8-38.8) | 21.8 (19; 26.5); (<i>n</i> = 20); (18-35.2) | 0.91 ² | 0.02 ² |
| Ethnicity | | | | | | |
| -African | 65 (47.1%) | 7 (25.0%) | 44 (52.4%) | 14 (53.8%) | 0.003 ³ | 0.05 ³ |
| -Asian | 16 (11.6%) | 7 (25.0%) | 4 (4.8%) | 5 (19.2%) | | |
| -White | 57 (41.3%) | 14 (50.0%) | 36 (42.9%) | 7 (26.9%) | | |
| Phases of infection | | | | | | |
| -HbeAg + chronic infection | 6 (4.3%) | 3 (10.7%) | 3 (3.6%) | 0 (0.0%) | < 0.0001 ³ | < 0.0001 ³ |
| -HbeAg + chronic hepatitis | 15 (10.9%) | 6 (21.4%) | 3 (3.6%) | 6 (23.1%) | | |
| -HbeAg-chronic infection | 60 (43.5%) | 1 (3.6%) | 57 (67.9%) | 2 (7.7%) | | |
| -HbeAg-chronic hepatitis | 57 (41.3%) | 18 (64.3%) | 21 (25.0%) | 18 (69.2%) | | |
| Diabetes | 9 (6.5%) | 5 (17.9%) | 4 (4.8%) | 0 (0.0%) | 0.04 ³ | 0.57 ³ |
| High blood pressure | 25 (18.1%) | 9 (32.1%) | 12 (14.3%) | 4 (15.4%) | 0.04 ¹ | 1.00 ³ |
| Renal insufficiency | 1 (0.7%) | 0 (0%) | 1 (1.2%) | 0 (0%) | 1.00 ³ | 1.00 ³ |
| Viral load | | | | | | |
| -PCR < 2000 UI/mL | 69 (61.6%) | 3 (15.8%) | 60 (77.9%) | 6 (37.5%) | < 0.0001 ³ | < 0.0001 ³ |
| -PCR ≥ 2000 et < 20000 UI/mL | 22 (19.6%) | 6 (31.6%) | 16 (20.8%) | 0 (0%) | | |
| -PCR ≥ 20000 UI/mL and < 7 (log) | 13 (11.6%) | 6 (31.6%) | 1 (1.3%) | 6 (37.5%) | | |
| PCR > 7 (log) | 8 (7.1%) | 4 (21.1%) | 0 (0%) | 4 (2.5%) | < 0.0001 ² | < 0.0001 ³ |
| ALAT UI/L | 25 (17; 36); (<i>n</i> = 133); (7- 214) | 40 (25; 57); (<i>n</i> = 27); (17- 148) | 19 (15; 26); (<i>n</i> = 83); (7- 89) | 46 (28; 70); (<i>n</i> = 23); (10- 214) | | |
| Fibrosis ⁴ | | | | | | |
| -F0/F1 ⁵ | 103 (84.4%) | 13 (56.5%) | 73 (94.8%) | 17 (77.3%) | < 0.0001 ³ | 0.0067 ³ |
| -F2 | 5 (4.1%) | 2 (8.7%) | 2 (2.6%) | 1 (4.5%) | | |
| -F2/F3 | 8 (6.6%) | 6 (26.1%) | 2 (2.6%) | 0 (0%) | | |
| -F3 | 1 (0.8%) | 0 (0.0%) | 0 (0%) | 1 (4.5%) | | |
| -F3/F4 | | | | | | |
| -F4 | 5 (4.1%) | 2 (8.7%) | 0 (0%) | 3 (13.6%) | | |
| Fibrosis F0/F1 vs F2 | | | | | | |
| -F0/F1 | 103 (84.4%) | 13 (56.5%) | 73 (94.8%) | 17 (77.3%) | < 0.0001 ³ | 0.02 ³ |
| -≥ F2 | 19 (15.6%) | 10 (43.5%) | 4 (5.2%) | 5 (22.7%) | | |
| Previous HBV therapy | 14 (10.1%) | 5 (17.9%) | 0 (0.0%) | 9 (34.6%) | 0.0007 ³ | < 0.0001 ³ |

| | | | | | | |
|-------------------|-----------|-----------|----------|----------|-------------------|-------------------|
| Nephrotoxic drugs | 12 (6.1%) | 4 (10.5%) | 6 (5.2%) | 2 (4.8%) | 0.22 ³ | 1.00 ³ |
|-------------------|-----------|-----------|----------|----------|-------------------|-------------------|

¹Chi² test.²Mann-Whitney test.³Fisher's exact test.⁴Evaluated by liver biopsy or FibroScan.⁵METAVIR classification. BMI: Body mass index; ALAT: Alanine aminotransferase; ETV: Entecavir; HbeAG: Hepatitis B e-antigen; HBV: Hepatitis B virus; Max: Maximum; Min: Minimum; Q1: First quartile; Q3: Third quartile; TDF: Tenofovir disoproxil.**Table 2 Clinical characteristics of the patients with no subclinical proximal tubulopathy at on day 0**

| | Global population (<i>n</i> = 138); median (Q1; Q3); (<i>n</i>) (min-max) | ETV group; (<i>n</i> = 28); median (Q1; Q3); (<i>n</i>) (min-max) | Naïve group; (<i>n</i> = 84); median (Q1; Q3); (<i>n</i>) (min-max) | TDF group; (<i>n</i> = 26); median (Q1; Q3); (<i>n</i>) (min-max) | <i>P</i> value; ETV vs naïve | <i>P</i> value; TDF vs naïve |
|---|--|--|--|--|------------------------------|------------------------------|
| Phosphatemia, mmol/L | 1.1 (1.0; 1.2); (<i>n</i> = 135); (0.6-1.4) | 1.1 (1.0; 1.1); (<i>n</i> = 26); (0.9-1.3) | 1.0 (1.0; 1.2); (<i>n</i> = 83); (0.7-1.4) | 1.0 (0.9; 1.2); (<i>n</i> = 26); (0.6-1.2) | 0.40 ¹ | 0.98 ¹ |
| Plasma creatinine, μmol/L | 73 (58; 85); (<i>n</i> = 137); (37.5-114.9) | 78.4 (66; 84); (<i>n</i> = 28); (51-114.9) | 71 (58; 87); (<i>n</i> = 83); (37.5-113) | 76.9 (57.5; 87); (<i>n</i> = 26); (38-98) | 0.23 ² | 0.77 ² |
| eGFR (MDRD), mL/min/1.73 m ² | 94.5 (82.6; 107.6); (<i>n</i> = 137); (58.6-169.1) | 91 (84.2; 101); (<i>n</i> = 28); (62.7-141.3) | 94.8 (80.7; 108.1); (<i>n</i> = 83); (58.6-151.1) | 95.4 (84.3; 108.4); (<i>n</i> = 26); (70.2-169.1) | 0.37 ² | 0.65 ² |
| 25(OH)D3, ng/mL | 15.9 (9.9; 22.2); (<i>n</i> = 130); (3.1-55.2) | 16.8 (12.6; 24.8); (<i>n</i> = 26); (5-55.2) | 14.8 (9.4; 21); (<i>n</i> = 81); (4-42.1) | 15.3 (9.8; 22.7); (<i>n</i> = 23); (3.1-36.9) | 0.27 ² | 0.95 ² |
| TmPi/eGFR, mmol/L | 1 (0.9; 1.1); (<i>n</i> = 181); (0.4-1.9) | 1 (0.9; 1.2); (<i>n</i> = 34); (0.7-1.8) | 1 (0.9; 1.1); (<i>n</i> = 111); (0.4-1.9) | 1 (0.8; 1.2); (<i>n</i> = 36); (0.6-1.5) | 0.24 ² | 0.86 ² |
| FEUA, % | 5.8 (4.5; 7.1); (<i>n</i> = 112); (2.2-9.7) | 5.9 (4.7; 7.5); (<i>n</i> = 23); (2.7-9.1) | 5.8 (4.4; 7); (<i>n</i> = 69); (2.2-9.3) | 5.5 (4.5; 6.7); (<i>n</i> = 20); (3.8-9.7) | 0.64 ¹ | 0.95 ¹ |

¹Student's test.²Mann-Whitney test. 25(OH)D3: 25-hydroxyvitamin D3; eGFR: Estimated glomerular filtration rate; ETV: Entecavir; FEUA: Fractional excretion rate of uric acid; Max: Maximum; Min: Minimum; Q1: First quartile; Q3: Third quartile; TDF: Tenofovir disoproxil.

Statistically significant differences in chronic hepatitis (*vs* infection), HBsAg-status, viremia levels, alanine aminotransferase (ALT) levels, and fibrosis stage were found between the treated groups (ETV or TDF) *vs* the naïve group. Some unexpected differences were also observed. Compared to the naïve group, the ETV group contained more Asian patients, and patients in the TDF group had lower BMIs. These differences were accounted for in the adjusted analyses.

Of the 138 patients without SPT at baseline, 45 had missing data at M24 and had to be excluded from the analysis of SPT prevalence at that timepoint. Therefore, the main analysis included data from 93 patients, with 62 in the naïve group, 19 in the ETV group, and 12 in the TDF group. Accordingly, the overall prevalence of SPT at M24 was 31.2% (*n* = 29/93; 95%CI: 22.0–41.6). Among the three treatment groups, the prevalence was 30.7% (*n* = 19/62; 95%CI: 19.6–43.7) in the naïve group, 21.1% (*n* = 4/19; 95%CI: 6.1–45.6) in the ETV group, and 50% (*n* = 6/12; 95%CI: 21.1–78.9) in the TDF group. No statistically significant differences were observed between the naïve group and the ETV (*P* = 0.42) or the TDF group (*P* = 0.42) (Table 3).

Adjusted analyses of SPT prevalence at 24 mo

Potential confounding factors among the different groups were assayed at baseline: Age, gender, ethnicity, virological status, diabetes, hypertension, potential nephrotoxic drugs, ALT and viremia levels, fibrosis stage, and previous HBV therapy (Table 1). Ethnicity was not included in the model because no Asian patient had SPT at M24. Table 4 contains the results of the univariate models given as raw odds ratios (ORs). Variables associated with *P* values of less than 0.20 (gender and age) were tested in a multivariate model comparing ETV and naïve groups. The effect of group on the presence or absence of SPT at M24 was not affected by any adjustment variables (OR = 0.60; 95%CI: 0.17–2.06; *P* = 0.42). No multivariate model could be built to compare TDF and naïve groups (no variable had a *P* value less than 0.20).

Finally, group membership had no significant effect on the presence or absence of SPT at M24 (OR = 2.26; 95%CI: 0.65–7.93; *P* = 0.20).

Table 3 Subclinical proximal tubulopathy prevalence at month 24 in the entecavir, naïve and tenofovir disoproxil groups

| | Global (<i>n</i> = 138); <i>n</i> (%) (95%CI) | ETV (<i>n</i> = 28); <i>n</i> (%) (95%CI) | Naïve (<i>n</i> = 84); <i>n</i> (%) (95%CI) | TDF (<i>n</i> = 26); <i>n</i> (%) (95%CI) | <i>P</i> value; ETV vs naïve | <i>P</i> value; TDF vs naïve |
|---|--|--|--|--|------------------------------|------------------------------|
| Missing values | 45 | 9 | 22 | 14 | | |
| SPT prevalence at M24; (<i>n</i> = 93) | 29 (31.2%); (22.0-41.6) | 4 (21.1%); (6.1-45.6) | 19 (30.7%); (19.6-43.7) | 6 (50.00%); (21.1-78.9) | 0.42 ¹ | 0.32 ² |

¹Chi² test.²Fisher's exact test. CI: Confidence interval; ETV: Entecavir; SPT: Subclinical proximal tubulopathy; TDF: Tenofovir disoproxil.**Table 4 Potential confounding factors at baseline susceptible to influence the prevalence of subclinical proximal tubulopathy at month 24 between the different groups in univariate analysis**

| | | HR (95%CI) | <i>P</i> value | Global <i>P</i> value |
|-----------------------|---------------------------|------------------|----------------|-----------------------|
| Fibrosis | ≥ F2 vs F0/F1 | 1.09 (0.32-3.67) | 0.89 | 0.89 |
| Group | ETV vs naïve | 0.41 (0.09-1.83) | 0.24 | 0.043 |
| | TDF vs naïve | 2.28 (0.98-5.30) | 0.05 | |
| Sex | Female vs male | 0.85 (0.38-1.87) | 0.68 | 0.68 |
| Ethnicity | African vs White | 0.91 (0.41-2.04) | 0.83 | 0.63 |
| | Asian vs White | 0.36 (0.05-2.84) | 0.33 | |
| Diabetes | Yes vs no | 0.63 (0.08-4.67) | 0.65 | 0.65 |
| Previous hypertension | Yes vs no | 1.26 (0.50-3.17) | 0.63 | 0.63 |
| Viral load | Low vs very low | 0.94 (0.30-2.89) | 0.91 | 0.46 |
| | Elevated vs very low | 2.38 (0.77-7.34) | 0.13 | |
| | Very elevated vs very low | 1.40 (0.40-4.93) | 0.60 | |
| Previous HBV therapy | Yes vs no | 1.11 (0.33-3.74) | 0.86 | 0.86 |
| Age at inclusion | | 1.02 (0.98-1.05) | | 0.35 |
| BMI at inclusion | | 0.98 (0.89-1.08) | | 0.67 |
| ALAT at inclusion | | 1.00 (0.99-1.01) | | 0.67 |

ALAT: Alanine aminotransferase; BMI: Body mass index; ETV: Entecavir; HBV: Hepatitis B virus; HR: Hazard ratio; TDF: Tenofovir disoproxil.

Cumulative incidence of SPT over 24 mo

The overall survival rate of SPT-free patients at M24 was 52.2% (95%CI: 38.3-71.2). Among the three groups, the survival rates were 57.6% (95%CI: 47.1-79.6) in the naïve group, 68.8% (95%CI: 38.1-100) in the ETV group, and 23.5% (95%CI: 5.3-100) in the TDF group.

The median survival time, corresponding to the time during which more than 50% of the patients remained SPT-free, was analyzable only in the TDF group. The median survival time in this group was 5.9 mo. The occurrence of SPT in the TDF group differed significantly from that in the other two groups (log-rank test; *P* = 0.0283; Figure 2).

Adjusted analysis of cumulative incidence of SPT over 24 mo

No multivariate analysis was conducted as no potential confounding factors had *P* < 0.20. The univariate model found no significant effects between ETV and naïve groups [hazard ratio (HR): 0.41; 95%CI: 0.09-1.83; *P* = 0.24]. The HR associated with the TDF group vs the naïve group was 2.28 (95%CI: 0.98-5.30; *P* = 0.0546). Thus, TDF treatment tended to be associated with TDF-induced tubular toxicity.

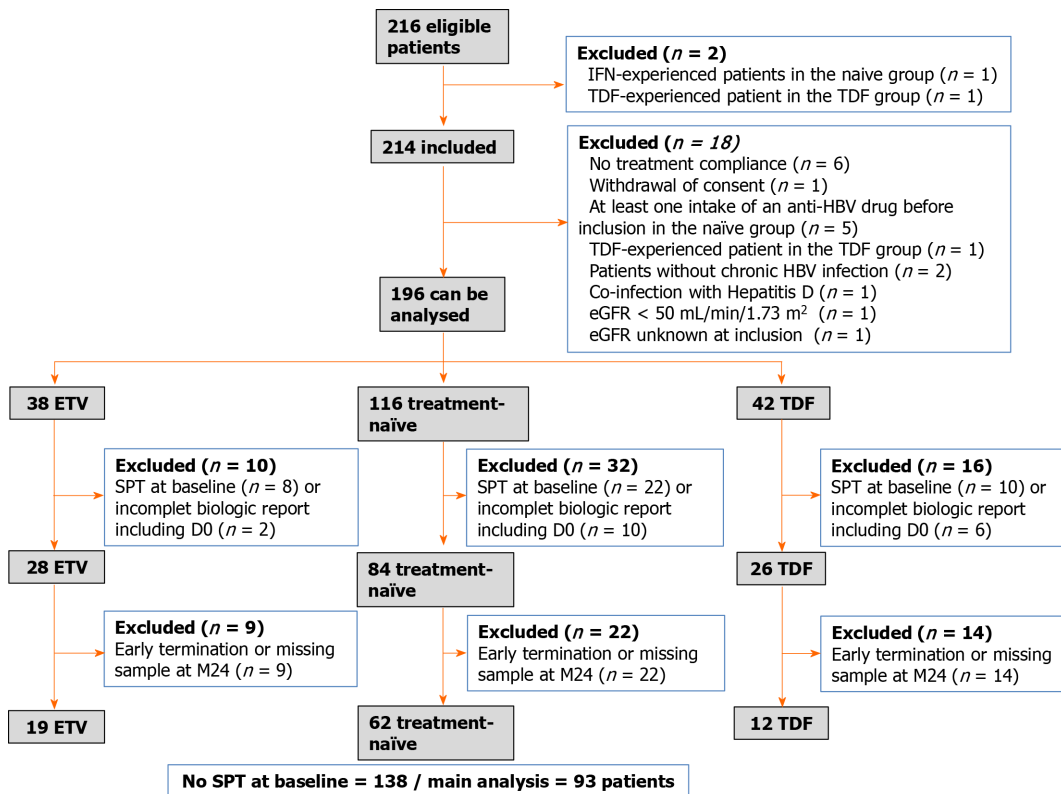


Figure 1 Data were obtained from 214 patients between December 2011 and December 2013; 18 were excluded from the analysis. eGFR: Estimated glomerular filtration rate; ETV: Entecavir; HBV: Hepatitis B virus; IFN: Interferon alpha; TDF: Tenofovir disoproxil; SPT: Subclinical proximal tubulopathy.

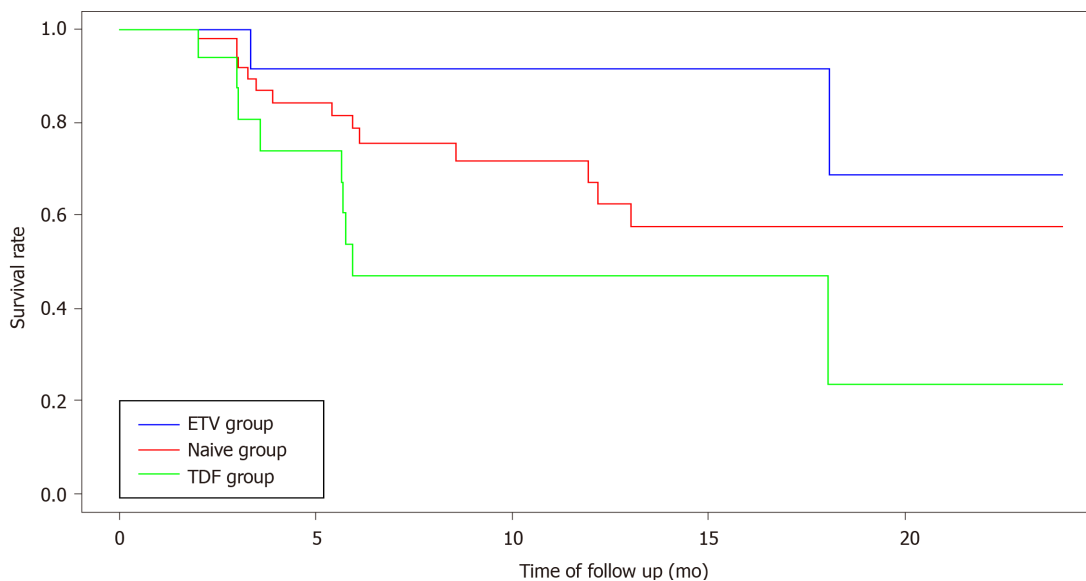


Figure 2 Kaplan Meier curves for free subclinical proximal tubulopathy survival among the different groups (entecavir, naïve, tenofovir disoproxil). ETV: Entecavir; TDF: Tenofovir disoproxil.

Prevalence of impaired renal function (eGFR < 50 mL/min/1.73 m²),

hypophosphatemia (< 0.48 mmol/L), and hypercalciuria (> 0.5 mmol/mmol) at M24

In patients without SPT at baseline, no renal function impairment or hypophosphatemia was observed at M24, regardless of whether they had developed SPT during follow-up. However, four patients (6.5%) experienced hypercalciuria at M24. Three (7.0%) did not develop SPT within 24 mo, whereas one (5.3%) developed SPT after M12 with simultaneous alterations of TmPi/eGFR and FEUA. This latter patient was an HBsAg-negative African female belonging to the naïve group and

presented with hypertension and grade I obesity.

DISCUSSION

Most of the studies investigating the renal tolerance of NAs have focused on glomerular markers (serum creatinine and eGFR) instead of tubular markers^[16-18]. Although data on the tubular toxicity caused by TDF in HIV-positive patients are widely available^[3-5], analogous data in HBV-monoinfected populations are sparse^[3,4].

This paper reports on the first prospective, multicenter study that evaluated the prevalence and incidence of SPT for an extended duration (24 mo) using early markers in a population of HBV-monoinfected patients starting treatment with ETV or TDF.

A strong point of this study was the comparison of the treated groups with a control naïve group. The latter allowed for an evaluation of the role of HBV on tubular function in the absence of any treatments. Additionally, the effects of confounding factors on the interpretation of SPT prevalence or incidence were limited, as the patient population was homogeneous, relatively young (median age: 37.5 years), and had very few renal comorbidities.

Tubular markers

Optimal markers of proximal tubulopathy are not agreed upon the literature. The most commonly used markers, whether early or late, are increased urinary α 1-microglobulin, urinary β 2-microglobulin, urinary retinol binding protein (RBP) or mixed proteinuria, fractional phosphate or uric acid excretion, non-diabetic glycosuria, hypophosphatemia, hypouricemia, hypokalemia, aminoaciduria, and renal tubular acidosis^[3,4,13]. None of these markers have demonstrated superiority in terms of sensitivity and specificity. The more sophisticated markers such as RBP or β 2-microglobulin are interesting, but they are expensive to analyze and not widely used. Kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin are markers of acute tubular injury, which is passed the early prevention stage^[19]. The markers chosen in this study, TmPi/eGFR and FEUA, are easy to use, inexpensive, repeatable over time, and thus ideal for routine follow-up.

TDF and SPT

In this study, the prevalence of SPT at M24 was higher in patients treated with TDF compared to naïve patients (50% *vs* 30%). However, this difference was not statistically significant. Nevertheless, the HR for the cumulative incidence of SPT in the TDF group *vs* the naïve group was 2.28, with a trend towards significance and TDF-induced tubular toxicity.

In the literature, many study designs are heterogeneous. Two main studies used the same population as this one, except they were cross-sectional. In the first, Tien *et al*^[20] compared the prevalence of SPT (defined as decreased TmPi/eGFR) in 146 HBV-monoinfected patients (60 naïve, 44 treated with ETV, and 42 treated with TDF), of whom fewer than 2% had an eGFR < 60 mL/min/1.73 m²^[20]. SPT prevalence was 30%, 23%, and 43% in naïve, ETV, and TDF patients, respectively. Differences among these groups were not statistically different. Nonetheless, in a subgroup of patients treated with ETV and TDF for more than 18 mo, the prevalence of SPT was significantly higher in the TDF-treated group than in the ETV-treated group (48.5% *vs* 12.5%; *P* = 0.005). The second study was the multicenter "MENTE" study consisting of 280 HBV-monoinfected patients (122 naïve, 89 ETV, and 69 TDF), which reported an association between the TDF group and the presence of SPT^[21]. Here, the urinary RBP/creatininuria ratio was used as an SPT marker^[21].

In brief, no study has rigorously demonstrated a causal link between TDF and SPT or directly compared patients treated with TDF and ETV. Moreover, no other study has prospectively evaluated SPT incidence according to treatment type (*i.e.* naïve, ETV, and TDF).

ETV and SPT

The prevalence and cumulative incidence of SPT in the ETV-treated group compared to those in the naïve group were not significantly different. This negative result reinforces the good renal safety profile of ETV in humans and mouse models^[22-24]. Accordingly, Viganò *et al*^[25] argued that SPT in TDF-treated patients improved after switching to ETV^[25].

HBV and SPT

As previously highlighted *in vitro*, HBV-specific tubular toxicity may result from HBV replication and transcription activity in proximal tubular cells. In tubular cell cultures, the serum of infected patients had potential apoptotic effects^[20,21,26,27]. Detection of SPT in our HBV-monoinfected naïve patients supports this hypothesis *in vivo*.

A limitation of this study is the absence of a matched control population not infected with HBV. However, the tubular markers chosen here had been in use for many decades and validated in populations of healthy subjects. For instance, the adult 95% reference range for TmPi/eGFR is 0.80–1.35 mmol/L. Independent of age, normal values are above 0.8 mmol/L in healthy subjects^[28]. The normal value of FEUA is approximately 8%; values above 10% are considered to reflect a reabsorption defect^[29]. Consequently, using as a reference the normal values as defined in healthy populations, the observation that nearly 30% of the naïve HBV-monoinfected population met the definition of SPT implies a link between SPT and HBV infection.

Renal insufficiency, hypophosphatemia, and hypercalciuria

In this study, SPT screened at baseline or during follow-up in the low renal risk population did not impact eGFR, phosphatemia, and urinary calcium at M24 of NA-therapy. Data from the literature are highly variable due to the heterogeneity of the populations in terms of renal risk factors, age, pre-existing renal insufficiency, concomitant nephrotoxic drugs, and/or HIV co-infection^[6,16,18].

In contrast to the results reported here, Tien *et al.*^[20] found that the eGFR was lower in the ETV- and TDF-treated groups compared to the naïve group ($P = 0.002$) but not significantly different between the ETV- and TDF-treated groups^[20]. However, the decline in eGFR correlated with age and not with antiviral treatment. Further, their study design did not allow for any conclusions regarding an association between the observed reduction in eGFR and changes in tubular function (TmPi/eGFR).

In a prospective, single-center study, Viganò *et al.*^[30] evaluated the prevalence and incidence of hypophosphatemia and hyperphosphaturia within a median duration of 27 mo in 156 NA-naïve patients receiving TDF^[30]. During the follow-up, hyperphosphaturia appeared *de novo* in 26% of the patients, of whom only 4% developed mild hypophosphatemia (≤ 2.5 mg/dL)^[30]. None of the hypophosphatemia patients developed a severe, diffuse stage of tubulopathy that is characteristic of Fanconi syndrome.

The occurrence of hypophosphatemia following a correction of 25(OH)D3 deficiency reflects major perturbation in proximal tubular function in which compensatory mechanisms are exceeded. Cases of Fanconi syndrome are exceptional in HBV-monoinfected patients and have been described only with nucleotide analogues (*e.g.*, adefovir and exceptionally, TDF)^[31–33]. Regarding bone toxicity, the "MENTE" study failed to find a clear association between SPT and abnormal markers of bone remodeling^[21].

In summary, the few studies focusing on SPT following NA treatment are mainly cross-sectional and consequently do not allow for the long-term evaluation of their effects on renal and bone health. This prospective study suggests that, in low renal risk patients, SPT does not have clinical impacts on renal or bone health at M24.

25(OH)D3 insufficiency

In this study, the prevalence of 25(OH)D3 insufficiency and severe deficiency was 66.9% and 25.4% at baseline and 84.7% and 7.1% at M24, respectively, despite iterative supplementation. These results are very similar to those reported in the literature. In the Maggi study, which evaluated renal and bone toxicity in chronic hepatitis B patients treated with lamivudine and adefovir, the prevalence of vitamin D insufficiency and severe deficiency was 72.2% and 20.4%, respectively^[34]. Vitamin D insufficiency is common in chronic liver disease irrespective of etiology^[35]. Additionally, 25(OH)D3 has been suggested to increase tubular reabsorption of phosphate, in particular by directly modifying the lipid structure of the cell membrane of proximal tubular cells^[36]. In line with this hypothesis, the patients in this study had their 25(OH)D3 levels measured and supplemented to limit renal phosphate loss and misinterpretation of TmPi/eGFR levels.

Limitations of the study

The main limitation of this study was the small number of patients who completed SPT markers follow-up. Also, some missing SPT markers were substituted with values from the nearest available date (< 3 mo). Moreover, the choice of the primary endpoint (TmPi/eGFR < 0.8 mmol/L and/or FEUA $> 10\%$) favored sensitivity over specificity.

When the two markers, $\text{TmPi}/\text{eGFR} < 0.8 \text{ mmol/L}$ and $\text{FEUA} > 10\%$, were combined, the prevalence of SPT was 2.6%, 0%, and 9.5% in the naïve, ETV, and TDF groups, respectively, with no significant differences among the groups.

The absence of randomization could have generated a selection bias as baseline parameters potentially influencing renal function might not have been well-balanced in the treatment assignments, which were selected by the investigator. However, these potential confounders were limited in the overall population, which was characterized by a young age (median, 37.5 years) and very few renal comorbidities.

The dose-dependence of tubular toxicity caused by NAs could have been explored, especially with TDF. Unfortunately, TDF dosages were not readily available and were not recommended at the time of this study. Gene polymorphisms in the transporter proteins involved in TDF elimination (ABCC2 or ABCC4 genes) have been linked to renal tubular damage, implying that overexposure to TDF could cause kidney tubular cell damage. In HIV-infected patients, Rodríguez-Nóvoa *et al.*^[37] reported that median TDF plasma trough concentration was higher in patients with SPT as defined by the same early markers used in this study. However, even if this result implies cumulative toxicity, whether elevated TDF plasma concentration causes the development of SPT could not be determined due to their cross-sectional analysis.

The overexposure of tenofovir has so far been suggested but not proven in terms of the mechanism of toxicity. Indeed, the mechanism underlying tubular toxicity is probably not singular and could involve a cumulative dose effect; a recent paper proposed progressive mitochondrial dysfunction as a mechanism of TDF tubular toxicity^[38].

TAF: An opportunity

TAF represents real progress in terms of renal tolerance, but it is not available in all countries for HBV-monoinfected patients, including France. It is similar to TDF, in that it is a tenofovir prodrug but has better renal and bone tolerance profiles, most likely due to its higher intracellular and much lower plasma concentrations.

Two recent randomized, double-blind phase 3 studies evaluated the utility of renal biomarkers in HBV-monoinfected patients treated with TAF or TDF. At 48 wk, glomerular and tubular proteinuria (RBP/creatininuria and β 2-microglobulinuria/creatininuria) was lower in the TAF group (percent change from baseline: 0.3% *vs* 25.1%; $P < 0.001$ and -3.5% *vs* 37.9%; $P < 0.001$, respectively)^[39]. The reversibility of SPT after TDF/TAF switching, as assayed with early tubular markers, remains unknown.

CONCLUSION

This prospective study did not find significant differences in SPT prevalence and incidence at M24 between low renal risk HBV-monoinfected patients treated with ETV or TDF and treatment-naïve patients. Nonetheless, the prevalence and incidence of SPT tended to be higher in the TDF group, which had a low survival time (5.9 mo) without SPT. The data presented here confirm that after 24 mo of NA therapy, patients exhibited a good renal safety profile irrespective of whether SPT was detected at baseline or during follow-up. However, these data should be treated with caution, as additional prospective studies involving large cohorts over several years are still warranted.

Current recommendations include monitoring phosphatemia, serum creatinine, and eGFR to screen renal toxicity, but these are late markers of tubular pathology. In clinical practice, proximal tubular damage would ideally be screened at an early stage using simple and inexpensive tools, especially in populations with renal risk (*e.g.*, patients with hypertension or diabetes or who underwent kidney transplantation). Indeed, the detection here of SPT markers in some HBV-monoinfected patients prior to any antiviral treatment confirms the hypothesis that HBV exerts specific toxicity on proximal tubular cells.

It has been suggested that at 1 year after stopping treatment, SPT could be reversible in approximately 80% of cases^[13]. Finally, TAF is a promising agent and should be used preferentially, at least in patients at risk of renal toxicity.

ARTICLE HIGHLIGHTS

Research background

Proximal tubular renal toxicity is a main concern in prolonged nucleot(s)ide analogue therapy in hepatitis B virus (HBV)-infected patients. Currently available data for HBV-monoinfected patients are either retrospective or cross-sectional. The recommended screening tools for renal toxicity, estimated glomerular filtration rate (eGFR) and phosphatemia, are late markers for subclinical proximal tubular (SPT) damage. Thus, early SPT detection with tools that are simple, inexpensive, and repeatable over time are needed. Moreover, preclinical studies have reported that HBV exhibits potential toxicity in proximal tubular cells before any antiviral treatment.

Research motivation

Early detection of tubulopathy could allow clinicians to choose less toxic therapeutic alternatives such as tenofovir alafenamide (TAF), particularly in patients with renal comorbidities. TAF is not available in all countries for HBV-monoinfected patients, but its use may be transitionally authorized. Clinical evidence in favor of HBV-induced renal toxicity may assist in improving interpretations of SPT markers over time, as well as explain why these markers improve under antiviral use.

Research objectives

The main objective was to determine the prevalence of SPT at month 24 (M24) in three populations: Treatment-naïve patients and patients starting entecavir (ETV) or tenofovir disoproxil (TDF) at M0. The secondary objectives were to evaluate the cumulative incidence of SPT over 24 mo in the three groups as well as the prevalence of SPT in the naïve population at baseline.

Research methods

This first real-life, prospective, multicenter, French study of patients with low renal risk aimed to determine SPT in three groups of HBV-monoinfected patients: Treatment-naïve and those starting ETV or TDF. Markers for SPT, the eGFR and phosphatemia, were assessed quarterly. SPT was defined using early and low-cost simple markers: TmPi/eGFR below 0.8 mmol/L and/or fractional excretion rate of uric acid above 10%. Confounding factors potentially impacting kidney function across the groups were assayed.

Research results

At M24, the prevalence of SPT was 30.7% in the naïve group, 21.1% in the ETV-treated group, and 50.0% in the TDF-treated group. However, differences in SPT prevalence between the naïve group and each treatment group (ETV and TDF groups) were not significantly different. In the multivariate analysis, no post-adjustment variables were identified. The incidence of SPT over 24 mo (25.5%, 13.3%, and 52.9% in the naïve, ETV-treated, and TDF-treated groups, respectively) tended to be higher in the TDF group compared to the naïve group (hazard ratio: 2.283; $P = 0.05$). The median survival time without SPT was 5.9 mo in the TDF group. In patients without SPT at baseline, no renal insufficiency or hypophosphatemia was observed at M24.

Research conclusions

This prospective, multicenter study is the first to evaluate the prevalence and incidence of SPT in low renal risk HBV-monoinfected patients using early markers. Patients were divided into treatment-naïve, ETV-treated, or TDF-treated groups. The prevalence of SPT at M24 was high (21%–50%), but it had no clinical impacts in terms of renal insufficiency or hypophosphatemia. The incidence of SPT tended to be higher in the TDF group. Moreover, the detection of SPT in HBV-monoinfected naïve patients supports the hypothesis of HBV-specific tubular toxicity.

Research perspectives

To better evaluate the clinical impacts of nucleot(s)ide analogue-induced SPT on renal function, future prospective studies tracking both simple and sophisticated SPT markers over a longer period of time are warranted. Furthermore and paradoxically, these early markers may be also used to evaluate treatment reversibility of HBV-induced SPT.

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Safety and efficacy of sofosbuvir/velpatasvir/voxilaprevir in post-liver transplant patients with previous direct-acting antiviral failure: Six case reports

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Abstract

BACKGROUND

Direct-acting antiviral (DAA) therapy regimens are highly effective at eliminating hepatitis C virus (HCV) infection but rates of sustained virologic response (SVR) are lower in patients with decompensated cirrhosis or hepatocellular carcinoma. Since many of these patients will be referred for liver transplant, they will require retreatment after transplantation. Sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX) is recommended by guidelines as the preferred regimen to treat HCV in DAA-experienced patients following liver transplant however there is limited data.

CASE SUMMARY

We present the cases of six liver transplant recipients who had previous treatment failure with sofosbuvir-based DAA therapy prior to transplantation and who then received SOF/VEL/VOX after transplant.

CONCLUSION

This case series demonstrate the real-world efficacy and safety of SOF/VEL/VOX in the post liver transplant setting. Treatment was successful with all patients achieving SVR, it was well tolerated, and there were minimal drug-drug interactions with their immunosuppressants.

Key Words: Sofosbuvir/velpatasvir/voxilaprevir; Hepatitis C; Liver transplant; Direct-

Checklist (2016).

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Core Tip: There have been limited reports published on the use of sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX) for the treatment of hepatitis C virus in post-liver transplant patients who had previous direct-acting antiviral failure prior to transplant. Herein, we present what we believe to be the largest case series of SOF/VEL/VOX use in these patients and highlight its efficacy and safety. More so, we discuss potential drug-drug interactions between SOF/VEL/VOX and common immunosuppression regimens.

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INTRODUCTION

Direct-acting antiviral (DAA) therapy regimens are highly effective at eliminating hepatitis C virus (HCV) infection but patients with decompensated cirrhosis or hepatocellular carcinoma (HCC) have been shown to have lower sustained virologic response rates (SVR)^[1-3]. Some of these patients with DAA treatment failure may eventually be referred for liver transplant and will require retreatment, which in many cases will be undertaken after transplantation. There is extensive published experience with DAA treatment of HCV after liver transplant, with most patients in these studies being treated with glecaprevir/pibrentasvir (G/P) and ledipasvir/sofosbuvir (L/S)^[4,5].

Post-transplant treatment of hepatitis C in patients who have experienced previous DAA failure has been less well studied. The American Association for the Study of Liver Diseases-Infectious Disease Society of America (AASLD-IDSA) recently updated practice guidelines to recommend sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX) as the preferred regimen to treat HCV in DAA-experienced patients following liver transplant^[1]. However, this recommendation is listed as level 1, C which is based on expert consensus as there is a paucity of published experience^[1]. Additionally, the safety of SOF/VEL/VOX in this population, including guidance on navigating drug-drug interactions (DDI) between SOF/VEL/VOX and commonly used immunosuppressants has not been well supported in published studies. As such, we report a series of six patients with previous DAA failure who were subsequently treated successfully with SOF/VEL/VOX after liver transplant. We report our experience to emphasize the efficacy of this regimen in this population while also highlighting the safety profile.

CASE PRESENTATION

Chief complaints

Case 1: A 51-year-old African American male who presented to hepatology clinic to discuss treatment options for HCV.

Case 2: A 68-year-old Caucasian male who presented to hepatology clinic to discuss treatment options for HCV.

Case 3: A 66-year-old African American male who presented to hepatology clinic to discuss treatment options for HCV.

Case 4: A 70-year-old African American male who presented to hepatology clinic to discuss treatment options for HCV.

Case 5: A 48-year-old African American male who presented to hepatology clinic to discuss treatment options for HCV.

Case 6: A 68-year-old African American female who presented to hepatology clinic to discuss treatment options for HCV.

History of present illness

Patients in cases 1-6 had no acute complaints on initial visit. All patients were post-liver transplant and had previously failed treatment of HCV.

History of past illness

Case 1: The patient had a past medical history of HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. He had HCV coinfection with genotypes 1a and 4 that was treated with 24 wk of L/S prior to transplant however SVR was not achieved. Additionally, he received transarterial chemoembolization and transarterial radioembolization prior to transplant to downstage his HCC to meet Milan criteria. He also had a history of latent tuberculosis.

Case 2: The patient had a past medical history of HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. He had HCV coinfection with genotypes 1 and 4 that was treated with 24 wk of L/S as well as interferon/ribavirin and sofosbuvir/ribavirin regimens prior to transplant, however SVR was never achieved. Additionally, he received transarterial chemoembolization and transarterial radioembolization prior to transplant to downstage his HCC to meet Milan criteria, however, following transplant, examination of explant revealed patient was not within Milan criteria. He also had a history of obesity, cataracts, anemia, and vitiligo.

Case 3: The patient had a past medical history of decompensated HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. Prior decompensations included ascites and prior esophageal variceal bleed. He had HCV genotype 1b that was treated with 12 wk of L/S prior to transplant, however SVR was never achieved. He also had a history of coronary artery disease, hypertension, chronic sinusitis and vitamin D deficiency.

Case 4: The patient had a past medical history of HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. The patient had HCV genotype 1b that was treated with 12 wk of L/S prior to transplant, however SVR was never achieved. He also had a history of hypertension, diabetes mellitus and latent tuberculosis.

Case 5: The patient had a past medical history of decompensated HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. Prior decompensations included ascites and portopulmonary hypertension. He had HCV coinfection with genotypes 1 and 4 that was treated with 24 wk of L/S as well as 12 wk of simeprevir/sofosbuvir prior to transplant, however SVR was never achieved. He also had a history of diabetes mellitus, latent tuberculosis, and prior clostridium difficile infection.

Case 6: The patient had a past medical history of HCV cirrhosis complicated by HCC who underwent orthotopic liver transplantation. She had HCV genotypes 1a that was treated with 12 wk of L/S prior to transplant, however SVR was never achieved. She was then treated with elbasvir/grazoprevir following transplant but she again failed to achieve SVR. She also had a history of diabetes mellitus, hypertension, and hyperlipidemia.

Baseline patient demographics are included in [Table 1](#) and patients' prior therapies are summarized in [Table 2](#).

Personal and family history

Case 1: The patient was a nonsmoker and did not drink alcohol. He had no relevant family history.

Case 2: The patient did not drink, smoke, or ever use intravenous drugs. Patient does not know his family history.

Case 3: Patient was a nonsmoker and denies alcohol use. His family history was negative for liver disease and otherwise noncontributory.

Table 1 Baseline demographics prior to sofosbuvir/velpatasvir/voxilaprevir therapy

| Patient No. | Age/Sex | Race | HCC | Decompensated cirrhosis | HCV RNA (IU/L) | Downstaged within Milan criteria | Within Milan criteria on explant |
|-------------|---------|------|-----|-------------------------|----------------|----------------------------------|----------------------------------|
| 1 | 51 M | AA | Yes | No | 27461835 | Yes | Yes |
| 2 | 68 M | O | Yes | No | 944857 | Yes | No |
| 3 | 66 M | AA | Yes | Yes | 894000 | No | Yes |
| 4 | 70 M | AA | Yes | No | 14700000 | No | Yes |
| 5 | 48 M | AA | Yes | Yes | 3150000 | No | Yes |
| 6 | 68 F | AA | Yes | No | 9024158 | ¹ | ¹ |

¹Liver transplant was performed at another hospital and pathology records were not available.

AA: African American; O: Identified as "other" race; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HCV RNA: Hepatitis C RNA viral load before sofosbuvir/velpatasvir/voxilaprevir therapy; Milan criteria: Did explant pathology satisfy Milan criteria.

Table 2 Patients who completed sofosbuvir/velpatasvir/voxilaprevir after liver transplant

| Patient No. | GT | Pre-transplant child-pugh | Previous therapies | LOT (wk) | Time to start (d) | SVR 12 | ACR |
|-------------|------|---------------------------|--------------------|----------|-------------------|--------|-----|
| 1 | 1a/4 | B | L/S | 24 | 31 | Yes | No |
| 2 | 1/4 | B | IFN/RBV SOF/RBV | | 106 | Yes | No |
| | | | L/S | 24 | | | |
| 3 | 1b | B | L/S | 12 | 132 | Yes | No |
| 4 | 1b | A | L/S | 12 | 256 | Yes | No |
| 5 | 1/4 | B | S/S | 12 | 120 | Yes | No |
| | | | L/S | 24 | | | |
| 6 | 1a | ¹ | L/S | 12 | 785 ³ | Yes | No |
| | | | E/G ² | 16 | | | |

¹Liver transplant was performed at another hospital and pathology records were not available.

²Indicates treatment was after transplant.

³Prolonged time as patient had been lost to follow-up between her transplant at a different institute and establishing care at our institute.

L/S: Ledipasvir/sofosbuvir; S/S: Simeprevir/sofosbuvir; E/G: Elbasvir/grazoprevir; SOF: Sofosbuvir; IFN: Interferon; RBV: Ribavirin; GT: Genotype; LOT: Length of treatment; Treatment start: Number of days after transplant that sofosbuvir/velpatasvir/voxilaprevir therapy was initiated; ACR: Acute cellular rejection after starting sofosbuvir/velpatasvir/voxilaprevir; SVR 12: Undetectable virus 12 wk after completion of therapy.

Case 4: He states he is a former intravenous drug user, smoker, and alcohol drinker. His family history is significant for diabetes mellitus and hypertension.

Case 5: The patient denies tobacco use and he will socially drink. He states both of his siblings have HCV as well.

Case 6: The patient denies recreational drug use, tobacco use, and alcohol use. Both of her parents had skin cancer.

Physical examination

On initial clinic visit, patients in cases 1-5 had physical exams notable for well-healed abdominal surgical scars. Their abdomens were soft, nontender, and without ascites. There was no jaundice or lower extremity edema. The remainder of exams was otherwise unremarkable. The patient in case 6 had similar exam but for a well-healing abdominal surgical scar with staples and Jackson-Pratt drain in still place after surgery.

Laboratory examinations

Relevant laboratory value just prior to initiation of SOF/VEL/VOX for cases 1-6 are as below. Of note, all liver grafts were from HCV antibody negative donors and nucleic acid testing for HCV was also negative.

Case 1: HCV RNA 27461835 IU/L, creatinine 1.38 mg/dL, total bilirubin 0.60 mg/dL, aspartate aminotransferase (AST) 30 units/L, alanine aminotransferase (ALT) 181 units/L, alkaline phosphatase 121 units/L.

Case 2: HCV RNA 944857 IU/L, creatinine 0.77 mg/dL, total bilirubin 0.20 mg/dL, AST 70 units/L, ALT 45 units/L, alkaline phosphatase 92 units/L.

Case 3: HCV RNA 894000 IU/L, creatinine 0.95 mg/dL, total bilirubin 0.20 mg/dL, AST 30 units/L, ALT 21 units/L, alkaline phosphatase 101 units/L.

Case 4: HCV RNA 14700000 IU/L, creatinine 0.98 mg/dL, total bilirubin 0.40 mg/dL, AST 19 units/L, ALT 14 units/L, alkaline phosphatase 84 units/L.

Case 5: HCV RNA 3150000 IU/L, creatinine 1.54 mg/dL, total bilirubin 0.18 mg/dL, AST 55 units/L, ALT 61 units/L, alkaline phosphatase 135 units/L.

Case 6: HCV RNA 9024158 IU/L, creatinine 0.74 mg/dL, total bilirubin 0.30 mg/dL, AST 18 units/L, ALT 26 units/L, alkaline phosphatase 60 units/L.

Imaging examinations

There was no imaging immediately prior to initiation of SOF/VEL/VOX for cases 1-6.

FINAL DIAGNOSIS

The patients in case 1-6 all had HCV that required treatment in the post-transplant setting.

TREATMENT

All six patients were treated with a 12-wk course of SOF/VEL/VOX, initiated at varying times in their post-transplant course with a median start date of 126 (IQR 87.3, 388.3) d after transplant.

OUTCOME AND FOLLOW-UP

After beginning treatment, 100% of the patients achieved undetectable HCV RNA levels within 4 wk. SVR12 was documented in all cases and no patient experienced a virologic relapse with mean follow up time of 505.3 ± 152.8 d. Since treatment was relatively early after transplant, no patients had suspected cirrhosis of their liver graft or clinical findings to suggest fibrosing cholestatic hepatitis, although no formal fibrosis (*i.e.*, liver biopsy or FibroScan) testing was performed. Additionally, no patients had severe renal dysfunction, which we defined as a GFR < 30 mL/min/1.73 m³.

No patient had a serious adverse event defined as death, life-threatening episode, hospitalization, or persistent or significant disability. All patient tolerated SOF/VEL/VOX well with no specific adverse effects reported. Most important, there were no episodes of acute cellular rejection or graft loss and no documented recurrence of HCC in any patient throughout the follow-up period. One patient received a hepatitis B core positive donor has been maintained medication to prevent hepatitis B reactivation.

All patients were on a regimen of tacrolimus and mycophenolate for the first six months following transplant at which point mycophenolate was discontinued and patients were continued on tacrolimus monotherapy as per our institute's immunosuppression protocol. Five of our patients required their tacrolimus dosing to be reduced. All changes were relatively minor and most were in the immediate post-transplant setting where dose changes are often necessary. No patient had toxic levels of tacrolimus, defined as greater than 20 ng/mL, the upper limit of normal for our

laboratory. **Table 3** outlines specific changes in tacrolimus dosing throughout their 12-wk course of SOF/VEL/VOX.

DISCUSSION

We present what we believe to be the first case series highlighting the safety and efficacy of SOF/VEL/VOX in post-liver transplant patients with prior DAA failure. Although DAAs typically have very high cure rates, in general, approximately five-percent of patients with HCV treated with DAAs will not achieve SVR; these patients often have evidence of decompensated cirrhosis or HCC, as the case with our cohort^[1-3]. Since some of these patients may eventually require a liver transplant, we expect there will be cases of transplant recipients with previous DAA failure requiring repeat treatment after the transplant.

SOF/VEL/VOX is a well-established option to treat recurrent HCV in DAA-experienced patients, however the two phase 3 trials from which this was founded did not include liver transplant recipients^[6]. Despite this, SOF/VEL/VOX is currently listed as the preferred regimen for DAA-experienced patients after transplant however, we believe the data in the post-transplant population to be very limited^[1]. To our knowledge, we are aware of only one case report highlighting its effectiveness and safety in this setting^[7]. Additionally, although our study involves a small cohort, we believe this to be a comparatively large sampling as DAA failures are an uncommon event. As such, we believe this case series contributes to our understanding of efficacy, safety profile, and potential DDI for SOF/VEL/VOX in this unique and under-studied population.

We also note that SOF/VEL/VOX has not been formally studied in post-transplant patients, and is therefore not FDA-approved for use in post-transplant population. We expected this regimen to be effective and well tolerated since both G/P and L/S have been previously shown to be safe and effective after transplant^[8,9]. In essence, SOF/VEL/VOX is a combination of both of these regimens in terms of its anti-HCV components. Our findings do show that all patients achieved SVR12 without serious adverse events. In addition, no patient reported any adverse effects that necessitated interruption or early termination of treatment.

We were very concerned about the possibility of DDI, specifically with regards to DAAs and immunosuppressant medications. Tacrolimus remains the backbone for most liver transplant anti-rejection regimens and that is the case at our institution. While we know tacrolimus levels will typically increase with initiation of DAA regimens containing NS3/4A protease inhibitors such as G/P and E/G, there seems to be minimal data on how tacrolimus levels are influenced by SOF/VEL/VOX^[1,10]. The AASLD-IDSA website currently does not suggest dose reduction citing lack of data^[1]. As such, we paid particularly close attention to monitoring tacrolimus levels not only during initiation of SOF/VEL/VOX but also at regular biweekly intervals until completion of treatment. **Table 3** shows changes to tacrolimus dosing for each patient during their 12-wk course of SOF/VEL/VOX. Five of our patients required a dose-reduction in their tacrolimus; this is consistent with other DAA regimens containing NS3/4A protease inhibitors^[10]. Most of the tacrolimus adjustments were minor, further emphasizing the safety profile of SOF/VEL/VOX. Of note, we do not typically use cyclosporine at our institution and as such we are unable to comment on DDI between it and SOF/VEL/VOX, although prior data suggest no dose adjustments are necessary^[10].

One potential limitation of this case series is the small patient cohort. Additionally, included patients are from similar demographics (gender, race, age, and city) possibility limiting generalizability of these findings. And finally, it is well known that patients who fail DAA regimens will frequently have multiple resistance association variants identified on resistance testing. We did not formally assess for these in our patients as their presence has not been shown to affect the ability to achieve SVR with SOF/VEL/VOX^[11].

CONCLUSION

In conclusion, our experience supports the efficacy of SOF/VEL/VOX as an effective therapy for post-transplant treatment of HCV in patients who had previously failed DAA therapy prior to liver transplant as all treated patients achieved SVR at 12 wk. In addition, SOF/VEL/VOX appears to be safe as there were no episodes of acute

Table 3 Immunosuppressive medication changes while receiving sofosbuvir/velpatasvir/voxilaprevir

| | 1 | 2 | 3 | 4 | 5 | 6 |
|------------------|--|--|---|------------------------------------|-----------------------------------|--|
| Baseline regimen | Tacrolimus 3/4 MMF 1000 mg BID Prednisone 20 mg QD | Tacrolimus 2/1 MMF 500 mg BID | Tacrolimus 2/2 MMF 500 mg BID | Tacrolimus 4/4 | Tacrolimus 5/5 | Tacrolimus 3/3 MMF 360 mg BID Prednisone 10 mg QD |
| Week 1 | TL: 14.5 ng/mL ↓ Tacrolimus 3/3 MMF 1000 mg BID Prednisone 15 mg QD | | | | | |
| Week 2 | TL: 12.9 ng/mL ↓ Tacrolimus 3/2 MMF 1000 mg BID Prednisone 15 mg QD | | | | | |
| Week 3 | TL: 8.3 ng/mL ↓ Tacrolimus 3/2 MMF 1000 mg BID Prednisone 10 mg QD | TL: 13.5 ng/mL ↓ Tacrolimus 1/1 MMF 500 mg BID | | TL: 8.6 ng/mL ↓ Tacrolimus 4/3 | | |
| Week 4 | | | | | TL: 8.9 ng/mL ↓ Tacrolimus 5/4 | |
| Week 5 | TL: 8.8 ng/mL ↓ Tacrolimus 3/2 MMF 1000 mg BID Prednisone 5 mg QD | | | TL: 10.9 ng/mL ↓ Tacrolimus 3/3 | | |
| Week 6 | TL: 11.5 ng/mL ↓ Tacrolimus 2/2 MMF 1000 mg BID Prednisone 5 mg QD | | | | TL: 8.7 ng/mL Tacrolimus 4/4 | |
| Week 7 | | | | | | |
| Week 8 | | | | | | |
| Week 9 | | MMF discontinued ² | TL: 2.7 ng/mL ↓ Tacrolimus 3/2 MMF 500 mg BID | | | |
| Week 10 | | TL: 10.7 ng/mL ↓ Tacrolimus 1/0.5 | | | | |
| Week 11 | TL: 14.1 ng/mL ↓ Tacrolimus 1/1 MMF discontinued ¹ Prednisone 5mg QD | | | | | |
| Week 12 | Tacrolimus 1/1 Prednisone 5 mg QD | Tacrolimus 1/0.5 | TL: 4.6 ng/mL ↓ Tacrolimus 3/3 MMF 500 mg BID | Tacrolimus 3/2 ³ | Tacrolimus 4/4 | TL: 2.2 ng/mL ↓ Tacrolimus 3/4 MMF 360 mg BID Prednisone 10 mg QD |

¹Patient developed sepsis from cholangitis.

²Per institution protocol at three months post-transplant.

³Dose adjusted preemptively as fluconazole was added.

MMF: Mycophenolate; TL: Tacrolimus trough level; BID: Two times daily; QD: Once daily.

cellular rejection or serious adverse events. Minor changes in tacrolimus dosing may be needed.

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Successful hepatic resection for recurrent hepatocellular carcinoma after lenvatinib treatment: A case report

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Abstract

BACKGROUND

Lenvatinib has been shown to be noninferior to sorafenib regarding prognosis and recurrence rate in patients with unresectable hepatocellular carcinoma (HCC) who have not received prior systemic chemotherapy. In patients treated with lenvatinib, 40% of cases achieved sufficient tumor reduction to make potential surgery possible. However, the outcomes of such surgery are unknown. We report a successful case of hepatic resection for recurrent HCC after lenvatinib treatment.

CASE SUMMARY

A 69-year-old man underwent right anterior sectionectomy for HCC in segment 8 of the liver. Ten months later, he was found to have an intrahepatic HCC recurrence that grew rapidly to 10 cm in diameter with sternal bone metastases. After confirming partial response to lenvatinib administration for 2 mo, a second hepatectomy was performed. Pathological examination showed that 80% of the tumor was necrotic. The patient did not develop any adverse effects under lenvatinib treatment. He was discharged at 25 d after surgery. Radiation therapy for bone metastases continued to be given under lenvatinib, and the patient has remained alive for 1 year after the second hepatectomy.

CONCLUSION

The prognosis of patients with recurrent HCC may be improved by liver resection combined with prior lenvatinib therapy.

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Core Tip: We report a case of intrahepatic hepatocellular carcinoma recurrence with rapid growth and sternal bone metastases after initial resection. A second surgery was successfully achieved after lenvatinib administration induced tumor shrinkage. Because the patient had bone metastases, multidisciplinary therapy, postoperative radiation therapy, and lenvatinib administration were performed, and the patient remains alive at 1 year postoperatively. In this case, lenvatinib induced a partial response for rapid growth of recurrent hepatocellular carcinoma with bone metastases, and conversion to surgery was successfully achieved for the purpose of controlling the intrahepatic lesion for the first time.

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INTRODUCTION

The treatment strategy for hepatocellular carcinoma (HCC) has been established in various guidelines, including the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan^[1], Barcelona Clinic Liver Cancer Guidelines^[2], American Association for the Study of the Liver Diseases Guidelines^[3], and European Association for the Study of the Liver European Organization for Research and Treatment of Cancer Guidelines^[4]. Hepatectomy is a potential curative treatment for early-stage HCC. However, the indications for liver resection are limited by tumor progression and liver function, and many cases are not eligible for resection. Transarterial chemoembolization (TACE) is commonly used for these unresectable cases, but the outcomes, especially of chemotherapy, have not been satisfactory compared with liver resection until recently^[5,6].

Against this background, the systemic chemotherapy landscape for HCC changed more than a decade ago, with sorafenib demonstrating a survival benefit in the first-line setting and becoming the first systemic therapy to receive approval for HCC. More recently, regorafenib and ramucirumab have been approved in the second-line setting after sorafenib, and lenvatinib has emerged in the first-line setting after a positive phase 3 study^[7-11]. The advent of these effective molecular targeted agents has allowed multidisciplinary treatments combined with chemotherapy and liver resection for HCC.

Regarding colorectal cancer, there are many reports on postoperative adjuvant therapy to prevent recurrence after resection and preoperative neoadjuvant chemotherapy for colorectal cancer and its liver metastases^[12-15]. Thus, conversion therapy in colorectal cancer has become a routine practice. Although conversion therapy for HCC has not yet been established, lenvatinib is expected to be a possible candidate agent because it exhibited a tumor-reducing effect in 40% of cases in the REFLECT study^[14]. There is a possibility that patients with unresectable or recurrent HCC can be converted to surgery after lenvatinib treatment. In the present case, lenvatinib induced a partial response (PR) for rapid growth of recurrent HCC with bone metastases, and conversion to surgery was successfully achieved for the purpose of controlling the intrahepatic lesion.

CASE PRESENTATION

Chief complaints

The patient complained of general malaise.

History of present illness

A 69-year-old man was referred to our facility with a diagnosis of recurrent HCC. He had undergone right anterior sectionectomy for two tumors in segment 8 at another hospital 10 mo previously. The pathological diagnosis of the two tumors was moderately differentiated HCC and poorly differentiated HCC, respectively, without vascular invasion. The pathological stage was stage III according to the Union for International Cancer Control 8th edition.

History of past illness

The patient had undergone appendectomy for appendicitis at 27 years of age.

Personal and family history

The patient's father had experienced lung cancer, and his sister had suffered from breast cancer.

Physical examination

On physical examination, the patient had a temperature of 36.6 °C, heart rate of 76 bpm, respiratory rate of 16 breaths/min, blood pressure of 143/86 mmHg, and oxygen saturation in room air of 98%. The patient's weight was 59 kg. He had no jaundice or anemia in the bulbar conjunctiva. There was an operative scar for an inverted L-shaped incision on the abdomen from the previous liver resection. No ascites and encephalopathy were detected.

Laboratory examinations

The preoperative liver functional reserve was good. Laboratory data revealed serum albumin of 3.5 g/dL, total bilirubin of 0.9 mg/dL, prothrombin time of 93%, and indocyanine green retention rate at 15 min of 5.2%. The Child-Pugh score/classification was 5/A and the albumin-bilirubin (ALBI) score/modified ALBI grade was -2.19/2b. The alpha fetoprotein value was within the normal range, while the protein-induced vitamin K absence or antagonist II value was elevated at 998 mAU/mL.

Imaging examinations

A computed tomography scan of the abdomen demonstrated a heterogeneous contrast-enhanced mass of 10 cm in size in segment 7, which had rapidly increased in 5 mo (Figure 1A and 1B). The magnetic resonance imaging (MRI) images at 5 mo before the second operation are shown in Figure 2 (A: T1 image; B: T2 image). There was no evidence of the recurrent tumor on these MRI images. A positron emission tomography examination showed elevated standardized uptake volume in the sternum and slight uptake in the liver (Figure 3A and 3B). The patient was diagnosed as recurrent HCC in segment 7 of the liver with metastasis in the sternum.

MULTIDISCIPLINARY EXPERT CONSULTATION

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As a treatment strategy, we should administer lenvatinib at a dose of 8 mg because the patient weighed less than 60 kg to suppress the rapidly increasing intrahepatic lesion, and if the intrahepatic lesion exhibited shrinkage, we should surgically remove the lesion. The reason for choosing lenvatinib was that in addition to the intrahepatic recurrence, there was a sternal metastasis. Therefore, we considered that systemic therapy would be better than transcatheter therapy, such as B-TACE. The reason for not choosing surgical resection without prior lenvatinib was that the site of recurrence was rapidly increasing, and it was thought that new lesions might appear in other parts of the liver immediately after surgical resection. In addition, after surgical treatment, we should perform postoperative radiation therapy and treatment using molecular targeted drugs for the metastatic bone lesions.

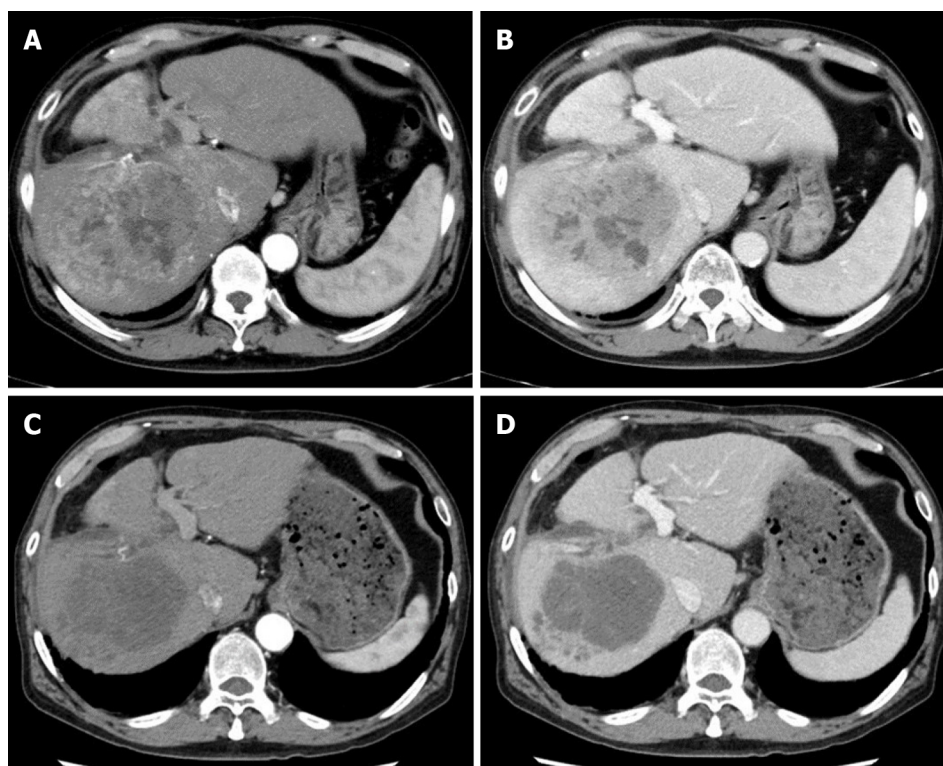


Figure 1 Preoperative computed tomography findings. Before lenvatinib administration, tumor stain was observed in the arterial phase and washout was observed in the portal phase. After lenvatinib administration, the tumor stain disappeared in the arterial phase, and the tumor size was found to have decreased slightly in the portal phase. A and B: Images of the arterial phase (A) and portal phase (B) before administration of lenvatinib; C and D: Images of the arterial phase (C) and portal phase (D) after lenvatinib administration.

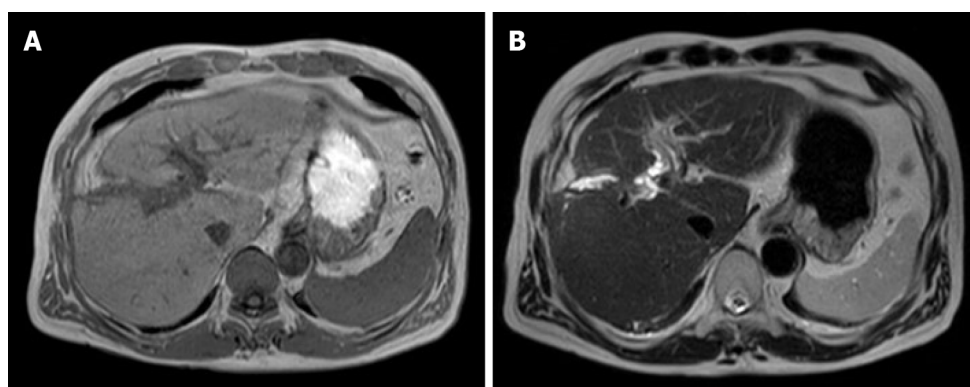


Figure 2 Magnetic resonance imaging findings at 5 mo before the second operation. There was no evidence of the recurrent tumor on the magnetic resonance images. A: T1 image; B: T2 image.

FINAL DIAGNOSIS

Computed tomography imaging after 1 mo of lenvatinib administration revealed that 90% of the tumor had lost the contrast effect. The response evaluation was PR, though close to complete response by the modified Response Evaluation Criteria in Solid Tumors^[6] (Figure 1C and 1D). Protein-induced vitamin K absence or antagonist II decreased to 434 mAU/mL at 2 mo after the treatment (Figure 4).

TREATMENT

Because the intrahepatic recurrent tumor was reduced by administration of lenvatinib, hepatectomy of segment 7 was performed, with blood loss of 3913 mL and operation time of 545 min. Segments 5 and 6 were successfully preserved without bile duct

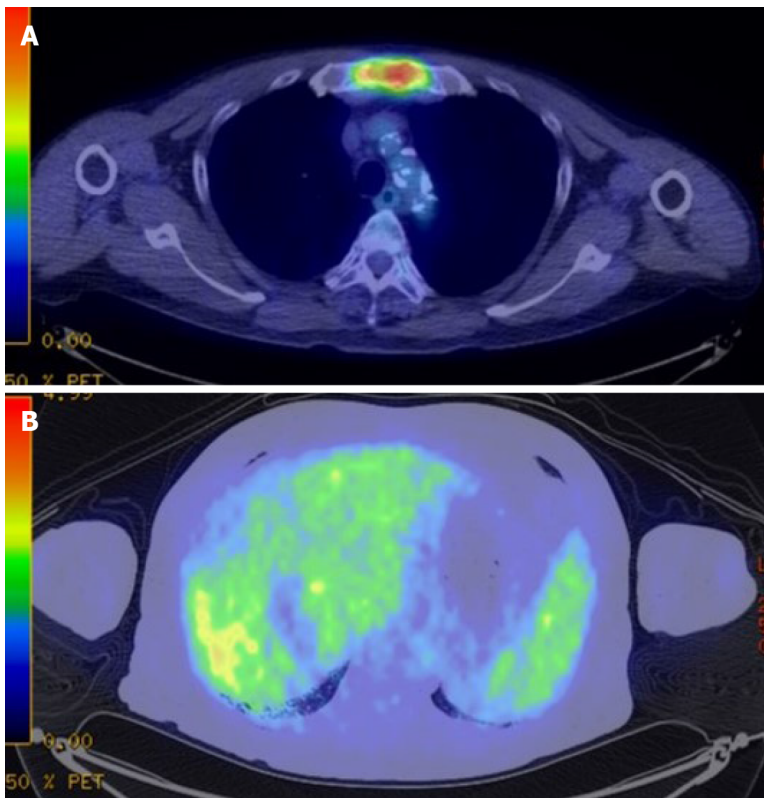


Figure 3 Preoperative fluorodeoxyglucose-positron emission tomography-computed tomography. A: The positron emission tomography-computed tomography scan revealed accumulation of fluorodeoxyglucose in the sternum. The diagnosis was bone metastases of hepatocellular carcinoma; B: Slight uptake was observed in the liver.

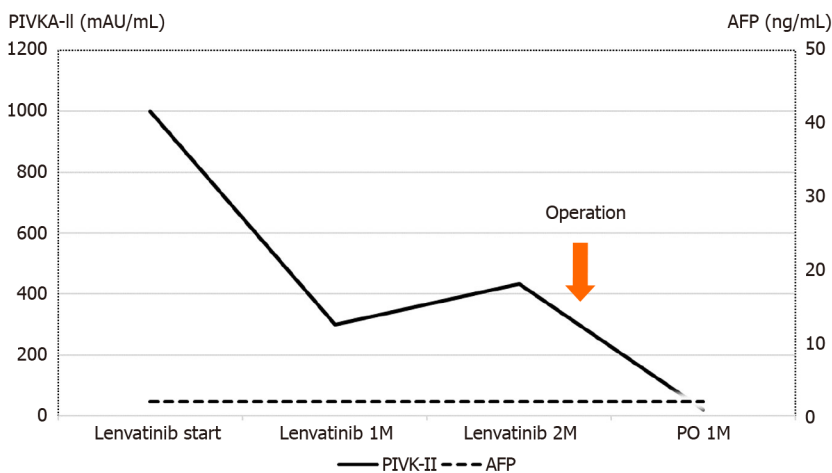


Figure 4 Perioperative tumor marker changes. The protein-induced vitamin K absence or antagonist II level before lenvatinib administration was high at 998 mAU/mL. The protein-induced vitamin K absence or antagonist II level decreased to 434 mAU/mL at 2 mo after lenvatinib administration, and further decreased to 20 mAU/mL at 1 mo after the second surgery. AFP: α -Fetoprotein; M: Month; PO: Post-operation; PIVKA-II: Protein-induced vitamin K absence or antagonist II.

injury (Figure 5). Histopathological findings showed that 80% of the tumor was necrotic, and the resected margin of the liver had no cancer component (Figure 6). The reason for choosing surgical resection after lenvatinib was that long-term administration of lenvatinib may result in decreased liver function such as decreased albumin, which can make surgery impossible. Early surgery was selected because it was unknown whether lenvatinib would result in a complete response, and it was better to aim for complete removal of the tumor by surgery.

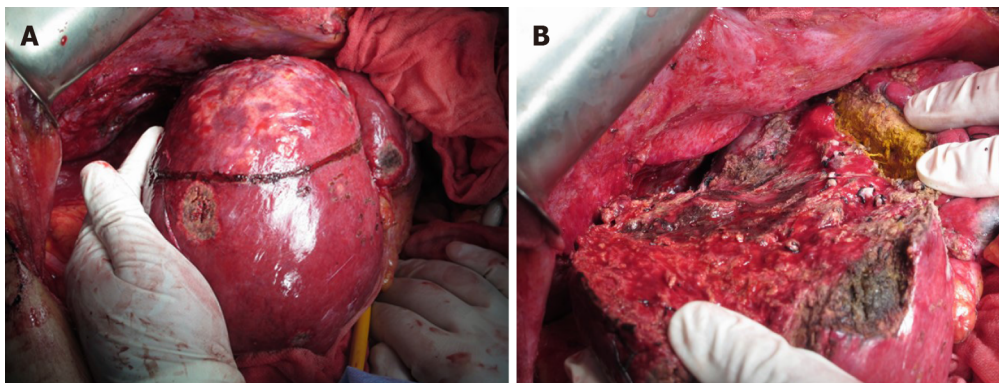


Figure 5 Surgical findings. Findings before (A) and after (B) the second hepatectomy.

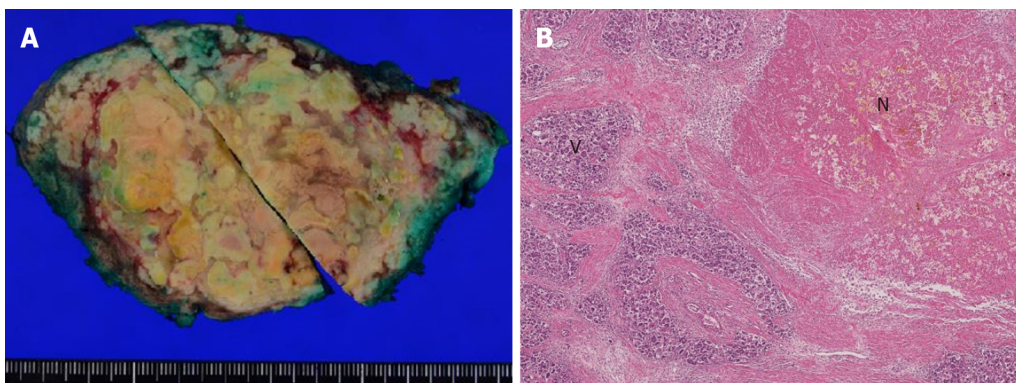


Figure 6 Macroscopic and pathological findings of the resected specimen. The findings revealed that 80% of the tumor was necrotic and the resected margin of the cut liver surface showed no cancer component. A: Macroscopic findings; B: Pathological findings. N: Necrotic lesion; V: Viable lesion.

OUTCOME AND FOLLOW-UP

No serious complications, such as bile leakage, were observed. The protein-induced vitamin K absence or antagonist II level decreased to the normal range after surgery (Figure 3). The patient was discharged on day 25 after the operation. Radiation therapy for bone metastases was performed by dividing 30 Gy irradiation into ten applications. At 1 mo after the end of radiotherapy, there was a small intrahepatic recurrence, and lenvatinib was immediately administered. PR was obtained, and the patient has remained alive on administration for 1 year after the second hepatectomy.

DISCUSSION

Here, we report a case in which lenvatinib was administered to a rapidly growing recurrent HCC, and conversion to hepatectomy was achieved after PR was obtained. There are previous case reports describing conversion to surgery for HCC after molecular targeted drug therapy with sorafenib and regorafenib^[17,18]. The present case report describing conversion to surgery for recurrent HCC after lenvatinib treatment appears to be the first case.

Numerous papers have been published on conversion to surgery for colorectal liver metastases^[19,20]. However, conversion therapy for HCC has not been established. Lenvatinib is expected to be a possible candidate agent and provides a new treatment perspective for recurrent HCC. To date, most reports have described cases of unresectable or recurrent HCC that were converted after local treatment, such as TACE and hepatic arterial infusion chemotherapy (HAIC), or after conventional anticancer drug treatment^[21,22]. For example, in a report from the MD Anderson Cancer Center^[23], the chemotherapy regimen was cisplatin, interferon α -1b, doxorubicin, and 5-fluorouracil, but the doses and administration schedules had two types (conventional and modified). The study compared and examined the resection rate (conversion rate to curative surgery) and survival rate between the two regimens. In

the results, the 3-yr survival rates were 70% for modified chemotherapy plus surgery ($n = 11$) compared with 20% for modified chemotherapy alone ($n = 22$) and 60% for conventional chemotherapy plus surgery ($n = 8$) compared with 5% for conventional chemotherapy alone ($n = 76$). Therefore, the cases with conversion to surgery showed better survival rates for both the conventional and modified regimens. There are various other reports on conversion to surgery. Hamaoka *et al*^[22] investigated the safety of hepatectomy and overall survival (OS) in 52 patients with unresectable advanced HCC treated with HAIC combined with three-dimensional chemoradiation therapy for portal vein tumor thrombus and compared the resection group with the nonresection group. As a result, OS was significantly higher in the resection group than in the nonresection group. A multivariate analysis identified conversion to surgery as an independent factor influencing OS.

A recent report described a case of HCC with hepatic vein tumor thrombosis protruding into the inferior vena cava that was successfully treated by surgery after second-line chemotherapy with regorafenib^[24]. Administration of sorafenib was initially started for this case, but 5 wk later the lesion had increased in size. Therefore, regorafenib was subsequently given as second-line therapy and administered for 12 mo. After shrinkage of the inferior vena cava-hepatic vein tumor thrombus, conversion to surgery was successful and a better prognosis was achieved. Meanwhile, Sato *et al*^[25] described a patient with initially unresectable HCC and an arteriportal shunt who underwent conversion hepatectomy after multidisciplinary treatment including lenvatinib^[25].

Thus, it is expected that the number of conversion cases will increase with the advent of molecular targeted drugs thereby contributing to improvement in the prognosis of HCC. Similar to previous reports, our patient obtained PR for recurrent HCC after lenvatinib administration and conversion to surgery was achieved. Therefore, it is anticipated that survival can be prolonged by continuing treatment with lenvatinib for bone metastases only.

The limitation is that the administration period for the molecular targeted drug before surgery and the timing of conversion to surgery are unknown. Therefore, it is necessary to collect more cases and perform further studies to clarify these points.

CONCLUSION

In conclusion, we have reported a case of recurrent HCC in which PR was obtained and conversion to surgery was achieved after lenvatinib administration. We believe that even for unresectable advanced and recurrent HCC, hepatectomy can be performed after administration of molecular targeted therapeutic drugs such as lenvatinib and can thus contribute to improvement of the prognosis.

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Hepatitis E virus re-infection accelerates hepatocellular carcinoma development and relapse in a patient with liver cirrhosis: A case report and review of literature

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Abstract

BACKGROUND

Hepatitis E virus (HEV) superinfection is a suspected promoting factor for hepatocellular carcinoma (HCC) in patients with chronic hepatitis and cirrhosis. However, to date, very few cases of HEV-related HCC have been reported. Nevertheless, the role of HEV re-infection in cirrhotic liver without other chronic hepatitis infections has rarely been explored.

CASE SUMMARY

A 53-year-old male farmer was diagnosed with liver cirrhosis and splenomegaly in August 2016, accompanied with negative HEV-IgM and positive HEV-IgG. No evidence of hepatitis B virus or hepatitis C virus infection was found. Since then the patient was evaluated for liver function and viral parameters every 3 mo. In June 2017, the patient presented severe fatigue with whole body itching and was diagnosed with HCC. Afterwards this patient experienced quick HCC development, progression, relapse, and metastasis in the following 8 mo, and presented persistent dual positivity of HEV-IgM and HEV-IgG. This patient had a long history of smoking and alcohol consumption.

CONCLUSION

This unique case invokes the importance of HEV surveillance and treatment

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among cirrhotic patients, HCC cases, and blood donors.

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Core Tip: The role of chronic hepatitis E virus (HEV) superinfection in hepatocellular carcinoma (HCC) progression in cirrhotic patients with negative hepatitis B virus (HBV) infection has not been studied. We present herein a unique chronic HEV case with liver cirrhosis who experienced repeated HEV re-infection and rapid HCC development and relapse. This case highlights the importance to investigate the association between HEV re-infection and rapid development of HCC and progression in liver cirrhosis cases, even in the absence of HBV infection. Moreover, routinely detecting HEV infection in high risk occupational group and all blood donors is warranted. Additionally, the treatment for symptomatic and asymptomatic chronic HEV infection is highly suggested.

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INTRODUCTION

To date, four pathogenic hepatitis E virus (HEV) genotypes have been identified. HEV genotypes 1 and 2 are transmitted mainly through the faecal-oral route while HEV genotypes 3 and 4 are likely to spread from infected animals to the human^[1,2]. HEV infection has been indicated in certain populations, such as immunocompromised patients or patients with chronic hepatitis B virus (HBV) infection accompanied with or without hepatic decompensation, either as a promoting factor or a cause for the progression of cirrhosis and hepatocellular carcinoma (HCC)^[3-6]. However, the impact of HEV infection on the risk of HCC development and progression in cirrhotic patients without HBV infection remains largely unknown. To date, only few chronic HEV-related HCCs have been reported^[7], with most of these having existing cirrhosis prior to HEV infection. In addition, to our knowledge the cases with repeated HEV infection have not been reported up to now, which are defined by persistent dual positivity for HEV-IgG and HEV-IgM. Here, we describe a unique case of HEV reinfection in a patient with liver cirrhosis who had rapid HCC development, progression, relapse, and metastasis.

CASE PRESENTATION

Chief complaints

A 53-year-old male farmer presented himself to the clinic of general surgery in June 2017 because of severe fatigue with whole body itching for 10 mo.

History of present illness

In August 2016, the patient was admitted to the hospital due to hematemesis and melena. He had been diagnosed with liver cirrhosis, confirmed by ultrasonography and computed tomography (CT), complicated with esophagogastric varices by gastroscopy and splenomegaly by ultrasonography and CT. This patient had splenectomy in September 2016 and blood transfusion during surgery. The pathological result suggested a chronic congestive splenomegaly. At that moment, the serum test determined that he was anti-HEV IgM negative and IgG positive.

History of past illness

He had no hypertension, diabetes, or other chronic diseases.

Personal and family history

The patient had a history of alcohol use with 200 mL daily intake and smoking 20 cigarettes a day for 20 years. Since June 2017 when HCC was diagnosed, the patient had quit alcohol drinking and smoking. Moreover, he had no family history of cancer.

Physical examination

Initial physical examination demonstrated pale skin with normal blood pressure (85/122 mmHg) and normal heart rate (80/min). No jaundice was observed in the skin and abdominal palpation elicited no pain.

Laboratory examinations

Serum anti-HEV IgG and IgM detection performed by enzyme-linked immunosorbent assay revealed that this patient was HEV-IgM and HEV-IgG double positive at admission in June 2017, and no evidence of hepatitis A virus (HAV), HBV, or hepatitis C virus (HCV) infection was detected (Table 1).

The biochemical test at admission showed normal alanine aminotransferase (ALT, < 40 U/L), slightly elevated aspartate aminotransferase (AST, 39-60 U/L) and alkaline phosphatase (ALP, 121-147 U/L), and highly elevated gamma glutamyl transferase (GGT, 154-186 U/L) and total bile acid (TBA, 27-34 μ mol/L) (Figure 1, Table 1, and Supplementary Material).

Imaging examinations

At admission in June 2017, the CT and magnetic resonance imaging (MRI) examinations detected multi-site liver masses (S5, S8), cirrhosis, portal hypertension, and esophageal and fundus varices (Supplementary Material).

FINAL DIAGNOSIS

The patient was diagnosed with HCC based on CT and MRI results^[8], accompanied with double positivity for HEV-IgM and HEV-IgG.

TREATMENT

In July 2017, the patient was classified with Barcelona Clinical Liver Cancer (BCLC) stage B, and transarterial chemoembolization (TACE) was conducted at S5 and S8 according to the international guidelines^[9,10].

OUTCOME AND FOLLOW-UP

The patient was then regularly followed every 3 mo upon discharge in August 2017. Since then, serum sample tests were persistently positive for HEV-IgM and HEV-IgG till April 2018, but HEV RNA was not detectable by quantitative real-time PCR.

During the follow-up period till April 2018, the patient presented significantly increased blood levels of GGT, type IV pro-collagen, and hyaluronidase. There was an obvious peak of ALT (135-139) and AST (67-167) in November 2017 during hepatectomy. Except for this perioperative elevation, ALT and AST remained normal or slightly increased (Figure 1). A normal range of alpha fetoprotein (AFP) was also observed during the whole admission period (Figure 2). Serum HEV RNA result was negative by quantitative real-time PCR (Supplementary Material).

One month after the discharge, CT examination suggested enhanced areas in S8 again. Then the patient was treated with radiofrequency ablation (RFA), which was commonly applied among patients with old age and/or accompanied by other diseases such as liver cirrhosis^[11], or used to shrink the tumor and reduce the surgical trauma probably caused by succeeding partial hepatectomy. Afterward, progressive HCC was evidenced by strengthened CT signal at the previously diagnosed site (S8) as well as a new site (S3) after 2 mo, in November 2017. A partial hepatectomy was

Table 1 Laboratory results of the patient from August 2016 to April 2018

| Time of admission (d) | 1 st | 2 nd | 3 rd | 4 th | 5 th | 6 th | 7 th |
|------------------------------|-----------------|-----------------|-----------------|---------------------|-----------------|-----------------|-----------------|
| Date of admission | 2016-08-19 | 2017-06-20 | 2017-09-26 | 2017-11-20 | 2018-02-07 | 2018-02-23 | 2018-04-16 |
| Diagnosis | Cirrhosis | HCC | HCC | HCC | HCC | HCC | HCC |
| Treatment | Splenectomy | TACE | RFA | Partial hepatectomy | | MWA | TACE and MWA |
| HEV IgM | Negative | Positive | Positive | | Positive | | Positive |
| HEV IgG | Positive | Positive | Positive | | Positive | | Positive |
| GLU (mmol/L) | 7.81 | 4.49 | 5.82 | 5.04 | 5.44 | 5.31 | 4.67 |
| BUN (mmol/L) | 13.1 | 3.1 | 3.54 | 4.35 | 4.3 | 4.2 | 3.7 |
| CO ₂ _CP (mmol/L) | 23.7 | 21.2 | 22.3 | 24 | 25.4 | 25.9 | 23.5 |
| UA (umol/L) | 334 | 271 | 312 | 293 | 282 | 289 | 300 |
| CREA (umol/L) | 65.5 | 58.4 | 72.8 | 65.25 | 65.7 | 61.1 | 62 |
| ALT (U/L) | 27 | 24 | 40 | 45 | 20 | 20 | 43 |
| GGT (U/L) | 166 | 154 | 224 | 232 | 67 | 65 | 195 |
| ALP (U/L) | 59 | 121 | 119 | 132 | 114 | 104 | 151 |
| ChE (U/L) | 3405 | 3926 | 4545 | 4393 | 3834 | 3515 | 3966 |
| TP (g/L) | 56.5 | 63.2 | 63.8 | 63.8 | 67.6 | 63.2 | 70.7 |
| ALB (g/L) | 27.2 | 29.3 | 30.8 | 31.3 | 30.6 | 29.2 | 32.5 |
| TB (umol/L) | 30.9 | 33.7 | 26.6 | 36.1 | 29.8 | 33.5 | 30.8 |
| DB (umol/L) | 8 | 7.7 | 5.4 | 7.7 | 7.7 | 7 | 7.8 |
| TBA (umol/L) | 2 | 27 | 46 | 22 | 76 | 41 | 32 |
| Na (mmol/L) | 141.6 | 141.2 | 140.7 | 141.9 | 139.3 | 141.4 | 139.9 |
| K (mmol/L) | 3.96 | 3.95 | 3.94 | 4.15 | 4.06 | 3.9 | 4.04 |
| Cl (mmol/L) | 109.6 | 109 | 107.1 | 108 | 109.9 | 109.3 | 107.7 |
| Ca (mmol/L) | 2 | 2 | 2.1 | 2.1 | 2.19 | 2.13 | 2.06 |
| PHOS (mmol/L) | 1.22 | 1.28 | 1.21 | 1.27 | 1.2 | 1.26 | 1.23 |
| Mg (mmol/L) | 0.81 | 0.7 | 0.81 | 0.82 | 0.83 | 0.81 | 0.84 |
| AST (U/L) | 38 | 39 | 49 | 67 | 38 | 37 | 55 |
| CIV (ng/mL) | 151.98 | | | | | 169.96 | 162.28 |
| LN (ng/mL) | 32.25 | | | | | 82.65 | 60.27 |
| PIIIP (ng/mL) | 10.9 | | | | | 17.33 | 15.82 |
| HA (ng/mL) | 841.13 | | | | | 592.83 | 553.67 |
| HBsAg (E) (COI) | 0.481 | 0.478 | | | 0.452 | 0.575 | 0.386 |
| Anti-HBs (E) (IU/L) | 494.2 | | | | 309.7 | 308.1 | 353.5 |
| HBeAg (E) (COI) | 0.102 | | | | 0.087 | 0.097 | 0.1 |
| Anti-HBe (E) (COI) | 1.27 | | | | 1.12 | 1.15 | 1.23 |
| Anti-HBc (E) (COI) | 0.008 | | | | 0.009 | 0.01 | 0.011 |
| HBV-DNA (FQ-PCR) (IU/mL) | 10 | | | | 10 | 10 | < 500 |
| HAV IgM | Negative | Negative | Negative | | Negative | | Negative |
| Anti-HCV (COI) | | 0.03 | | | 0.04 | 0.04 | |
| HIV COM (COI) | | 0.21 | | | 0.22 | 0.24 | |

| | | | | | | | |
|-----------------|------|------|------|------|------|------|------|
| Syphilis (COI) | | 0.08 | | | 0.07 | 0.08 | |
| AFP (A) (ng/mL) | 4.12 | 3.6 | 4.57 | 8.59 | 2.7 | 3.79 | 3.58 |
| CEA (A) (ng/ml) | 4.74 | 4.86 | 4.32 | 5.47 | 4.71 | 5.89 | 5.65 |
| PSA (A) (ng/mL) | 0.1 | | | | | | |

HCC: Hepatocellular carcinoma; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; MWA: Microwave ablation; HEV: Hepatitis E virus; IgM: Immunoglobulin M; IgG: Immunoglobulin G; GLU: Glucose; BUN: Blood urea nitrogen; CO₂_CP: Carbon dioxide combining power; UA: Uric acid; CREA: Creatinine; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transferase; ALP: Alkaline phosphatase; ChE: Cholinesterase; TP: Total protein; ALB: Albumin; TB: Total bilirubin; DB: Direct bilirubin; TBA: Total bile acids; AST: Aspartate aminotransferase; CIV: collagen IV; LN: Laminin; PLIP: Precollagen type III peptidase; HA: Hyaluronate; HBsAg: Hepatitis B surface antigen; Anti-HBs: Hepatitis B surface antibody; HBeAg: Hepatitis B e Antigen; Anti-HBe: Hepatitis B e antibody; Anti-HBc: Hepatitis B core antibody; Anti-HCV: Hepatitis C virus antibody; HIV COM: Combination of HIV antibody; AFP: Alpha fetoprotein; CEA: Carcino-embryonic antigen; PSA: Prostate specific antigen; PHOS: Phosphorus; COI: Cut off index.

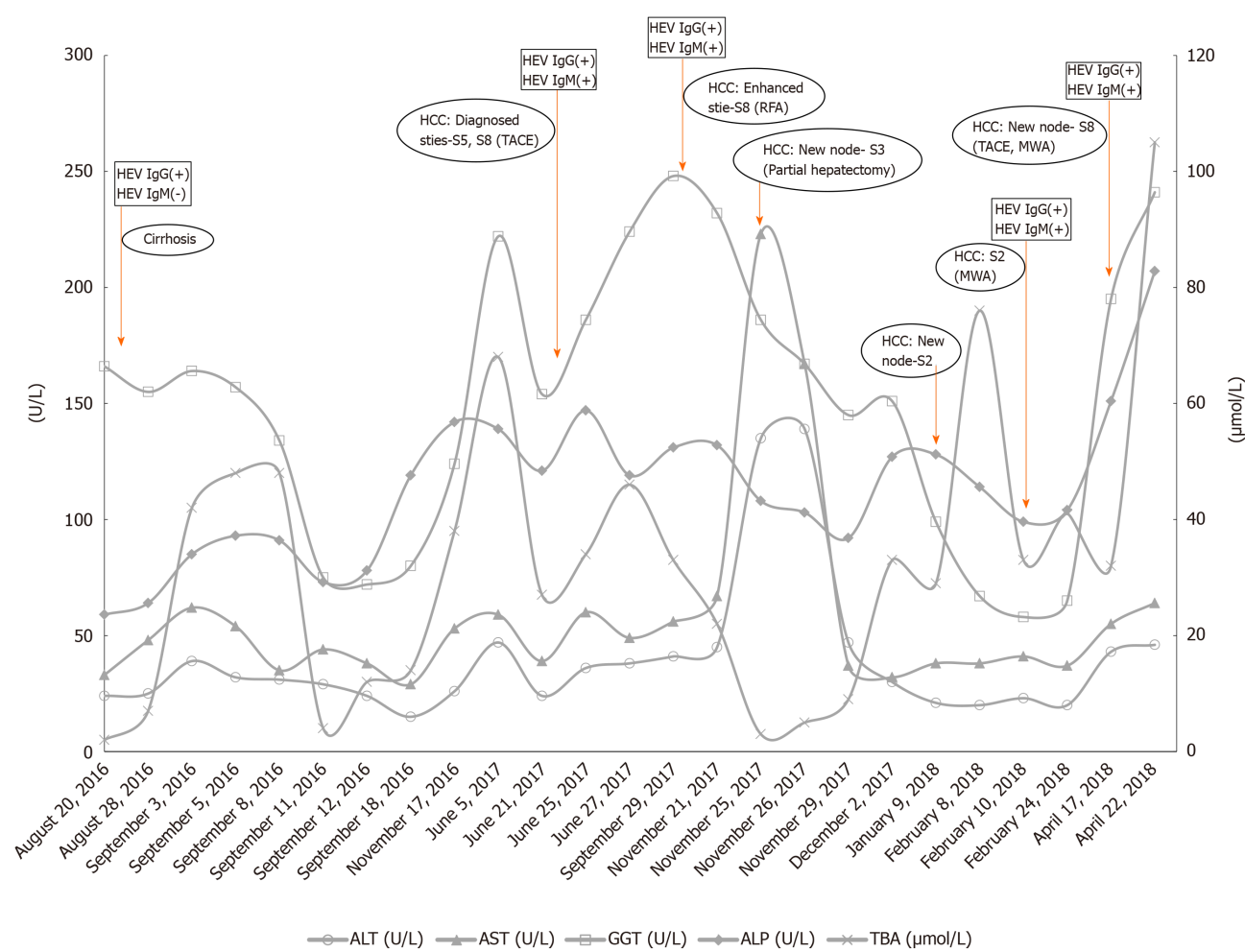


Figure 1 Biological measurements of liver function of the patient from August 2016 to April 2018. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma glutamyl transferase; ALP: Alkaline phosphatase; TBA: Total bile acid; HCC: Hepatocellular carcinoma; HEV: Hepatitis E virus.

conducted to remove the relapsed tumors at the previous treatment site (S8) and the new site (S3). The histological examination of surgically resected tissue confirmed cirrhosis and multi-site HCC grading from I to II, with sizes ranging from 0.6 cm to 0.9 cm. The histopathological examination revealed that tumors were CD10, CD34, CEA, and Glypican III positive (Figure 3, Supplementary Material).

In the following 2 mo, a newly developed liver tumor was identified by CT at S2 and a suspected right upper zone lung metastasis was also indicated. Microwave ablation (MWA) treatment was applied at S2 for HCC relapse. In April 2018, suspected tumor nodes at S8 were detected by CT again, followed by TACE and MWA treatment at S8 (Supplementary Material).

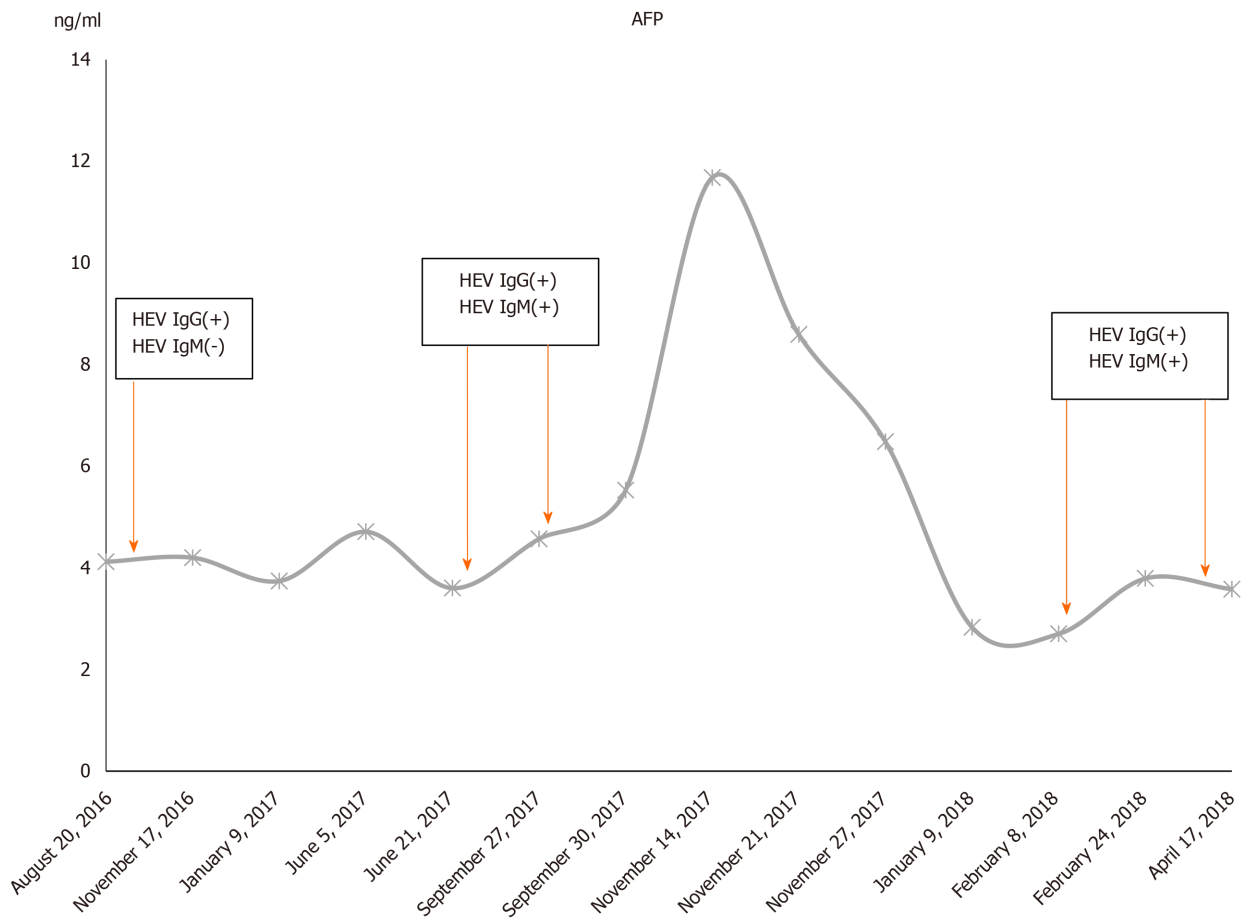


Figure 2 Alpha fetoprotein test results of the patient between August 2016 and April 2018. AFP: Alpha fetoprotein; HEV: Hepatitis E virus.

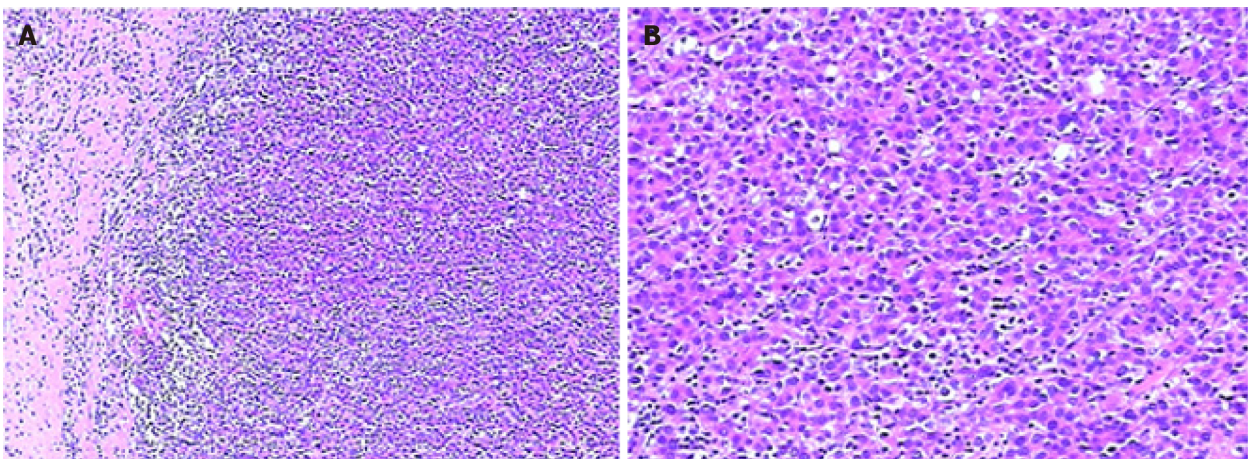


Figure 3 Histopathologic characterization of hepatocellular carcinoma. A and B: Haematoxylin and eosin staining of the patient's pathological tissue.

This patient was treated with MWAs in July 2018 and December 2018 due to newly identified HCC relapses.

During the whole process, no anti-viral medications including interferon-based therapeutics were prescribed to the patient. And, no further HEV infection examination was performed after April 2018.

DISCUSSION

At the first presentation to the clinic, this patient suffered from chronic HEV infection, evidenced by positive HEV-IgG and negative HEV-IgM, and cirrhosis simultaneously, but no chronic HBV or HCV infection. In the following 20 mo, this patient was suspected with repeated HEV infection, supported by persistent dual positivity for HEV-IgM and HEV-IgG. More importantly, in the following 20 mo, the patient experienced rapid HCC development, progression, multiple times of relapse, and metastasis.

The patient is speculated to have initial infection of HEV in his farm where farm animals are a suspected source of HEV infection^[12]. A previous study has defined farmers as a high risk group of HEV infection due to the potential dissemination of HEV infection in Chinese farms^[13]. However, the presumed cause of HEV re-infection is blood transfusion during splenectomy. Previous studies have indicated that the likelihood of developing clinically relevant HEV infection after transfusion of a HEV positive blood product can be as high as approximately 50%^[14,15]. And in immunosuppressed patients, receiving HEV-RNA positive blood products might lead to or prompt the development of fatal acute-on-chronic liver failure^[16,17]. Regarding to this, the blood authorities in Europe have advocated to implement HEV screening among blood donors^[18]. Unfortunately, currently in China HEV is not tested on blood donations, which therefore lets the patients on the risk of HEV infection from blood transfusion. In this specific case, pre-existed liver cirrhosis predisposes the patient to HEV reinfection, as well as subsequent HCC development. Additionally, the persistent HEV re-infection might also be a result of the lack of anti-viral treatment for HEV chronic infection, especially after the patient has developed HCC and during the progression of HCC. In consequence, a small amount of virus in the liver is able to repeatedly reactivate or cause infection, and invoke weak immune response which is deficient to eliminate the viruses regardless of the production of a small amount of anti-HEV antibodies.

The patient had the history of alcohol drinking and smoking, which might be the underlying causes of cirrhosis^[19-21], and the functional decompensated liver predisposes the patient to HEV re-infection and rapid HCC development, progression, relapse, and metastasis^[22-24]. It was reported that long-term tobacco exposure would increase the levels of hepatic cancer stem cell-like markers and variate the expression of inflammatory factors IL-33^[25]. Similarly, chronic and acute HEV infection would increase the levels of some inflammatory factors and compromise liver function. On the other hand, alcohol intake would lead to chromosomal loss, DNA methylation aberration, genetic susceptibility, oxidative stress, and retinoic acid level decrease in the liver^[26]. Previous investigations revealed that excess alcohol consumption was associated with high seroprevalence of HEV in cirrhosis cases, indicating that the alcohol-decompensated liver would be more susceptible to HEV infection^[22,27]. All abovementioned risk factors are exhibited in this patient. Thus, we have rationale to speculate a synergic effect evolved from proinflammatory state, genetic instability, and hepatic decompensation leading to accelerated malignant progression in an HEV-infected and re-infected cirrhotic liver. Nevertheless, the definite association between these factors and liver carcinogenesis remains to be further investigated.

Our study has several limitations. First, HEV genotype was not determined because this test is not routinely performed for patients infected with HEV in clinical practice in China. It has been well documented that in China genotype 4 is the most dominant type in human chronic HEV infection, and the cases infected with other genotypes have been reported but remain sporadic in China^[28-30]. Nevertheless, in future a routine diagnosis of HEV genotype should be implemented in both clinical and research settings to acquire a deep insight into the association between chronic HEV infection and development of HCC. Second, other potential HCC serum biomarkers such as PIVKA were not screened. This case presented a normal range of AFP during the whole admission period, which warned us the importance of utilizing other biomarkers to assist in early detection of HCC, especially for cases with an uncommon etiology. Further studies should set effort to develop a set of multiple biomarkers, complementary with AFP, for clinical diagnosis of HCC.

Recent studies have suggested that immunocompromised patients are predisposed to HEV infection^[31,32], and HEV might promote the progression of HCC in patients with chronic HBV infection and/or cirrhosis^[33,34]. However, the role of HEV re-infection in patients with hepatic decompensation with or without chronic HBV infection has not yet been explored, to our knowledge. Our observation in this unique case has indicated that, regardless of chronic HBV infection, in patients with liver cirrhosis HEV superinfection might promote not only HCC development and

progression, but also relapse and metastasis. Our report provides new knowledge to HEV-related carcinogenesis and clinical management of HEV-associated liver pathologies. Future studies should emphasize on the mechanisms underlying HEV re-infection accelerated malignant transformation of cirrhotic liver in the presence or absence of chronic HBV infection. The potentially distinct effect on HCC progression exposed by unique sequential acquirement of HEV infection and cirrhosis is an important question to address in future study as well.

CONCLUSION

This unique case highlights an urgent need to investigate the effect of HEV re-infection on rapid HCC development and progression in cirrhotic liver, despite the presence of chronic HBV infection. Our report also reveals the importance of routine screening HEV in blood donations. Further, antiviral treatment for symptomatic and asymptomatic HEV infection to cirrhosis and HCC patients is highly suggested.

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Autophagy related protein 9A increase in hepatitis B virus-associated hepatocellular carcinoma and the role in apoptosis

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Abstract

The majority of hepatocellular carcinoma (HCC) cases are associated with the hepatitis B virus (HBV) infection. Autophagy related protein 9A (ATG9A) is a transmembrane protein required for autophagosome formation. In order to investigate the role of ATG9A in HBV-associated HCC, ATG9A protein expression was determined in tumor liver tissues and compared with adjacent nontumor tissues from HCC patients with or without HBV infection. In HBV-associated HCC tissues, ATG9A protein level was increased in tumor liver tissues, but not in cases of non-HBV HCC. Our findings suggested that ATG9A might be involved in HBV and cancer cell survival. Therefore, we aimed to analyze the function of ATG9A in HBV replication using RNA interference to evaluate the HBV DNA level using real-time PCR. In the present study, there were no significant differences between shATG9A-transfected HepG2.2.15 cells and the mock control. However, we found that silencing ATG9A affected apoptosis in HepG2.2.15 and HepG2 cell lines. Our results indicated that ATG9A might be partly involved in the survival of HCC. Thus, the inhibition of ATG9A together with other targets might be a potential drug target for HCC treatment.

Key Words: Autophagy; Hepatitis B virus; Hepatocellular carcinoma; Autophagy related protein 9A; Apoptosis; HBx

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Core Tip: Autophagy related protein 9A (ATG9A) protein expression was increased in

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tumor liver tissues compared to adjacent nontumor tissues from hepatocellular carcinoma (HCC) patients with hepatitis B virus infection. We showed that silencing ATG9A increased cell apoptosis of HepG2.2.15 and HepG2 cells. These results suggested that ATG9A protein is involved in the survival of HCC. The inhibition of ATG9A combined with other targets might be a potential drug target for HCC treatment.

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TO THE EDITOR

Autophagy related protein 9A (ATG9A) is a transporter membrane molecule required for initial autophagosome formation in the autophagy pathway^[1]. ATG9A has been identified as having the function of a stimulator of interferon (IFN) genes (STING)inhibition. A loss of ATG9A results in enhanced assembly of STING/TANK-binding kinase 1 complexes in response to dsDNA, leading to an increase in innate immune responses^[2]. Silencing of ATG9A in macrophages increases STING-mediated IFN- β production and promotes cell viability^[3]. Our previous study reported that gene and protein expressions of ATG9A were upregulated in HepG2 and HepG2.2.15 cells compared with a THLE-2 hepatic cell line^[4]. Thus, in this study we investigated the role of ATG9A in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) tissues. We found that ATG9A protein levels were highly increased in tumor liver tissues in HBV-associated HCC (9 of the 10 sample pairs). In the case of non-HBV HCC, ATG9A protein levels were decreased or slightly increased in tumor liver tissues (Figure 1). Therefore, we hypothesized that HBV induces the upregulation of ATG9A to benefit its replication. To determine the effect of ATG9A on HBV replication, HBV DNA was quantified from shATG9A-transfected cells and compared to mock cells. We observed no significant difference in shATG9A transfected cells (Figure 2).

HBV induces autophagy *via* the HBx protein and is directly involved in starvation-induced autophagy *via* upregulation of Beclin-1 expression^[5]. HBx also binds and activates phosphatidylinositol 3-kinase class III for autophagy induction^[6]. Our study showed that overexpression of HBx did not affect ATG9A expression (Figure 3), suggesting that the function of ATG9A may not involve HBV replication or viral clearance.

Autophagy is involved in tumor progression and tumor suppression. Several studies have shown that HBV induces autophagy for cell survival in an unsuitable environment^[7]. In order to search for the effect of ATG9A on apoptosis, we performed flow cytometry in HepG2.2.15 cells and compared against HepG2 cells after ATG9A silencing. We found that silencing ATG9A increased apoptosis in both cell lines (Figure 4), suggesting that ATG9A is involved in cell apoptosis related to HCC.

In conclusion, we provide information that ATG9A is highly expressed in HBV-associated HCC tissue samples and plays a role in cell apoptosis. Further studies are needed to investigate the mechanism of ATG9A-mediated inhibition of apoptosis in HCC.

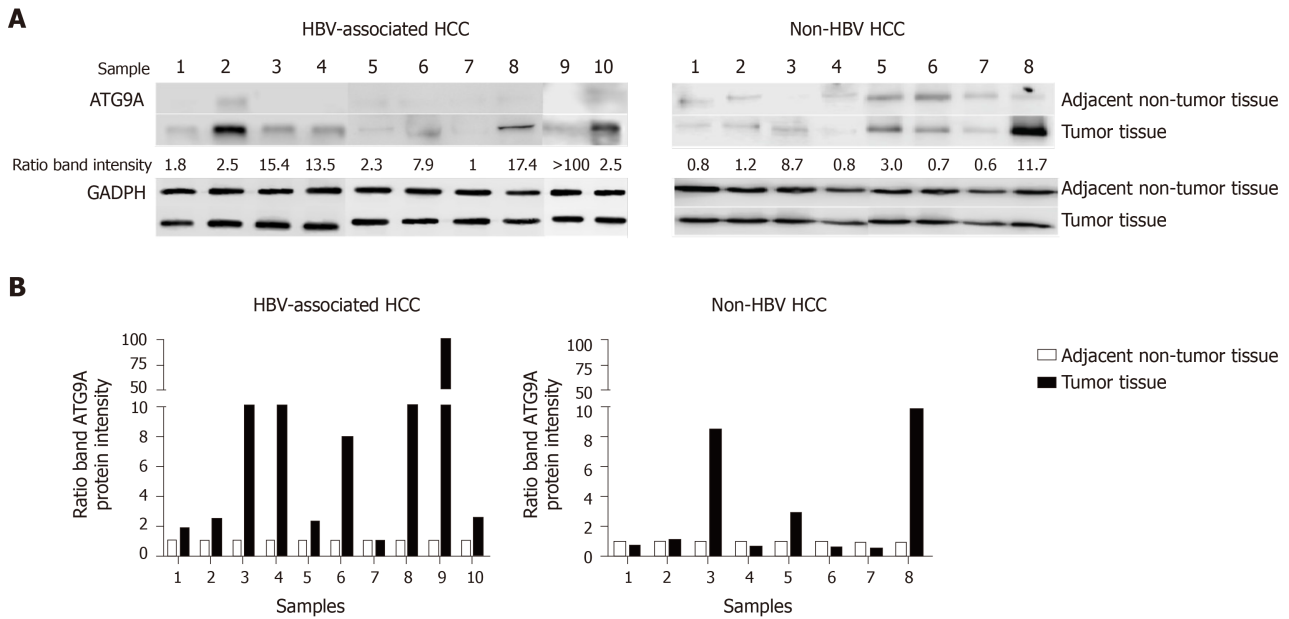


Figure 1 Quantification of autophagy related protein 9A protein levels from hepatitis B virus-infected hepatocellular carcinoma patients and nonhepatitis B virus hepatocellular carcinoma patients. A: Western blotting with specific antibodies was used to analyze autophagy related protein 9A (ATG9A) protein expression in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) and nonHBV-HCC. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a protein loading control; B: Graphs showing the intensity band ratio (tumor tissue/adjacent nontumor tissue) quantified using the LI-COR® image system for western blot analysis.

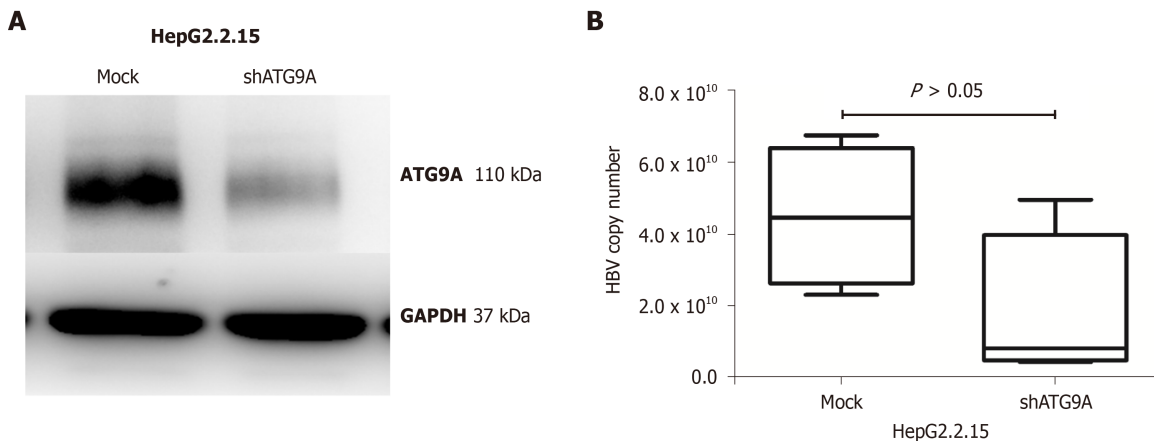


Figure 2 Silencing of autophagy related protein 9A by RNA interference and detection of hepatitis B virus DNA level. A: The western blot method was applied to analyze autophagy related protein 9 (ATG9A) protein levels against a mock treatment (control) and ATG9A knockdown (shATG9A) HepG2.2.15 cells; B: Quantitation of hepatitis B virus (HBV) DNA by real-time PCR. Total purified DNA from mock treatment (mock) and ATG9A knockdown (shATG9A) in HepG2.2.15 cells was amplified using preS1 specific primers. Hepatitis B virus preS1 plasmid was used as standard copy number. Data is shown as mean \pm standard error of the four independent experiments. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

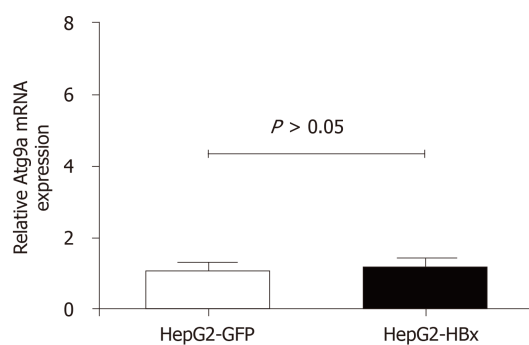


Figure 3 Quantitative real-time reverse transcriptase-PCR analysis of autophagy related protein 9A mRNA expression in HepG2-GFP and HepG2-HBx transfected cell lines. β -actin was used as an internal control. Data represent the mean \pm standard error in the three independent experiments. ATG9A: Autophagy related protein 9A.

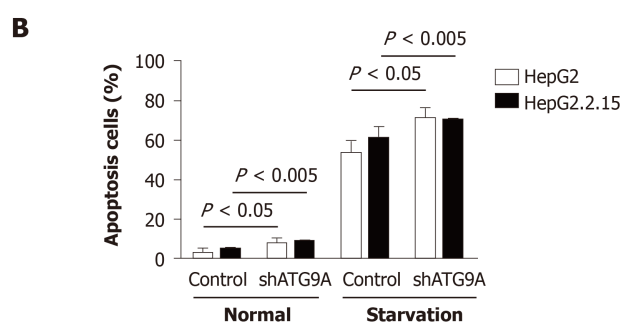
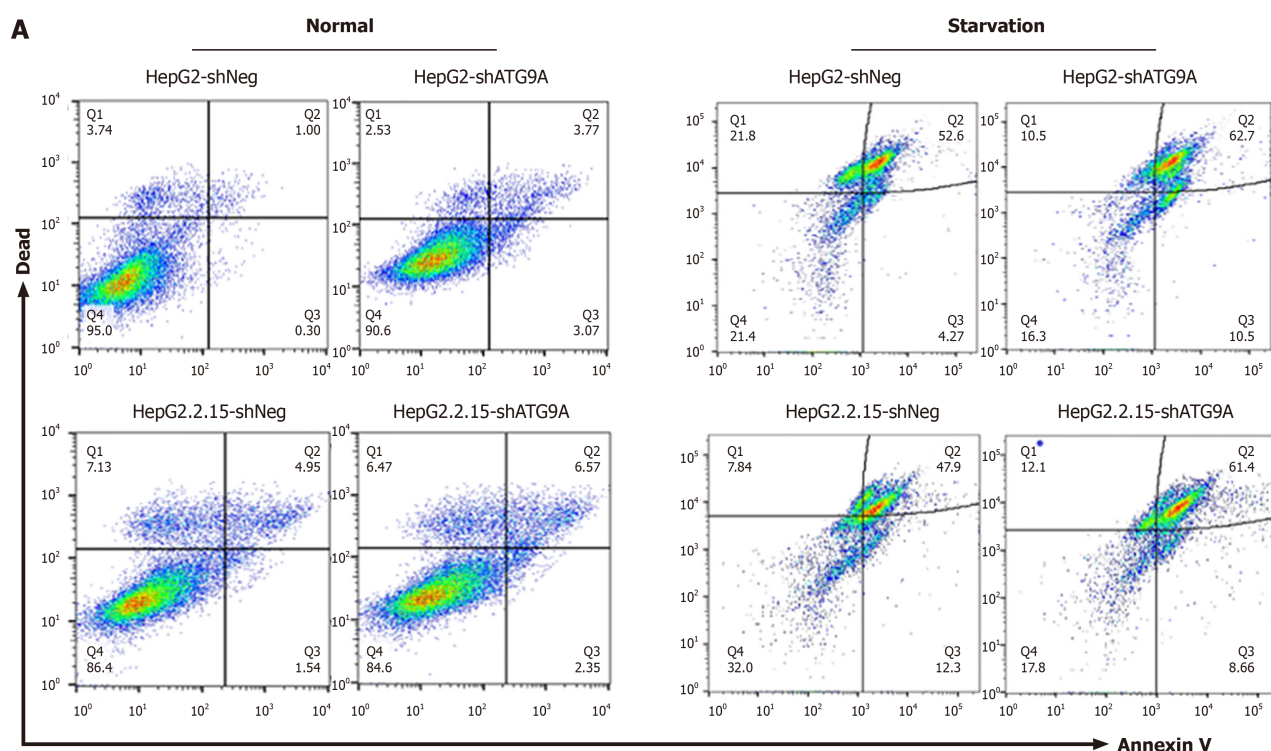


Figure 4 Apoptosis assays of HepG2 and HepG2.2.15 transfected with sh-autophagy related protein 9A or shNeg (control). A: Cells were transiently transfected with shRNA plasmids for 72 h and then cultured in starvation medium for 4 h; B: Bar graphs showing the percentage of total apoptotic cells detected by Annexin V binding. Data represent the mean \pm standard error from the three independent experiments. ATG9A: Autophagy related protein 9A.

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