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MINIREVIEWS

How to assess the efficacy or failure of targeted therapy: Deciding when to stop sorafenib in hepatocellular carcinoma

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Abstract

Sorafenib is thus far the only systemic treatment for hepatocellular carcinoma (HCC) based on the results of two randomized controlled trials performed in Western and in Eastern countries, despite a poor response rate (from 2% to 3.3%) following conventional evaluation criteria. It is now recognized that the criteria (European Association of the Study of the Liver criteria, modified response evaluation criteria in solid tumors) based on contrast enhanced techniques (computed tomography scan, magnetic resonance imaging) aimed to assess the evolution of the viable part of the tumor (hypervascularized on arterial phase) are of major interest to determine the efficacy of sorafenib and of most antiangiogenic drugs in patients with HCC. The role of alpha-fetoprotein serum levels remains unclear. In 2016, in accordance with the SHARP and the Asia-Pacific trials, sorafenib must be stopped when tolerance is poor despite dose adaptation or in cases of radiological and symptomatic progression. This approach will be different in cases of available second-line therapy trials. Some recent data (in renal cell carcinoma) revealed that despite progression in patients who received sorafenib, this drug can still decrease tumor progression compared to drug cessation. Then, before deciding to continue sorafenib post-progression or shift to another drug, knowing other parameters of post-progression survival (Child-Pugh class, Barcelona Clinic Liver Cancer, alpha-fetoprotein, post-progression patterns in particular, the development of extrahepatic metastases and of portal vein thrombosis) will be of major importance.

Key words: Tumor evaluation; Response evaluation criteria in solid tumors; Sorafenib; Hepatocellular

carcinoma; Modified response evaluation criteria in solid tumors

Core tip: The response rate of sorafenib in hepatocellular carcinoma is low using standard parameters and is better assessed using new criteria based on tumor vascularization (European Association of the Study of the Liver criteria, modified response evaluation criteria in solid tumors). In case of minor progression, if sorafenib is well tolerated, knowing the predictors of post-progression survival will be of value in deciding whether to continue or stop sorafenib.

Raoul JL, Adhoute X, Gilabert M, Edeline J. How to assess the efficacy or failure of targeted therapy: Deciding when to stop sorafenib in hepatocellular carcinoma. *World J Hepatol* 2016; 8(35): 1541-1546 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i35/1541.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i35.1541

INTRODUCTION

In the majority of solid tumors, assessing the efficacy or failure of a systemic treatment is based on the tumor size, which is measured either bidimensionally using the World Health Organization criteria (WHO) criteria or unidimensionally using response evaluation criteria in solid tumors (RECIST). The response rate is used as a surrogate marker of drug efficacy in clinical trials, and in clinical practice, the evolution of tumor size is a major parameter to decide whether to stop or continue treatment. In a palliative setting, a treatment is continued as long as the disease is controlled (stable disease or response) and the regimen is tolerated. This approach is less simple with targeted therapies. In gastrointestinal stromal tumors, the efficacy of imatinib was associated with modifications in tumor content and not always with a decrease in tumor size. That finding leads researchers to propose new response criteria[1] not only based on tumor size but also based on combining size and density shown on computed tomography (CT) scans. The efficacy of bevacizumab that is associated with chemotherapy is also underestimated under the standard criteria. In a large series, Chun *et al*[2] demonstrated that CT scan-based morphologic criteria correlated better with the histological response than the response by RECIST in patients with liver metastases of colorectal cancer treated with bevacizumab-containing chemotherapy. In hepatocellular carcinoma (HCC), despite a low and disappointing response rate (2%) using conventional criteria in a phase Ⅱ trial[3], sorafenib is thus far the only systemic treatment[4,5] that has been demonstrated to improve overall survival. In this era of great expectations regarding new drugs, we would like to briefly review these response evaluation criteria used in patients with HCC and the determination of when to continue or stop sorafenib treatment.

CONVENTIONAL CRITERIA FOR EVALUATING TUMOR RESPONSE: WHO AND RECIST

The WHO criteria for defining a response to treatment are based on bidimensionally measured lesions (*i.e.*, the product of the greatest tumor diameter and the greatest perpendicular distance summed over all measured tumors). The RECIST guidelines were published in 2000, with the major change being that the RECIST 1.0 uses unidimensional measurements of the sum of the longest diameters of the tumors. All unmeasurable lesions are considered to be "non-target" lesions, and lymph nodes are not distinguished from extranodal lesions. Progression is defined by an increase of at least 20% of the sum of the longest diameter and the appearance of new lesions or the progression of a non-target lesion. In 14 studies, the application of the WHO criteria and RECIST to the same patients with a large range of cancers has shown similar results[6]. A few years later, the RECIST 1.1 criteria were published[7], which better defined the minimal target size and reduced the number of allowed target lesions to 2 per organ and to a total of 5. It was also stated that a lymph node was considered as a target only if the short axis was larger than 15 mm. Ascites, pleural effusion, and lymph nodes from 10 mm to 14 mm on the short axis were considered as non-measurable lesions. Progression of non-target lesions was, by definition, considered to be a sign of disease progression. In a comparison of RECIST 1.0 with RECIST 1.1 in patients with lung cancer treated by erlotinib, the latter group demonstrated a slightly better performance[8].

However, all these criteria were subject to failure in HCC. Ascites or pleural effusions are usually related to the underlying liver cirrhosis, lymph nodes are frequent and may be large in the case of viral hepatitis, and the appearance of non/malignant small liver nodules is common. Moreover, most non-surgical treatments target tumor vascularization (chemo-embolization, radio-embolization, antiangiogenic drugs), and efficacy might be poorly reflected by size only.

NEW CRITERIA SPECIFICALLY DEDICATED TO HCC

Thus, new, more appropriate criteria were required to assess treatment efficacy in patients with HCC. European Association of the Study of the Liver (EASL) criteria were introduced during the EASL conference in Barcelona in 2000. They were based on bidimensional WHO criteria and the targeting of viable tumors, which were defined as those that showed contrast material-enhancing areas in the arterial phase of a dynamic CT

scan[9]. These criteria were later adapted to RECIST[10]; in addition to this new definition of target lesions, non-target lesions were revisited, and new hepatic nodules were considered as evidence of progression only if they had typical imaging and a longest diameter of at least 10 mm. Cytopathological confirmation of the neoplastic nature of any effusion that appeared or worsened was required. These new parameters, named modified RECIST (mRECIST), were considered to be a better tool for assessing HCC tumors[11]. Several Japanese authors proposed response evaluation criteria in cancer of the liver (RECICL), based on the bidirectional measurement of tumors showing arterial enhancement and considering non-hypervascularized tumors[12]. In a series of 156 patients receiving sorafenib for more than 30 d, response rates and the evaluation of overall survival by mRECIST and RECICL were similar. Recently, mRECIST was prospectively validated[13] in a phase 3 study (brivanib in second-line treatment). In this study comparing 395 patients who progressed after sorafenib was administered or were intolerant (brivanib to placebo; 2:1 ratio), tumor assessment every 6 wk by contrast-enhanced CT or magnetic resonance imaging was performed by a central review using mRECIST. A partial response was achieved in 8% of patients who received brivanib and 2% of patients who received placebo; the median overall survival was 16.4 mo for mRECIST responders and 8.3 mo for non-responders, and mRECIST evaluation had a prognostic value in multiparametric analysis.

Another way to evaluate tumor vascularization is contrast-enhanced ultrasound. In a short series of 19 patients (16 who received sorafenib and 3 who received sunitinib), this technique seemed effective at distinguishing progressors from non-progressors at 1 mo[14]. In a prospective series of 37 patients treated with sorafenib and explored by contrast-enhanced ultrasound before treatment and on days 7, 14 and 28, Sugimoto *et al*[15] found that this technique was not only predictive of tumor response (tumor vascularization) but also of major adverse events (liver parenchyma vascularization). Additional data are still necessary to validate these results.

The impact of alpha-fetoprotein (AFP) evaluation is unclear. In a series of 72 patients who had an elevated baseline AFP and were treated with different antiangiogenic drugs (thalidomide, bevacizumab), a decline of > 20% from the baseline AFP level within the first 4 wk (early AFP response) was associated with a higher response rate and a longer PFS and OS[16]. In contrast, in patients who received brivanib[17], a longer survival rate was not associated with either an early AFP response (*i.e.*, a decrease by more than 20% from baseline within the first 4 week) or an AFP response (*i.e.*, an AFP decrease by more than 50% from baseline). In a Japanese retrospective study[18], the best way to assess prognosis was a combination of mRECIST and AFP ratio (AFP under treatment/AFP before treatment), but this ratio (< or > 1) was only associated with survival at 8 wk.

COMPARISON OF THESE RESPONSE EVALUATION CRITERIA IN HCC CASES

After transarterial chemoembolization (TACE) and percutaneous ablation in 55 patients, Forner *et al*[19] demonstrated that RECIST missed all complete responses (including patients treated by curative options) and underestimated the extent of tissue necrosis. The authors concluded that RECIST should not be used and that dynamic imaging techniques and evaluations must become the standard for assessing treatment efficacy. In a series of 143 patients with HCC who underwent TACE, a comparison of various response criteria showed that volumetric functional imaging is better correlated with outcome than other parameters and that AFP serum levels[20] and new 3D-imaging approaches are of great value in differentiating the responders from the non-responders to TACE[21] and can be used early to predict outcome after initial TACE. Shim *et al*[22] compared WHO, RECIST, EASL and mRECIST in a cohort of 332 patients with intermediate HCC treated by TACE. They concluded that the enhancement models (EASL guidelines and mRECIST) were the best independent predictors of overall survival after chemoembolization. Similarly, the same results were found in an English series of 83 patients[23]. Thus, measuring the viable part of the tumor seems to be the best option after loco-regional treatment of HCC.

In the seminal SHARP[4] and AP[5] trials, the response rates using RECIST were 2% and 3.3% for patients who received sorafenib and 1% and 1.3% for those who received placebo, respectively; however, the overall survival analysis was clearly in favor of sorafenib, showing a discrepancy between the response rate by RECIST and outcome, with sorafenib efficacy being related to an increase in the time to progression. Many retrospective series have analyzed tumor responses using different criteria for patients receiving sorafenib. Their common features were that the evaluation of the viable part of the tumor based on arterial enhancement provided better results than the usual parameters and showed a real response rate and, thus, should be used for assessing treatment efficacy. Edeline *et al*[24], in a series of 53 patients, determined that 1 out of 10 patients considered as PD by RECIST was scored as SD using mRECIST. Forty-two patients evaluated as stable by RECIST were reassessed as complete response in 1 case, partial response in 10 cases, SD in 29 cases and PD in 2 cases using mRECIST. Then, the objective response rate of 1.9% by RECIST increased to 22.6% with mRECIST. The mRECIST result was associated with outcome, as those initially considered as SD by RECIST but as responders (n = 11), stable (n = 29) or progressive (n = 2) by mRECIST had different median overall survival rates of 17.1, 9.7 and 3.7 mo, respectively. However, there was no difference between these two criteria regarding the median time to progression. Another retrospective study[25] compared RECIST 1.1 with vascularization-based criteria

Table 1 Parameters of post-progression survival for patients receiving sorafenib

Performance status
Child-Pugh class
BCLC class
CLIP score
Macroscopic venous invasion
AFP serum level
TTP on sorafenib
Pattern of progression

BCLC: Barcelona Clinic Liver Cancer staging classification; CLIP: Cancer of the Liver Italian Program; AFP: Alpha-fetoprotein; TTP: Time to progression.

(Choi criteria, EASL criteria, and mRECIST). The response rates were 3%, 51%, 28% and 28%, respectively, in a cohort of 64 patients treated using sorafenib. The tumor response following RECIST 1.1 did not correlate well with the overall survival rate, whereas other criteria were more appropriate to identify responders with longer survival rates. In two phase Ⅱ trials (101 patients) evaluating brivanib, an independent review compared the outcomes between the WHO criteria and mRECIST[17]. The response rates were higher with mRECIST *vs* WHO in both cohorts, and PD assessed by mRECIST, was associated with a poorer overall survival rate than when assessed using the WHO criteria.

Thus, these vascularization-based criteria are better than size-only criteria to categorize responders. However, the essential problem exists: How do we define when sorafenib treatment is no longer effective? Progression can be related to an increase in tumor size (or of its viable part) and also to the appearance of new liver nodules (considering vascularization, size, and evolution), effusion and ascites (cytology required), and lymph nodes (size and vascularization). These parameters are listed in a recent paper from the BCLC[26]. However, is progression a strict criterion to stop sorafenib treatment?

IN 2016, WHEN TO STOP SORAFENIB?

In the SHARP trial, treatment was continued until both radiological and symptomatic progression or unacceptable toxicity occurred. In our experience, many patients seem to clinically benefit from the drug despite progression; in clinical practice, progression is not always a clear indication to stop sorafenib, particularly if there is no second-line trial available. In patients with poor prognostic factors at progression (worsening of performance status or of Child-Pugh status), cessation of the drug is recommended. In contrast, if the patients are candidates for second-line therapies, then inclusion is the best option if available. In other cases, we can postulate that sorafenib may retain some efficacy in certain instances despite tumor progression and that cessation of the drug might lead to an acceleration of tumor growth. In metastatic renal cell cancer, some data

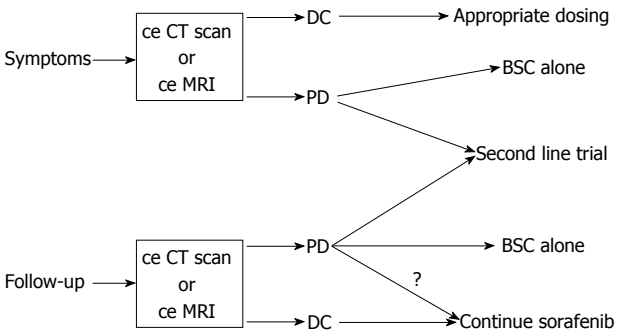

Figure 1 Proposed algorithm for deciding to continue or stop sorafenib in patients with hepatocellular carcinoma. ce CT scan: Contrast-enhanced computed tomography scan; ce MRI: Contrast-enhanced magnetic resonance imaging; DC: Disease controlled; PD: Progressive disease; BSC: Best supportive care.

show that, at progression, the tumor growth rate is lower than before initiation of the treatment using sorafenib and lower than will be observed after cessation of the drug. More interestingly, in renal cell carcinoma, this persistent activity beyond progression with an apparent flare-up effect after drug discontinuation of the drug was observed only with sorafenib and not with everolimus[27]. Then, even after progression, this treatment can participate in slowing down the disease. However, continuing sorafenib treatment after progression can be of interest only for patients who have a reasonable life expectancy and an excellent tolerance of the drug. Analysis of post-progression survival (Table 1) showed that, in addition to performance status, Child-Pugh score, and macrovascular invasion at progression, some other parameters are valuable. These include AFP, time to progression (correlation between time to progression using sorafenib and post-progression survival)[28], and pattern of progression[29]. Post-progression survival is significantly worse for patients who developed new extrahepatic lesions compared to patients with intra- or extra-hepatic growth or new intrahepatic lesions. These data in a Spanish cohort were later confirmed in Asian patients[30,31]. Thus, continuing sorafenib is a possibility if second-line trials are unavailable or if the patient cannot be included. This is particularly relevant for patients who had mild intrahepatic progression, who had a good PS with no worsening in BCLC or the Child-Pugh scores, and who had progressed very slowly (Figure 1).

CONCLUSION

Contrast-enhanced imaging techniques using mRECIST criteria are the best objective approach to appreciate the efficacy of vascularization targeting agents, particularly sorafenib. The value of AFP serum levels is not clear and not sufficient to impact therapeutic decisions. The enrollment of progressing patients in second-line trials is the best option. If this is not possible, then sorafenib must be discontinued if patients have poor prognostic factors or poor tolerance. In contrast, if patients do not have worsening PS or Child-Pugh classification or

if macrovascular invasion occurs, then sorafenib can be pursued; however, we must consider the important prognostic values of the progression pattern.

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ORIGINAL ARTICLE

Basic Study

Primary liver injury and delayed resolution of liver stiffness after alcohol detoxification in heavy drinkers with the *PNPLA3* variant I148M

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Abstract

AIM

To investigate the influence of *PNPLA3* genotype in heavy drinkers on serum markers and liver stiffness (LS) during alcohol withdrawal and its association with histology.

METHODS

Caucasian heavy drinkers (n = 521) with a mean alcohol consumption of 192.1 g/d (median alcohol consumption: 169.0 g/d; 95%CI: 179.0-203.3) were enrolled at the Salem Medical Center, University of Heidelberg. LS was measured by transient elastography (Fibroscan, Echosens SA, Paris, France). LS and serum markers were prospectively studied in these patients with all stages

of alcoholic liver disease (steatosis, steatohepatitis, fibrosis) prior and after alcohol detoxification with a mean observation interval of 6.2 ± 3.2 d. A liver biopsy with histological analysis including the Kleiner score was obtained in 80 patients.

RESULTS

The *PNPLA3 rs738409* genotype distribution for CC, CG and GG was 39.2%, 52.6% and 8.2%. GG genotype primarily correlated with histological steatohepatitis (r = 0.404, P < 0.005), ballooning (r = 0.319, P < 0.005) and less with steatosis (r = 0.264, P < 0.05). Mean LS was lowest in CC carriers (13.1 kPa) as compared to CG and GG carriers (17.6 and 17.2 kPa). Notably, LS primarily correlated with fibrosis stage (r = 0.828, P < 0.005), ballooning (r = 0.516, P < 0.005), steatohepatitis (r = 0.319, P < 0.005) but not with steatosis. After alcohol withdrawal, LS did not change in CC carriers, significantly decreased in CG-carriers from 17.6 to 12.7 kPa but to a lesser extent in GG carriers from 17.6 to 14.5 kPa. This was due to prolonged resolution of inflammation with significantly elevated aspartate transaminase levels after alcohol withdrawal in GG carriers. Non-invasive fibrosis assessment by LS in all patients showed a significantly higher F0 rate as compared to the biopsy cohort (47% *vs* 6%) with 3.8% more CC carriers while 3.7% less were seen in the F4 cirrhosis group. Thus, about 20% of patients with alcoholic liver cirrhosis would be attributable to *PNPLA3* G variants. The OR to develop cirrhosis corrected for age, gender and body mass index was 1.295 (95%CI: 0.787-2.131) for CG + GG carriers.

CONCLUSION

In heavy drinkers, *PNPLA3* GG primarily correlates with ballooning/steatohepatitis but not steatosis resulting in a delayed inflammation-associated resolution of LS. Consequently, sustained ballooning-associated LS elevation seems to be a potential risk factor for fibrosis progression in *PNPLA3* GG carriers.

Key words: Liver stiffness; Alcoholic liver disease; Adiponutrin; *PNPLA3*; Transient elastography; Alcohol withdrawal; Inflammation

Core tip: The role of the *PNPLA3* rs738409 variant (CG and GG) on histology and liver stiffness in response to alcohol detoxification was studied in a large monocentric cohort of heavy drinkers with various stages of ALD. About 20% of our patients with alcoholic liver cirrhosis were attributable to *PNPLA3* G variants with an OR to develop cirrhosis of 1.295. Our data further show that *PNPLA3* GG carriers primarily develop ballooning and not steatosis causing a delayed resolution of liver stiffness after alcohol withdrawal. We suggest that the delayed ballooning-associated stiffness elevation may contribute to fibrosis progression (see also the sinusoidal pressure hypothesis).

Rausch V, Peccerella T, Lackner C, Yagmur E, Seitz HK, Longerich T, Mueller S. Primary liver injury and delayed resolution of liver stiffness after alcohol detoxification in heavy drinkers with the *PNPLA3* variant I148M. *World J Hepatol* 2016; 8(35): 1547-1556 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i35/1547.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i35.1547

INTRODUCTION

Alcoholic liver disease (ALD) is the most common chronic liver disease in the Western world[1]. ALD encompasses a broad spectrum of disorders ranging from simple steatosis to severe forms of liver injury, including alcoholic steatohepatitis, fibrosis and cirrhosis. Although the majority (80%-90%) of heavy drinkers with an alcohol consumption > 80 g/d develop steatosis, only 35% show signs of inflammation and about 8%-20% progress to cirrhosis[2]. The underlying molecular mechanisms of disease progression, especially why some patients rapidly progress to severe liver disease, are still poorly understood. In addition, it remains unclear whether steatosis necessarily precedes steatohepatitis or is a coinciding bystander. The role of environmental factors that affect disease progression such as drinking habits and comorbidities has been known for many years[3]. However, twin studies, the enhanced sensitivity of female drinkers and the fact that only a minority of patients progress to cirrhosis despite heavy drinking clearly suggest a genetic pre-disposition[4,5].

Recent studies in multiethnic populations with non-alcoholic fatty liver disease (NAFLD) and ALD have demonstrated that the single-nucleotide polymorphism, the rs738409 variant, that encodes for an isoleucine to methionine substitution at position 148 (I148M) in the patatin-like phospholipase-3 (*PNPLA3/Adiponutrin*) gene is a strong disease modifier by influencing steatosis, liver enzymes and fibrosis progression[6-12]. So far, the function of *PNPLA3* and the effect of the amino acid substitution remain controversial. *PNPLA3* is closely related to *PNPLA2/ATGL*, the major hormone-sensitive lipase of adipose tissue, sharing 56% amino acid identity in the patatin-like domain[13,14]. *PNPLA3* is expressed in adipocytes, hepatocytes and hepatic stellate cells[15-18]. Despite many efforts, the physiologic role of *PNPLA3* and its direct action in the liver is still incompletely understood and it remains unclear whether the I148M substitution in *PNPLA3* directly causes steatosis, lipotoxicity, or both.

PNPLA3 GG carriers not only more rapidly progress toward fibrosis but also show elevated liver stiffness (LS)[19]. Non-invasive measurement of LS by ultrasound-based elastographic techniques such as transient elastography (TE) are increasingly used to screen for liver fibrosis[9,20-25]. However, various conditions have been shown to increase LS in the absence of fibrosis including inflammation and liver damage[26-28], congestion[29], cholestasis[30], arterial pressure[31] food intake[32,33] or amyloidosis[34,35]. For these reasons, we here study in

Table 1 Patient characteristics before and after alcohol withdrawal

Parameters	Before withdrawal (*n* = 521)	After withdrawal (*n* = 370)
Demographic characteristics		
Male (%)	72.1	
Age (yr)	50.2 ± 11.3	
Risk factors		
BMI (kg/m^2)	25.2 ± 4.6	
H/W ratio	1.0 ± 0.1	
Alcohol consumption (g/d)	192.1 ± 139.7	
Duration (yr)	19.9 ± 13.3	
Smoker (%)	70.9	
Diabetes (%)	10.0	
Coronary heart disease (%)	5.1	
RR (%)	34.5	
Ascites (%)	9.0	
F0 (%)	47.4	
F1-2 (%)	17.1	
F3 (%)	10.8	
F4 (%)	24.7	
Noninvasive parameters		
Hepatic steatosis (0-3, US)	1.9 ± 0.9	
Liver stiffness (kPa)	15.8 ± 21.1	12.6 ± 18.1
Laboratory parameters		
AST (U/L)	101 ± 108	54 ± 48
ALT (U/L)	70 ± 79	52 ± 46
GGT (U/L)	398 ± 577	268 ± 360
AP (U/L)	109 ± 76	88 ± 55
Bilirubin (mg/dL)	1.3 ± 2.8	0.9 ± 2.3
Albumin (g/dL)	5.0 ± 6.0	
INR	1.2 ± 3.4	1.2 ± 5.1
Urea (mg/dL)	22.6 ± 16.6	23.7 ± 12.5
Creatinine (mg/dL)	0.7 ± 0.3	0.8 ± 0.2
Hemoglobin (g/dL)	14.2 ± 2.2	13.8 ± 1.8
Platelets (/nL)	209 ± 87	215 ± 82
Glucose (mg/dL)	109.1 ± 36.4	
HbA1C (%)	5.6 ± 1.0	
Triglycerides (mg/dL)	195.7 ± 206.6	
Cholesterol (mg/dL)	215.9 ± 58.2	
HDL cholesterol (mg/dL)	72.3 ± 36.9	
LDL cholesterol (mg/dL)	112.6 ± 45.6	
Lipase (U/L)	63.6 ± 164.8	60.7 ± 56.3
Ferritin (ng/mL)	580.6 ± 650.5	
CRP (mg/dL)	6.1 ± 15.9	7.1 ± 12.5

Data are presented as mean ± SD or in %. BMI: Body mass index; H/W ratio: Hip to waist ratio; RR: Hypertension; F: Fibrosis stage; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl-transpeptidase; AP: Alkaline phosphatase; INR: International normalized ratio (Prothrombin); HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; US: Ultrasound.

detail the impact of *PNPLA3* I148M substitution on LS and histology in a large population of heavy drinkers primarily admitted to the hospital for alcohol withdrawal. We further analyze the impact of alcohol withdrawal on LS depending on *PNPLA3* status. Our data further suggest that the sustained and drinking-associated LS elevation in *PNPLA3* GG carriers is most likely associated with ballooning and seems to contribute to fibrosis progression.

MATERIALS AND METHODS

Patients

Caucasian heavy drinkers (*n* = 521, 148 females/369 males, age range 22-87 years) with a mean alcohol consumption of 192.1 g/d (median alcohol consumption: 169.0; 95%CI: 179.0-203.3) were enrolled at the Department of Gastroenterology, Salem Medical Center in Heidelberg. Patients presented primarily for alcohol detoxification with a mean duration of chronic alcohol consumption of 19.9 years. Patient's characteristics are given in Table 1, a more refined *PNPLA3* genotype-associated data presentation is shown in Table 2. All patients underwent careful clinical examination, standard laboratory routine (venous blood sampling), abdominal ultrasound and liver stiffness measurement. Inclusion criteria were daily alcohol consumption > 60/80 g/d, age > 18 years, and successful assessment of LS. Other causes of liver diseases (exclusion criteria) were ruled out serologically in all patients by screening for AMA, ANA, HCV and HBV. The study protocol was reviewed and approved by the local Ethics Committee of the University of Heidelberg and all patients gave written informed consent prior to inclusion. Laboratory parameters, TE were performed both at day of admission and release with a mean observation interval of 6.2 ± 3.2 d.

Liver histology and immunostainings

Eighty patients (15.4%) underwent liver biopsy using the Menghini technique (mean biopsy lengths 15.6 mm). Specimens were fixed in formalin and embedded in paraffin. Two experienced pathologists (TL and CL) blinded to the patient's data analyzed all liver biopsies independently. For histological analysis, 4 μm sections were dewaxed and stained with hematoxylin and eosin, Chromotrop-Anilinblue and Sirius-Red using standard procedures. Histological semiquantitative scoring of macro- and microvesicular steatosis, lobular inflammation, hepatocellular ballooning, Mallory-Denk bodies and apoptosis as well as fibrosis staging was performed exactly as described by Kleiner *et al*[36]. In addition, fibrosis was also assessed using the semiquantitative method of Chevallier *et al*[37] and collagen content was quantified by computer-assisted image analysis of Sirius-Red stained sections (morphometry). The histological diagnosis of steatohepatitis was based on the minimal criteria of steatosis (any degree), lobular inflammation and ballooning[38].

TE and non-invasive fibrosis assessment in ALD patients

LS was measured by TE (Fibroscan, Echosens SA, Paris, France) using the M[39] or XL probe[40,41]. TE was performed by physicians with at least 12 mo of experience in abdominal ultrasound and transient elastography on the right lobe of the liver in intercostal position according to established protocols[25]. Fibrosis stages were determined using the recently established aspartate transaminase (AST)-adapted cut-off values[42]. In patients with two measurements prior and after alcohol detoxification, the second measurements were used with less pronounced steatohepatitis and transaminase elevation, since such conditions correlate better with histology[9,25]. In addition, liver size, spleen size, ascites formation and semiquan-

Table 2 Characteristics of alcoholic liver disease sub-cohorts (*n* = 521) based on genotype distribution of rs738409 polymorphism

Parameters	*PNPLA3* CC (*n* = 204)	*PNPLA3* CG (*n* = 274)	*PNPLA3* GG (*n* = 43)	*PNPLA3* CG + GG (*n* = 317)
Demographic characteristics				
Patients (%)	39.2	52.6	8.2	60.8
Age (yr)	49.5 ± 11.0	50.7 ± 11.8	50.1 ± 9.7	50.7 ± 11.5
Risk factors				
BMI (kg/m^2)	25.4 ± 4.9	25.1 ± 4.5	25.6 ± 3.9	25.2 ± 4.4
H/W ratio	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Alcohol consumption (g/d)	194.0 ± 136.1	190.8 ± 146.2	181.2 ± 116.1	189.4 ± 142.0
Duration (yr)	18.3 ± 13.3	20.9 ± 13.1	17.2 ± 14.2	20.4 ± 13.3
Smoker (1 = yes)	0.7 ± 0.4	0.7 ± 0.5	0.6 ± 0.5	0.7 ± 0.5
Diabetes (1 = yes)	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.2	0.1 ± 0.3
Coronary heart disease (1 = yes)	0.1 ± 0.2	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
Noninvasive parameters				
Hepatic steatosis (0-3, US)	1.8 ± 0.9	2.0 ± 0.8	1.9 ± 0.8	2.0 ± 0.8
Liver stiffness (kPa)	13.1 ± 17.7	17.6 ± 23.0[a]	17.2 ± 22.2	17.5 ± 22.9[a]
Laboratory parameter				
AST (U/L) before detox	95.2 ± 100.8	102.8 ± 111.4	113.1 ± 116.8	104.0 ± 111.9
AST (U/L) after detox	47.8 ± 32.9	52.6 ± 46.0	82.8 ± 89.5[a]	56.2 ± 53.5
ALT (U/L) before detox	66.0 ± 59.4	71.9 ± 93.0	76.4 ± 60.4	72.5 ± 89.2
ALT (U/L) after detox	47.5 ± 35.9	52.4 ± 50.9	67.7 ± 55.0[a]	54.2 ± 51.5
GGT (U/L) before detox	406.3 ± 572.2	365.9 ± 516.1	537.7 ± 869.6	389.6 ± 578.9
GGT (U/L) after detox	254.8 ± 290.9	261.7 ± 347.3	389.7 ± 671.6	276.9 ± 399.6
AP (U/L) before detox	105.5 ± 76.2	111.6 ± 75.8	112.7 ± 72.6	111.8 ± 75.3
AP (U/L) after detox	83.3 ± 45.1	90.5 ± 59.2	97.5 ± 68.4	91.3 ± 60.2
Bilirubin (mg/dL)	1.2 ± 2.8	1.4 ± 3.0	0.9 ± 1.1	1.3 ± 2.8
Albumin (g/dL)	4.7 ± 4.7	5.3 ± 7.2	4.5 ± 0.5	5.2 ± 6.7
INR	1.4 ± 5.4	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.4
Urea	20.6 ± 10.8	24.6 ± 20.2[a]	20.1 ± 9.9	24.0 ± 19.2[a]
Creatinine	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.7 ± 0.3
Hemoglobin (g/dL)	14.2 ± 1.8	14.2 ± 2.5	14.6 ± 2.0	14.2 ± 2.4
Platelets (/nL)	216.7 ± 92.7	201.1 ± 80.0[a]	224.2 ± 91.4	204.5 ± 82.0
Glucose (mg/dL)	112.0 ± 46.2	107.7 ± 28.5	110.7 ± 34.6	108.1 ± 29.3
HbA1C (%)	5.6 ± 1.1	5.6 ± 0.8	5.8 ± 1.3	5.6 ± 0.9
Triglycerides (mg/dL)	190.6 ± 202.2	192.0 ± 205.8	240.9 ± 230.4	198.7 ± 209.6
Cholesterol (mg/dL)	219.9 ± 55.0	213.1 ± 61.1	222.9 ± 53.4	214.4 ± 60.1
HDL cholesterol (mg/dL)	73.2 ± 35.9	71.4 ± 37.6	75.6 ± 37.3	71.9 ± 37.5
LDL cholesterol (mg/dL)	113.5 ± 46.3	112.4 ± 45.5	118.0 ± 44.7	113.0 ± 45.3
Lipase (U/L)	48.5 ± 45.9	75.9 ± 216.5	45.3 ± 26.0	72.0 ± 202.7
Ferritin (ng/mL)	546.1 ± 611.6	599.6 ± 668.3	685.2 ± 708.2	610.8 ± 673.1
CRP (mg/dL)	4.7 ± 11.1	7.1 ± 18.9	6.0 ± 12.0	7.0 ± 18.1

Data are presented as mean ± SD or in %; significant paired T tests ([a]$P < 0.05$) with CC. BMI: Body mass index; H/W ratio: Hip to waist ratio; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl-transpeptidase; AP: Alkaline phosphatase; INR: International normalized ratio (Prothrombin); HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; US: Ultrasound; PNPLA3: Adiponutrin.

titative liver steatosis (0-3) were assessed by abdominal ultrasound.

PNPLA3 genotyping

Genomic DNA was isolated from EDTA anti-coagulated blood using standard protocols. The *PNPLA3* coding SNP I148M was genotyped using tetra-primer ARMS polymerase chain reaction (PCR) technique on the GeneAmp PCR System 2400 (Applied Bioscience) using standard protocol. Primers were designed using Batch Primer 3 software[43], synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are available upon request. PCR reactions were performed in a total volume of 25 μL, containing approximately 30-50 ng of template DNA, 1 × PCR buffer, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 2 nmol/L of outer primer and 20 nmol/L inner allele-specific primers and 1U of Taq polymerase (Roche, Penzberg, Germany). Post-PCR allelic discrimination was carried out using horizontal non-denaturing polyacrylamide gel (10%) electrophoresis followed by ethidium bromide staining and visualization on a UV transilluminator. To ensure genotyping quality, we included negative controls and DNA samples with known *PNPLA3* genotypes as internal controls.

Statistical analysis

We used descriptive statistics to compute equally distributed data, including means, standard deviations and frequencies. Not normally distributed data were log transformed before statistical analysis. Comparisons of the genotype distribution of CC, GG and combined CG and GG were performed and the Spearman correlation or χ^2 test for non-parametric variables (regression coefficient *r*, *P*) was used to determine the associations between laboratory findings, LS, histological scores and the genotypes. To determine whether there are significant

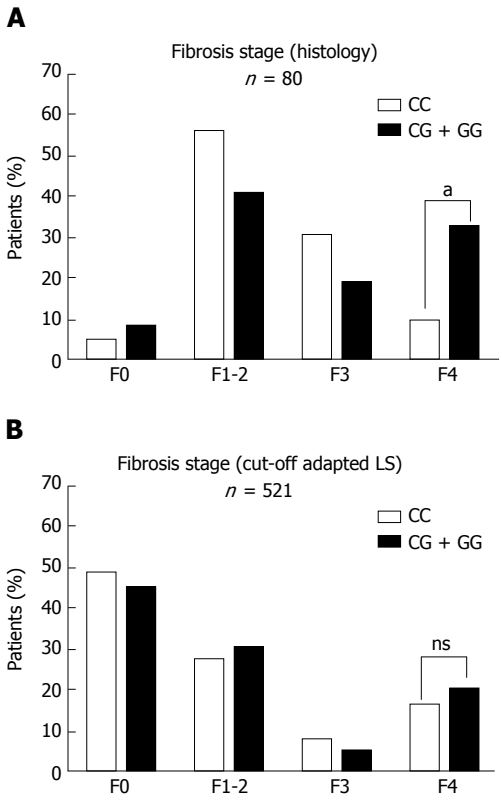

Figure 1 Distribution of fibrosis stages using (A) histology (Kleiner fibrosis score F0-4) or (B) non-invasive liver stiffness measurement (aspartate transaminase-adapted cut-off values). [a]*P* < 0.05. ns: Not significantly; LS: Liver stiffness.

differences between the variants (CC, CG, GG or CG combined with GG) we used a two-sample Student's *t*-test when the data were normally distributed. Binary logistic regression analysis was calculated to proof possible effects of genotype, gender, age and body mass index (BMI) on the outcome of AST-adapted cut-off values for fibrosis staging. Statistical calculations were performed with SPSS (version 21.0, IBM, SPSS) or SAS (version 9.4, SAS) software and two-sided *P* values < 0.05 were considered statistically significant. Statistical methods of this study were reviewed by Thomas Bruckner from Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany.

RESULTS

PNPLA3 rs738409 GG carrier show more cirrhosis

The *PNPLA3 rs738409* genotype distribution in our cohort of 521 ALD patients was 39.2%, 52.6% and 8.2% (*n* = 204, 274 and 43) for CC, CG and GG (Table 2). Notably, fibrosis distribution differed markedly in the non-invasively (*n* = 521) *vs* histologically (*n* = 80) assessed cohorts (Figure 1), histologically characterized patients showed only a small fraction of F0 stages (6%, *n* = 5). In contrast, the F0 fraction was much higher in the non-invasively assessed cohort by LS (47%, *n* = 245, Figure 1B). In both approaches, CG + GG carriers had more F4 cirrhosis as compared to CC carriers as shown in Figure 1A (9.3% *vs* 32.4%) and 1B (16.3% *vs* 20.0%). CC carriers represented 42.1% of the F0 cohort but 35.5% of the F4 cohort. In other words, about 3.8% more CC carriers had F0 while they were 3.7% less frequent in the non-invasively assessed F4 cohort. Both cohorts did not differ significantly with respect to age and mean drinking duration (approximately 20 years). Linear regression analysis corrected for age, gender and BMI calculated an OR of 1.295 (95%CI: 0.787-2.131) for CG + GG carriers to develop F4 cirrhosis (Table 3). Taken together, our study indicates a *PNPLA3*-attributable effect on fibrosis stage. Notably and as could be expected, the non-invasively characterized cohort had a much larger proportion of non-fibrotic patients.

Table 3 Risk factors associated with F4 cirrhosis

Factor	OR	95%CI	*P* value
PNPLA3 G (CG + GG)	1.295	0.787-2.131	> 0.05
Gender	0.855	0.496-1.475	> 0.05
Age	1.040	1.017-1.064	< 0.001
BMI	1.037	0.983-1.093	> 0.05

BMI: Body mass index; OR: Odds ratio; PNPLA3: Adiponutrin.

PNPLA3 rs738409 GG carriers have no pronounced metabolic phenotype

Since *PNPLA3* rs738409 SNP has been primarily identified in NAFLD patients, we next characterized typical features of the NAFLD phenotype. No significant differences were observed between CC, CG and GG carriers with regard to BMI (25.4 *vs* 25.1 *vs* 25.6), HbA1c (5.6% *vs* 5.6% *vs* 5.8%), and serum fasting glucose concentrations (112 mg/dL *vs* 108 mg/dL *vs* 111 mg/dL). This was also the case with regard to coronary heart disease, type Ⅱ diabetes, smoking habits (assessed by pack years) and arterial hypertension (Table 2 and data not shown). Likewise, no significant differences were observed between levels of high-density lipoprotein and low-density lipoprotein cholesterol and triglycerides (TG) although TG levels were notably higher in GG carriers (Table 2). In summary, in this large cohort of heavy drinkers, GG is associated with advanced fibrosis in the absence of a typical NAFLD-associated metabolic phenotype.

Ballooning/steatohepatitis is the predominant histological feature of PNPLA3 rs738409 GG carrier

To learn more about histological association with the *PNPLA3* carrier status, we assessed steatosis, inflammation and fibrosis using the Kleiner and the semiquantitative Chevallier score. Interestingly, GG genotype primarily correlated with steatohepatitis ($r = 0.404$, $P < 0.005$), ballooning ($r = 0.319$, $P < 0.005$), less with steatosis ($r = 0.264$, $P < 0.05$) but not significantly with fibrosis (Table 4). In line with this, CC genotype correlated negatively with ballooning ($r = -0.221$, $P < 0.05$). These data were mirrored in the direct comparison of the genotypes. More fibrosis and ballooning was

Table 4 Spearman rank correlation of *PNPLA3* carrier status and liver stiffness with histological parameters

Parameter (n = 80)	*PNPLA3* CC (n = 43)	*PNPLA3* CG (n = 29)	*PNPLA3* GG (n = 8)	Liver stiffness (kPa)
Steatohepatitis (score 0-2)	-0.163	-0.099	0.404[b]	0.391[b]
Microgranulomas (score 0-1)	-0.095	-0.139	0.357[b]	0.387[b]
Ballooning (score 0-2)	-0.221[a]	0.020	0.319[b]	0.516[b]
Glycogenated nuclei (score 0-1)	-0.124	-0.080	0.316[b]	0.335[b]
Steatosis (score 0-3)	-0.125	-0.045	0.264[a]	0.096
Lobular inflammation (score 0-3)	-0.142	-0.003	0.227[a]	0.420[b]
Megamitochondria (score 0-1)	-0.121	-0.005	0.198	0.278[b]
Large lipogranulomas (score 0-1)	0.134	-0.238[a]	0.145	0.144
Acidophil bodies (score 0-1)	-0.016	-0.072	0.133	0.285[b]
Pericellular fibrosis (score 0-3)	-0.224	0.141	0.131	0.567[b]
Chevallier fibrosis score (SSS)	-0.189	0.112	0.131	0.828[b]
Ballooning k8/18 stain (score 0-2)	-0.537[b]	0.490[b]	0.089	0.692[b]
Kleiner fibrosis score (score 0-4)	-0.163	0.148	0.035	0.745[b]
Mallory Denk Bodies (score 0-1)	-0.121	0.110	0.026	0.530[b]
Apoptosis M30 stain (score 0-3)	-0.039	0.031	0.014	0.490[b]
Pigmented macrophages (score 0-1)	0.003	0.012	-0.022	-0.009
Portal inflammation (score 0-1)	-0.027	0.099	-0.106	0.427[b]
Liver stiffness (kPa)	-0.045	0.017	0.037	1.000

Liver stiffness primarily correlates with fibrosis and liver damage but not significant with steatosis. In contrast, GG carrier status is tightly associated with liver injury and weakly with steatosis. [a]$P < 0.05$ *vs* [b]$P < 0.01$. PNPLA3: Adiponutrin.

present in the CG + GG carriers (Supplemental Table 1). Interestingly, neither a significant association was found with serum markers of liver damage (data not shown), with signs of liver cirrhosis in the ultrasound and with LS. Taken together, liver injury such as ballooning and steatohepatitis are the primary histological features associated with GG genotype in heavy drinkers while fibrosis and steatosis are less pronounced.

LS is predominantly associated with fibrosis and ballooning/steatohepatitis but not steatosis

Since previous studies indicated a higher LS in carriers of the *PNPLA3* risk allele (CG + GG) in various liver diseases and LS is increasingly used to screen for liver fibrosis, we next carefully analyzed the correlation of LS with histological subscores and the *PNPLA3* status (Table 4). As expected, LS showed a very tight and significant association with fibrosis stage (r = 0.828, P < 0.005) but also with ballooning (r = 0.692, P < 0.005) and steatohepatitis (r = 0.391, P < 0.005). Notably, no correlation was observed with steatosis (r = 0.096, ns). In addition, no significant correlation was seen between LS and *PNPLA3* genotype. Taken together, in a cohort of heavy drinkers, LS is correlated with fibrosis, liver injury and inflammation but not with steatosis and the *PNPLA3* status.

Elevated LS in PNPLA3 rs738409 GG carriers and a delayed resolution after alcohol withdrawal

Mean LS was lowest in CC carriers (13.1 kPa) and significantly higher in CG carriers (17.6 kPa, Figure 2). LS was likewise elevated in GG carriers (17.2 kPa) without reaching statistical significance due to the limited number of patients (8.2%). Interestingly, almost no change was observed in CC carrier after alcohol withdrawal (12.0 kPa, LS2). In contrast, LS significantly decreased in CG carriers to comparable 12.7 kPa after withdrawal from alcohol. Despite a longer observation interval of 6.6 d, LS decreased slower in GG and remained higher (14.5 kPa). This was most likely due to sustained inflammation/ballooning as reflected by elevated AST levels, which were significantly higher after alcohol withdrawal (Figure 2B, Table 2). In summary, GG-associated liver damage results in a reversible, inflammation-associated increase of liver stiffness. In addition, GG carriers show a slower resolution of liver damage and LS after withdrawal from alcohol.

DISCUSSION

We here show in a large monocenter cohort of histologically and non-invasively characterized heavy Caucasian drinkers that the SNP rs738409 in *PNPLA3* (CG and GG) is primarily associated with ballooning/steatohepatitis but less with steatosis. Importantly and as seen previously, G carriers (CG + GG) had higher initial LS values as compared to CC carriers. Notably and in some contrast to the genotype analysis, LS was primarily correlated with fibrosis stage, ballooning/steatohepatitis but not at all with steatosis. GG carriers showed a slower resolution of liver damage and LS after withdrawal from alcohol. Since AST levels were significantly elevated in GG carriers after withdrawal from alcohol, we attributed this to delayed resolution of inflammation/ballooning.

Several findings of this study are unexpected and shed new light on the function of *PNPLA3* and its link to inflammation and fibrosis development. First of all, we see clear differences of the fibrosis distribution between the biopsy and non-invasively characterized cohorts. While only 6% showed no fibrosis (F0) in the biopsy cohort this number increased drastically to 47% in the non-invasive cohort. These numbers are especially

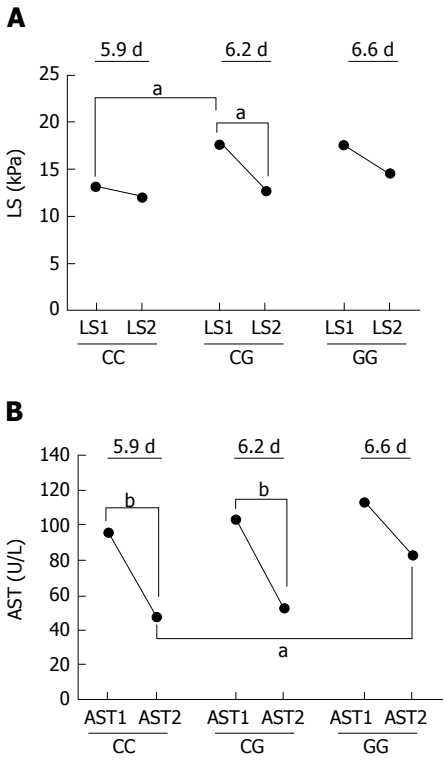

Figure 2 PNPLA3 carrier status and its effect on liver stiffness (A) and aspartate transaminase (B) levels prior and after alcohol detoxification (1 and 2). Mean observation of detoxification periods in days are indicated for each genotype. $^{a}P < 0.05$, $^{b}P < 0.01$. AST: Aspartate transaminase; ALT: Alanine transaminase; LS: Liver stiffness.

impressive with regard to the high negative predictive values of transient elastography[24,25]. We believe that these findings clearly indicate an often underestimated selection bias of biopsies in ALD study cohorts. Obviously, significantly less patients with no or mild liver disease are asked or motivated to undergo liver biopsy, whereas more severe patients are willing to agree with the invasive procedure. We believe that this observation is a strong argument to enforce well non-invasively characterized ALD cohorts in future studies.

Second, another interesting finding of the non-invasively characterized cohort is the almost symmetric, mirror-like distribution of CC *vs* G (CG + GG) carriers in the F0 and F4 population over almost 20 years of alcohol consumption. Circa 4% less CC carriers were seen in the cirrhosis group, an equating circa 4% more CC carriers were observed in the F0 group. Thus, about 20% of patients with alcoholic liver cirrhosis would be attributable to *PNPLA3*-G variants. The odds ratio to develop F4 cirrhosis was 1.3 for our cohort, which corresponds to earlier reports[12,44]. Notably and in line with previous reports[45], the genotype distribution did not follow the Hardy-Weinberg equilibrium which could point to phenotype (GG)-related increased mortality, *e.g.*, due to complications of cirrhosis such as primary liver cancer (HCC)[46].

Third, the histological findings are intriguing and partly surprising. Up to date, our study presents the most detailed histological analysis with respect to *PNPLA3* carrier status and ALD since previous GWAS studies had primarily relied on retrospective samples with laboratory tests such as transaminases and diagnosis of steatosis by ultrasound[10,12]. Our data clearly show that signs of liver injury such as steatohepatitis or ballooning are the major and predominant features of GG carriers. In contrast, other widely discussed findings such as steatosis or fibrosis are less pronounced. Our study suggests that rather ballooning and not steatosis is the key feature of the *PNPLA3* GG phenotype in heavy drinkers that later develop ALD. Whether steatosis is either just a consequence of apoptotic liver damage or a bystander needs to be further clarified.

Fourth, special novel insights are seen with the detailed analysis of LS prior and after alcohol withdrawal. It is especially surprising that *PNPLA3* status and LS are differentially associated with histology. These data may also serve as explanation for the rather weak effect of the *PNPLA3* status on LS and less pronounced results in the past[19]. Thus, LS is highly associated with fibrosis stage (Kleiner and Chevallier) (r = 0.79) and with steatohepatitis/ballooning (r = 0.4-0.7) but not at all with steatosis (r = 0.09). In contrast, the GG status primarily correlates with liver injury (ballooning, steatohepatitis) (r = 0.3-0.5) and weaker with steatosis (r = 0.26). Moreover, a striking feature of the protective CC status is the fast resolution of transaminase levels after alcohol detoxification without notable changes of LS. We can only speculate why CC carriers do not respond with a significant LS decrease after alcohol withdrawal despite an almost normalization of liver transaminases. One explanation could be that only 30% of ALD patients with elevated transaminase levels show a change of LS after alcohol withdrawal[42]. In other words, liver injury as assessed by elevated AST levels not necessarily increases LS in all patients. It rather suggests that ballooning as predominant histological finding of GG carriers may not necessarily cause an increase of transaminase levels. Indeed, ballooning was not significantly associated with elevated AST levels. We therefore believe that GG carriers not only have higher inflammation but also seem to have a slower resolution of liver damage/ballooning. One possible explanation could be that *PNPLA3* directly affects pressure-mediated LS elevation according to the recently introduced pressure hypothesis of cirrhosis that also encompasses mechano-signaling[24]. In line with this the co-presence of steatosis in GG carriers could lower LS since steatosis and LS seem not to associate directly (tissue softening of fat).

One of the limitations of our study is the fact that the exact time point of stopping drinking cannot always be determined with absolute correctness nor the adherence to abstaining from alcohol. In addition, the individual response of both laboratory parameters and LS to alcohol withdrawal may also vary considerably. Nevertheless, we strongly feel that the delayed resolution of alcohol-induced inflammation and LS in GG carriers could contribute to fibrosis progression in drinkers who typically show a pulsatile exposure to alcohol and in line with the

recently proposed sinusoidal pressure hypothesis[47]. Consequently, GG carriers could have a longer overall exposure to liver inflammation and elevated LS finally resulting in fibrosis progression.

Taken together, liver damage (inflammation/ballooning) with increased LS appears to be the primary event in GG carriers in response to heavy alcohol consumption, which resolves after alcohol withdrawal. Interestingly, GG carriers require a longer period of medical care in the hospital for alcohol detoxification showing advanced liver fibrosis and pointing toward more severe alcohol-related health problems. However, as demonstrated by our non-invasive fibrosis assessment of the whole study population, *PNPLA3* carrier status accounts only for circa 20% of alcoholic cirrhosis corresponding to about 4% of our overall study cohort and suggesting additional other, hitherto not recognized pro-fibrogenic factors. On a final note, we would like to emphasize the importance of non-invasive characterization of ALD study cohorts in the light of potential study bias of solely biopsy-based designs.

COMMENTS

Background

Polymorphisms of *PNPLA3* gene (Adiponutrin) have been identified as important genetic progression factor both of nonalcoholic fatty liver disease and alcoholic liver disease (ALD), the most common liver diseases worldwide. However, *PNPLA3* function and its molecular role in liver fibrosis are still unsettled.

Research frontiers

Several studies in different populations have confirmed the association of a *PNPLA3* polymorphism with chronic liver disorders ranging from steatosis, inflammation to fibrosis progression and even hepatocellular carcinoma. It has also been shown that *PNPLA3* I148M elevates liver stiffness, an increasingly used non-invasive parameter to screen for liver cirrhosis.

Innovations and breakthroughs

This is the first study, which investigated in detail the impact of *PNPLA3* I148M status, first, on detailed histological subscores in heavy drinkers, and, second, on liver stiffness and other laboratory parameters in response to alcohol withdrawal.

Applications

In heavy drinkers, *PNPLA3* GG primarily correlates with ballooning/steatohepatitis but not steatosis resulting in a delayed inflammation-associated resolution of liver stiffness (LS). Consequently, sustained ballooning-associated LS elevation seems to be a potential risk factor for fibrosis progression in *PNPLA3* GG carriers. Significantly more patients without fibrosis (F0) were seen in the non-invasively characterized cohort as compared to the liver biopsy cohort (47% *vs* 6%) underlining the potential bias of biopsy-based studies.

Terminology

ALD is the most common chronic liver disease in the Western world. ALD encompasses a broad spectrum of disorders ranging from simple steatosis to severe forms of liver injury, including alcoholic steatohepatitis, fibrosis and cirrhosis. It has been shown, that the SNP rs738409 in *PNPLA3* encoding for an isoleucine to methionine substitution at position 148 (I148M) is a strong liver disease modifier responsible for disease progression.

Peer-review

Rausch *et al* analyzed the influence of *PNPLA3* genotype in heavy drinkers on serum markers and LS during all stages of alcoholic liver disease (steatosis, steatohepatitis and fibrosis) prior and after alcohol detoxification. This is a study of great interest that can help the researchers in evolving in this field.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis C eradication with sofosbuvir leads to significant metabolic changes

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Author contributions: Morales AL designed the study; Morales AL and Junga Z collected and analyzed the data, and drafted the manuscript; Singla MB performed statistical analysis and manuscript review; Sjogren M and Torres D revised the manuscript for important intellectual content, and help with development of the manuscript and study design; all authors have read and approved the final version to be published.

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Abstract

AIM

To assess the effect of sofosbuvir (SOF) based regimens on glycemic and lipid control.

METHODS

This is a retrospective analysis of hepatitis C virus (HCV)-infected patients treated and cured with a SOF regimen [SOF/ribavirin/interferon, SOF/simeprevir, or SOF/ledipasvir (LDV) ± ribavirin] from January 2014 to March 2015. Patients with hemoglobin A1C (HbA1C) and lipid panels within six months before and six months after therapy were identified and included in our study. Due to the known hemolytic effect of ribavirin, HbA1C was obtained a minimum of three months post-treatment for the patients treated with a ribavirin regimen. Medical history, demographics, HCV genotype, pre-therapy RNA, and liver biopsies were included in our analysis. The patients who started a new medication or had an adjustment of baseline medical management for hyper-

lipidemia or diabetes mellitus (DM) were excluded from our analysis.

RESULTS

Two hundred and thirty-four patients were reviewed, of which 60 patients met inclusion criteria. Sixty-three point three percent were male, 26.7% were Caucasian, 41.7% were African American and 91.7% were infected with hepatitis C genotype 1. Mean age was 60.6 ± 6.7 years. Thirty-nine patients had HbA1C checked before and after treatment, of which 22 had the diagnosis of DM type 2. HbA1C significantly decreased with treatment of HCV (pretreatment 6.66% ± 0.95% *vs* post-treatment 6.14% ± 0.65%, $P < 0.005$). Those treated with SOF/LDV had a lower HbA1C response than those treated with other regimens (0.26% ± 0.53% *vs* 0.71% ± 0.83%, $P = 0.070$). Fifty-two patients had pre- and post-treatment lipid panels; there was a significant increase in low-density lipoprotein (LDL) and total cholesterol (TC) after treatment (LDL: 99.5 ± 28.9 mg/dL *vs* 128.3 ± 34.9 mg/dL, $P < 0.001$; TC: 171.6 ± 32.5 mg/dL *vs* 199.7 ± 40.0 mg/dL, $P < 0.001$). Pre-treatment body-mass index (BMI) did not differ from post-treatment BMI ($P = 0.684$).

CONCLUSION

Eradication of HCV with a SOF regimen resulted in a significant drop in HbA1C and an increase in LDL and TC post therapy.

Key words: Hepatitis C; Sofosbuvir; Hyperlipidemia; Hemoglobin A1c; Low-density lipoprotein

Core tip: In our retrospective study, we evaluated the changes in glucose and lipid metabolism in a group of hepatitis C patients treated and cured with a sofosbuvir-containing regimen. We used hemoglobin A1c (HgA1c) and lipid panels to assess those two parameters. Six months post eradication, we found a statistically significant drop in HgA1c and an increase in low-density lipoprotein and total cholesterol. The use of HgA1c, although not perfect, is easy to understand and is frequently used by primary care doctors as a tool to assess glucose control.

Morales AL, Junga Z, Singla MB, Sjogren M, Torres D. Hepatitis C eradication with sofosbuvir leads to significant metabolic changes. *World J Hepatol* 2016; 8(35): 1557-1563 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i35/1557.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i35.1557

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, with a prevalence of infection in the United States of approximately 1.6%[1]. It remains the leading cause of death from liver disease and is the leading indication for liver transplantation in the United States despite recent medical advances in HCV therapy. HCV can cause major alterations in insulin resistance (IR) and lipid homeostasis[2-4]. HCV infection has been associated with the development of diabetes mellitus type 2[5,6] as well as a 3.5-fold increase in the prevalence of glucose alterations in non-diabetics[7]. The pathogenesis between HCV and IR seems to be multifactorial, with cytokine upregulation and direct interactions between viral particles and insulin signaling pathways[8-12]. In the traditional pegylated interferon (PegIFN) based regimens, IR was associated with decreased sustained virological response (SVR)[13]; multiple studies have shown an association between viral suppression or clearance and improvement of IR[14-17].

Along with IR, steatosis is also very common in patients infected with HCV[18]. The exact mechanism has not been fully elucidated, but host lipid alterations seem to play a major role. The virus utilizes very low-density lipoproteins (LDLs) to infect hepatocytes and several other lipid secretory mechanisms to perpetuate replication[4]. Several proteins, including Seipin and the HCV core protein, have been shown to alter the production of free fatty acids, as well as the proper excretion of lipids, increasing steatosis in the host[19-21]. Hypocholesterolemia is another finding that seems to be closely related to HCV replication mechanisms. After successful treatment with interferon-based therapy, it has been shown that hypocholesterolemia was resolved, with significant increases in LDL, triglycerides, and cholesterol[22,23].

The era of direct-acting antiviral (DAA) agents has increased SVR rates to over 90%, with dramatically improved side effect profiles[24-30]. IR and lipid alterations do not seem to affect treatment outcomes, and there is limited data on the effects of DAA therapy on metabolic and lipid profiles. A recent study evaluating the effects of sofosbuvir (SOF) and ribavirin (RBV) therapy in a mostly non-diabetic population demonstrated fluctuations in LDL levels throughout treatment, with elevations in LDL in patients achieving SVR as well as a small decrease in hemoglobin A1C (HbA1C) levels (5.58% ± 0.08% to 5.45% ± 0.91%; $P = 0.0046$)[31]. Additional data regarding metabolic alterations after therapy with the new DAA is scarce. In this retrospective study, the effects of HCV eradication on glucose and lipid metabolism in patients treated with SOF-based regimens at WRNMMC from 2014 to 2015 were assessed.

MATERIALS AND METHODS

Eligibility criteria

Patients aged 18 years or older with confirmed infections with HCV (by RNA) treated and cured at our institution with any combination of a NS5B inhibitor (SOF), NS5A inhibitor [ledipasvir (LDV)], protease inhibitor (Simeprevir), RBV and PegIFN from January 2014 to March 2015 were eligible for the study. Electronic records were reviewed to look for patients with a HbA1C and/or lipid panel drawn

before and after therapy. A total of 234 patient charts were reviewed.

HbA1C

The HbA1C closest to starting day of HCV therapy (up to six months pre-therapy) and the closest HbA1C post-therapy (up to six months) were included in our analysis. RBV is known to cause hemolysis, with remarkable drops in hemoglobin. Since HbA1C is closely related to red blood cell lifespan and could be altered by RBC destructions and anemia, the HbA1C in this population was selected between three to six months post therapy. Hemoglobin levels were reviewed pre-therapy and post-therapy and added to the analysis.

In patients with the diagnosis of diabetes mellitus type 2, a review of concomitant hypoglycemic medications was performed. All clinic encounters up to a year prior to starting HCV therapy, during therapy, and up to six months post therapy were reviewed, looking for adjustments of medications that could have altered HbA1C values. Those patients who started a medication or had an adjustment of baseline medical management were removed from the analysis. Patients on stable doses of oral hypoglycemic medications or insulin regimens during the study period were included. Attempts to account for diet and exercise regimens were beyond the scope of this analysis.

Lipids

Lipid panels closest to the starting and end dates of therapy (up to six months pre and post therapy) were included. All samples were drawn during the morning. However, due to the retrospective nature of the analysis, fasting could not be confirmed for all patients. Patients on stable doses of lipid-lowering agents were included, while patients started on new medications or with adjustments during the study period were removed from the analysis.

Data collection

Basic demographic and clinical information was collected for all patients, including age, gender, race, body-mass index (BMI) pre-and post-HCV therapy, and specific HCV anti-viral therapy used. Pre-therapy aspertate amino-transferase (AST), alanine transaminase (ALT), alkaline phosphatase, total bilirubin, albumin, total protein, Hepatitis C RNA, Hepatitis C genotype, and liver biopsy staging were also included. Patients with other liver diseases, such as hemochromatosis, Wilson disease, and alcoholic liver disease, were excluded from the analysis. A total of 60 patients were included in the final analysis. The study protocol was approved by the Institutional Review Board of Walter Reed National Military Medical Center.

Statistical analysis

Data were collated and analyzed using statistical software package IBM SPSS Statistics 21.0 (IBM, Armonk, New York). Continuous data was reported as means ± SDs. Paired *t*-test was used to compare variables measured before and after HCV treatment. Student's *t* test was used to analyze between group comparisons of continuous data including the change in HbA1C, HA1C, LDL, and high density lipoprotein (HDL) over the HCV treatment. A probability value of less than 0.05 was considered statistically significant. The statistical review of the study was performed by a biomedical statistic.

RESULTS

A total of 234 patients were treated for HCV during the study period. Of these, 60 patients met the inclusion criteria. Their average age was 60.6 ± 6.7 years; 26.7% were Caucasian, 41.7% were African American, and 63.3% were male. Clinical history in the cohort was significant for diabetes (38.3%) and hyperlipidemia (HLD) (33.3%). Patients had a mean viral load of 4.7×10^6 ± 7.6×10^6; 50.0% were infected with genotype 1a, and 26.7% were infected with genotype 1b. The mean pre-treatment AST was 61.0 ± 49.3 units/L, ALT 72.1 ± 56.2 units/L, alkaline phosphatase 102.3 ± 71.3 units/L, total bilirubin 0.8 ± 1.5 mg/dL, albumin 4.2 ± 0.4 g/dL, and total protein 7.4 ± 0.7 g/dL.

All patients were treated with a SOF-based regimen. Of the 23 patients with diabetes, 15 were treated with a stable dose of anti-diabetic medications, most commonly metformin. Of the 20 patients with HLD, 12 were taking a statin, and only three had their statins held during therapy (Table 1).

A total of 39 patients had pre- and post-treatment HbA1C measured. Overall, there was a significant drop in HbA1C during treatment (Figure 1). This was not accompanied by a significant decrease in BMI (pre-treatment 28.86 ± 5.15 kg/m^2, post treatment 28.48 ± 4.72 kg/m^2, P = 0.683). There was no significant difference in HbA1C effect between males and females (P = 0.793). There was no significant difference in HbA1C drop between genotype 1a and 1b (P = 0.605). Although not statically significant, patients with a history of diabetes tended to have a larger drop in HbA1C than those without diabetes, and Caucasians tended to have a larger drop in HA1C than African Americans. Patients aged 65 and older were less likely to have a drop in their HbA1C with treatment (younger than 65, 0.68% ± 0.75%, 65 and older, -0.01% ± 0.47%, P = 0.0187). Sixteen of these patients were treated in conjunction with ribavirin; this did not have a significant effect on HbA1C change (drop in HbA1C with ribavirin 0.44% ± 0.76%; without ribavirin 0.68% ± 0.74%, P = 0.342). Patients with a high viral load (> 6000000 copies) tended to have a larger drop in HbA1C with treatment (high VL 0.87% ± 0.97%, low VL 0.40% ± 0.62%, P = 0.080).

Fifty-two patients had pre- and post-treatment lipid panels measured. Overall, there was a significant increase in LDL and total cholesterol (TC) with minimal change in HDL (pre 52.8 ± 18.3 mg/dL, post 51.4 ± 18.5 mg/dL, P = 0.699) and triglycerides (pre 132.1 ± 99.7 mg/dL, post 129.8 ± 80.8 mg/dL, P = 0.853) (Figure 2). Patients with a history of HLD did not have

Table 1 Patient characteristics (n = 60)

Male (n = 38)	63.3%
Female (n = 22)	36.7%
Race	
Caucasian (n = 16)	26.7%
African American (n = 25)	41.7%
Hispanic (n = 3)	5.0%
Asian (n = 2)	3.3%
Not listed (n = 14)	23.3%
Mean age ± SD	60.6 ± 6.7
Diabetic (n = 23)	38.3%
Hyperlipidemia (n = 20)	33.3%
Hypertension (n = 42)	70.0%
Treatment	
Sofosbuvir/ribavarin/interferon (n = 21)	35.0%
Sofosbuvir/simeprevir (n = 11)	18.3%
Sofosbuvir/ledipesvir (n = 23)	38.3%
Sofosbuvir/ribavirin (n = 4)	8.3%
Sofosbuvir (n = 1)	1.7%
Biopsy stage (n = 49)	
1 (n = 8)	13.3%
2 (n = 21)	35.0%
3 (n = 5)	8.3%
4 (n = 15)	25.0%
Statin use (n = 20)	20.0%
Statin held during tx (n = 3)	5.0%
Mean viral load	4746471 ± 7641768
Mean ALT	72.1 ± 56.2
Genotype	
1a (n = 30)	50.0%
1b (n = 16)	26.7%
1 undistinguished (n = 9)	15.0%
2 (n = 2)	3.3%
3 (n = 3)	5.0%

ALT: Alanine transaminase.

significantly larger increase in LDL than those without a history of hyperlipidemia (HLD 27.8 ± 30.7 mg/dL, non-HLD 30.4 ± 44.6 mg/dL, P = 0.810). Patient's age 65 or older did not have a significantly larger increase in LDL than younger patients (65 or older 22.4 ± 32.3 mg/dL, younger 30.4 ± 37.1 mg/dL, P = 0.511). Caucasians and African Americans had similar increases in LDL (Caucasians 35.7 ± 36.0 mg/dL, African Americans 33.4 ± 35.5 mg/dL, P = 0.847). Those treated with SOF + ledipisvir tended to have a larger increase in LDL than those treated with other regimens (SOF + LED 36.7 ± 39.3 mg/dL, other therapy 22.1 ± 33.1 mg/dL, P = 0.157). Treatment regimen including interferon did not affect LDL increase (P = 0.755). High VL (> 6000000 copies) prior to treatment did not affect significantly impact the increase in LDL (P = 0.221). Patients with hepatitis C genotype 3 (n = 3) on average had an increase in LDL of 59 mg/dL (pre 87.3 mg/dL, post 146.3 mg/dL) and an increase in TC of 60 mg/dL (pre 148.7 mg/dL, post 208.7 mg/dL).

DISCUSSION

The main finding of this retrospective study was a significant decrease in HbA1C up to six months post-HCV eradication. The mechanism responsible for this

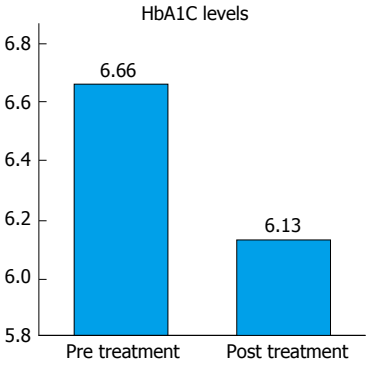

Figure 1 Effects of hepatitis C eradication on hemoglobin A1C. Vertical axis represents HbA1C levels in mg/dL. HbA1C significantly decreased after eradication of hepatitis C virus (pretreatment 6.66 ± 0.95 mg/dL *vs* post-treatment 6.14 ± 0.65 mg/dL, P < 0.005). HbA1C: Hemoglobin A1C.

improvement in glycemic control is unknown although likely multifactorial. It is well known that HCV alters glucose metabolism by inducing inflammatory cascades and promoting IR. Defects in pathways important in hepatic gluconeogenesis such as Pi3K and AKT phosphorylation have been reported in patients infected with HCV. Insulin receptor substrates 1 and 2 are closely related to the Pi3K/AKT pathways; these two receptors are key components in the development of IR in patients infected with HCV. The virus can degrade these two receptors, directly affecting the PI3K/AKT pathways[32-34]. Eradication of the virus restores homeostasis of these pathways, leading to an improvement in IR.

In the interferon/RBV era, several studies have demonstrated an improvement of IR with SVR. Early work by Thompson *et al*[17] demonstrated a 10% decrease in IR in genotype1 patients who achieved SVR, which was supported by the more recent results from Chien *et al*[35] that showed a significant decrease in HOMA-IR at EOT after eradication of the virus with this combination. Similarly, a study by Meissner *et al*[31] demonstrated a small but significant decrease in HbA1c in patients treated with SOF/RBV (5.58% ± 0.08% to 5.45% ± 0.91%; P = 0.0046). While the majority of these patients were non-diabetic or pre-diabetics, the patients included in this analysis had a significantly higher rate of diabetes, at 56%. When compared to the non-diabetic patients, the diabetics had a greater improvement in HbA1C. Gender, race, HCV genotype, and HCV RNA did not affect HbA1C drop.

In a subgroup analysis, patients treated with the SOF/LDV had a lower drop in HbA1C when compared to SOF/RBV and SOF/SIM groups. One possible explanation is the relationship between the new DAA and its target. The non-structural proteins of the virus NS5A and NS5B are key components in the activation of inflammatory cascades promoting insulin resistance[34]. It is plausible that the interaction of the medication or the duration of therapy alters the effects of insulin resistance, although further study is required.

Although this study was not designed to identify the

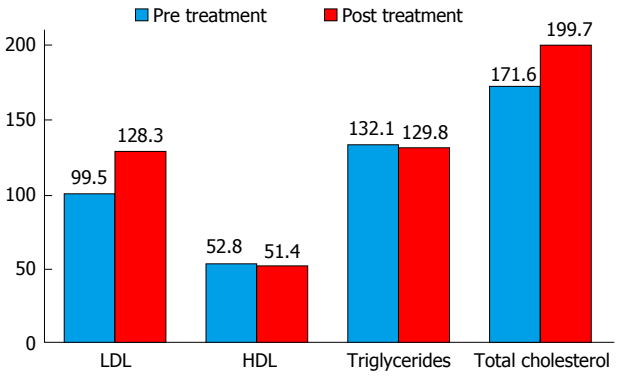

Figure 2 Effects of hepatitis C eradication on lipids. Vertical axis represents lipid levels in mg/dL. LDL and total cholesterol were significantly higher post hepatitis C eradication (LDL: 99.5 ± 28.9 mg/dL *vs* 128.3 ± 34.9 mg/dL, $P < 0.001$; total cholesterol 171.6 ± 32.5 mg/dL *vs* 199.7 ± 40.0 mg/dL, $P < 0.001$). No significant changes were noted for HDL and triglycerides. LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

long-term implications of hepatitis C eradication in glucose control, it is possible that these changes could have long-term implications regarding medical management. One of 16 patients on medical therapy for diabetes required a decrease in insulin therapy post viral eradication and another was taken off completely of therapy. The savings from a drop of even 0.5% of HbA1c are significant, and many oral hypoglycemics maximum efficacy is only a 1% improvement in Hba1c. Even more important than potential cost savings are the implications of better glucose control in the development of microvascular and macrovascular disease as small drops in HbA1C can alter the course of these complications. Primary care physician should monitor diabetic patients post HCV eradication to assess if changes in medical management are required and to prevent complications such as hypoglycemia.

The implications of insulin resistance, especially in diabetic patients infected with HCV, are well established. Huang *et al*[36] showed an increased risk of liver disease progression to cirrhosis in HCV-infected patients with diabetes. Hui *et al*[3] demonstrated that insulin resistance was an independent predictor for the degree of fibrosis and fibrosis progression in HCV-infected patients. Everhart *et al*[37] showed that not only hepatic steatosis was associated with liver disease progression, but also the degree of insulin resistance. They suggested that addressing these two issues might modify disease progression[37]. Taking into account this information and the results of our study, we should consider adding diabetic HCV-infected patients to the high-risk group that would benefit from priority in treatment.

These results also correlate with previous studies evaluating the effects of HCV eradications and lipids. An increase in TC and LDL post therapy was demonstrated irrespective of anti-viral therapy or genotype. Chronic infection with HCV has been implicated in the development of hypolipidemia[38,39]. A reversal of these findings has been reported in patients treated with INF/RBV regimens, as well as SOF/RBV regimens that have achieved SVR, suggesting this is most likely related to viral clearance rather than a medication effect[22,23,31]. The implications of these alterations in cardiovascular and cerebrovascular disease are beyond the scope of this retrospective study but should be further investigated.

The study does have several limitations including its retrospective nature and the small number of patients. Even though all lipids were drawn during the morning time, fasting was unable to be confirmed. Other parameters that could have altered the results, such as dietary changes and exercise, were not available. Medication reconciliation was not directly obtained, but an evaluation of several encounters from the electronic medical record from different providers was performed, looking for adequate medication reconciliation. The length of analysis was also limited to six months post-HCV therapy, so an analysis of the long-term implications of these results cannot be made.

This analysis did strengthen the knowledge pertaining to the metabolic effects of SOF-based regimens and confirmed that eradication of the virus could have extra-hepatic benefits. Even though HOMA-IR is a more direct measurement of IR, HbA1C is a more practical parameter that can be used to assess glucose control, and this study confirmed an improvement in HgA1c with SVR.

In conclusion, this study showed a significant drop in HbA1C up to six months after the eradication of HCV with SOF-based regimens. Future studies are needed to see if this change is sustainable. The effects of virus eradication on lipid panels were also determined, and they confirmed previous analyses that showed an increase in lipid panels, including LDL and TC, with SVR. This study suggests that physicians treating HCV patients should reassess preventive medicine measures after therapy, as the benefits of eradicating HCV may extend beyond eliminating the effects of chronic liver inflammation.

COMMENTS

Background

The hepatitis C virus (HCV) is a leading cause of chronic liver disease, with a prevalence of infection in the United States of approximately 1.6%. It is the leading cause of death from liver disease and is the leading indication for liver transplantation in the United States. Chronic hepatitis C infection (CHC) is known to induce systemic changes regarding glucose control and lipid metabolism. Glycemic balance can be affected by direct effect over insulin activation cascades and as a systemic response to inflammatory cytokines. Patients with CHC developed diabetes mellitus earlier than non-infected patients. Lipid metabolism is also affected due to known impaired lipid secretions associated with the infectious mechanism of the virus (possible use of lipid receptor to infect hepatocytes). Steatosis is another major finding in patients infected with CHC.

Research frontiers

Hepatitis C therapy has changed drastically in the last four years. The authors are now able to achieve cure rates of over 90%, with minimal side effects. The long-term implications of these new agents are still unclear, and previous studies have shown mixed results regarding alterations in glucose and lipid control after eradication. There is limited data regarding the newer anti-viral agents and their effects on metabolic derangements. The study attempts to assess the metabolic changes associated with these new agents.

Innovations and breakthroughs

Similar studies evaluating metabolic changes associated with hepatitis C eradication have used HOMA-IR as a surrogate of glucose homeostasis. Although this is a very accurate way of assessing glucose changes post hepatitis C eradication, its use on a daily clinic encounter is limited. In the study, the authors used HgA1c as a surrogate for glucose homeostasis. This laboratory test is easy to use and is well known by non-gastroenterology/hepatology providers. This laboratory test is more practical for daily clinic encounters. Previous studies on patients treated and cured with interferon and Ribavirin have shown alterations in lipid homeostasis, similar to the results. As the data on lipid alterations with the new direct antiviral agents is limited, the study adds to the knowledge on non-hepatic effects associated with a sustained virological response.

Applications

As reported in the study, several patients required adjustments in their hyperglycemic regimens, and one patient was completely taken off medication. The study suggests that shortly after completing hepatitis C therapy, primary care doctors should monitor diabetic patients, under medical management, to assess if changes to their medications are needed. Although the study was not meant to assess long-term effects, changes in HbA1c, as seen in the study, can add benefits in cost savings, as well as prevent microvascular and macrovascular disease. The changes seen in lipid homeostasis are worrying and require further investigation. These patients are still at risk of developing other liver diseases, such as non-alcoholic fatty liver disease. Primary care doctors should implement close monitoring of lipids after hepatitis C eradication, and those who meet the criteria for therapy should be treated accordingly.

Terminology

NS5A: Non-structural protein 5A. This protein plays a key role in HCV replication. It is one of the main targets for some of the new direct acting antiviral agents; NS5B: Non-structural protein 5B. Involved in Hepatitis C RNA replication. Main target for some of the new anti-viral agents, such as Sofosbuvir; Sustain virological response: Patients with undetectable hepatitis C viral load 12 wk after completing hepatitis C therapy.

Peer-review

The manuscript is well presented and of interest and the results can contribute to increase the knowledge of this topic.

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ORIGINAL ARTICLE

Retrospective Study

Is cirrhosis associated with lower odds of ischemic stroke: A nationwide analysis?

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Abstract

AIM

To determine the association between cirrhosis and ischemic stroke in a large nationally representative sample.

METHODS

A retrospective cross-sectional study of all hospitalized patients during 2012 and 2013 in the United States was performed using the National Inpatient Sample database. Hospitalizations with acute stroke, cirrhosis and other risk factors were identified using ICD-9-CM codes.

RESULTS

There were a total of 72082638 hospitalizations in the United States during the years 2012 and 2013. After excluding hospitalizations with missing demographic variables, that there were a total of 1175210 (1.6%) out of these were for acute ischemic stroke. Cirrhosis was present among 5605 (0.4%) cases of ischemic stroke. Mean age among the cirrhotic and non-cirrhotic groups with ischemic stroke were 66.4 and 70.5 years, respectively. Prevalence of risk factors among the two groups was also calculated. After adjusting for various known risk factors the odds of having an ischemic stroke (OR = 0.28, $P < 0.001$) were 72% lower in cirrhotics compared to non-cirrhotics.

CONCLUSION

Our study suggests that in a large, nationally representative sample of the United States population, cirrhosis

is associated with a lower likelihood of stroke.

Key words: Cirrhosis; Ischemic stroke; Cerebrovascular accident; National Inpatient Sample

Core tip: Our study demonstrates that in a large, nationally representative sample, cirrhosis is associated with a lower likelihood of having an ischemic stroke, after adjusting for known risk factors. Although the odds of having a stroke are lower in cirrhotics, the mortality is significantly higher in them compared to non-cirrhotics.

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INTRODUCTION

Cirrhosis is among the top ten leading causes of death in the United States[1,2]. With recent advances in the management of various complications of cirrhosis, it has become one of the most prevalent chronic conditions, that patients live with for a considerable duration of time[3]. For instance, there were 633323 patients living with cirrhosis in 2010[4].

Due to altered homeostasis and hemodynamics in cirrhosis it is reasonable to assume that the risk of an ischemic cerebrovascular event [acute ischemic stroke (AIS)] in cirrhotics would be different from that of the general population[5-8]. The question whether cirrhosis is associated with a reduced risk of stroke has been a source of controversy for a long time. There have been various studies reporting increased incidence of carotid plaques and atherosclerosis in patients with advanced liver disease, both known risk factors for ischemic stroke[9-11]. On the other hand, it is also well known that liver disease causes thrombocytopenia and coagulopathy which should in turn be protective against an ischemic cerebrovascular accident (CVA)[12]. Recently, Chen *et al*[12] and Berzigotti *et al*[13] showed that patients with liver cirrhosis may be at a lower risk of experiencing an ischemic CVA[12-14]. However, due to predominance of one ethnic group in the former and the relatively small sample size in the latter, the impact of cirrhosis on risk of stroke still remains inconclusive.

We therefore aim to define the impact of cirrhosis and extent of its association with ischemic stroke by using the largest national database for hospitalized patients in the United States.

MATERIALS AND METHODS

Data source

The National Inpatient Sample (NIS) formerly known as Nationwide Inpatient Sample database is an administrative database developed by the Agency of Healthcare Research and Quality for Healthcare Cost and Utilization Project (HCUP). It is the largest all-payer database of hospitalized patients in the United States. NIS is a 20% stratified sample of all discharges from United States community hospitals[15]. Thus, manufacturer provided sampling weights were used to produce national estimates. We used NIS databases for the years 2012 and 2013 in this study. The NIS database provides de-identified information regarding the demographic characteristics (age, gender, race), mortality, principal and secondary diagnoses, *etc*., for each hospitalization. It however does not contain any lab values, imaging or other advanced diagnostic information.

Study design

This is a retrospective cross-sectional study using a national inpatient database. We used International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) codes 571.2 (Alcoholic cirrhosis of liver), 571.5 (biliary cirrhosis) and 571.6 (cirrhosis without mention of alcohol) to identify the patients with cirrhosis[3,16]. ICD-9-CM codes 433.x1, 434.x1, 435 and 436 listed as principal diagnoses were used to identify hospitalizations for acute ischemic cerebrovascular events. These ICD-9-CM codes with modifiers have been previously validated and used to identify AIS in administrative databases with good accuracy[17-20]. All the patients with missing age, gender or race information were excluded. The patients with missing age and gender information constituted < 1% of the included population. The hospitalization with missing race were more prevalent, however they were due to non-participation of some states in reporting ethnic information and thus did not result in underrepresentation of any particular ethnic group. The basic demographic characteristics for different sub-groups have been described in Table 1. We used ICD-9-CM codes to identify the known risk factors for ischemic stroke[21-25]. Prevalence of these risk factors was also calculated for different subgroups (Table 1). Since, this study was conducted using a de-identified commercially available database Institutional Review Board approval was not required.

Statistical analysis

Stata 13.1 (Stata Corp, College Station TX) and SPSS 23.0 (SPSS Inc., Chicago, Ill) were used for statistical analysis. National estimates were produced by using the sampling weights provided by HCUP. χ^2 test and Independent-samples *t*-test for means were used to determine statistical significance of differences in the prevalence of risk factors and demographic variables among the two groups. Due to the binary nature of the outcome/dependent variable, *i.e.*, presence of ischemic stroke, multivariate logistic regression model was used to assess the association between cirrhosis and ischemic stroke while controlling for known risk factors (as listed in Table 1) of ischemic stroke. Wald's

Table 1 Demographic characteristics and prevalence of risk factors in patients with ischemic stroke among cirrhotic and non-cirrhotic groups

	Cirrhotic	Non-cirrhotic	*P* value
Mean age (SD)	66.4 (11.9)	70.5 (14.3)	< 0.001
Age categories			
Age < 40	0.8	2.3	< 0.001
Age 40-64	46.6	30.6	
Age > 65	52.6	67.1	
Gender			
Male	55.1	46.5	< 0.001
Female	44.9	53.5	
Race			
Caucasian	66.6	70.8	< 0.001
African-American	14.9	15.9	
Other races	18.5	13.3	
Hospital characteristics			
Teaching status			
Teaching	51.3	48.3	0.13
Non-teaching	48.7	51.7	
Location			
Rural	9.3	10.9	0.04
Urban	90.7	89.1	
Bed size			
Small	10.3	12	0.46
Medium	26.8	26.7	
Large	62.9	61.3	
Risk factors			
Hypertension	52.2	62.5	< 0.001
Diabetes	45.1	37.8	< 0.001
Tobacco use	37.2	28.8	< 0.001
CHF	18.9	13.1	< 0.001
Personal history of stroke	13.1	14.8	< 0.001
CAD	25.3	27.7	< 0.001
Peripheral artery disease	8.7	6.9	< 0.001
Atrial fibrillation	23.8	21.9	0.001
Anticoagulation use	4.4	6.8	< 0.001
Dyslipidemia	34.4	56.5	< 0.001
Alcohol abuse	28.8	3.8	< 0.001
Family history of stroke	1.5	2.3	< 0.001

The numbers in each cell represent percentage of patients with particular characteristics/risk factor in each group. SD: Standard deviation; CAD: Coronary artery disease; CHF: Congestive heart failure.

test was used to determine statistical significance of association between the factors used in the regression model and the outcome, *i.e.*, stroke. *P*-value less than 0.05 was considered statistically significant. The coefficient obtained as a result of the regression model was converted to OR for ease of understanding and is being reported here along with 95%CI. Since the prevalence of our outcome (Stroke) was less than 10%, the OR provides a good estimate of the relative risk[26]. The biostatistical methods and tests used in this study were reviewed by a biomedical statistician.

RESULTS

There were a total of 72082638 hospitalizations in the United States during the year 2012 and 2013. After excluding hospitalizations with missing demographic variables (age, gender, and race), a total of 1175210 (1.6%) hospitalizations were for AIS. Out of these, 5605 (0.4%) were identified to have co-existing cirrhosis of liver. Decompensated cirrhosis which was defined by presence of variceal hemorrhage, ascites, spontaneous bacterial peritonitis or hepatic encephalopathy, constituted 14.3% of the cirrhotic group[27]. Mean age among cirrhotic and non-cirrhotic patients were 66.4 (SD = 11.9) and 70.5 (SD = 14.3) years, respectively. Proportion of males among the two groups was 55.1% and 46.5% respectively. The racial distribution was similar among the two groups with 66.6% and 70.8% Caucasians; and 14.9% and 15.9% African-Americans in cirrhotic and non-cirrhotics respectively. The prevalence of some risk factors (Figure 1) for AIS like - diabetes (45.1% *vs* 37.8%), hypertension (52.2% *vs* 62.5%), congestive heart failure (18.9% *vs* 13.1%), smoking (37.2% *vs* 28.8%), atrial fibrillation (23.8% *vs* 21.9%), alcohol use (28.8% *vs* 3.8%) and peripheral vascular disease (8.7% *vs* 6.9%), were all higher among cirrhotics compared to non-cirrhotics (Table 1). Whereas, others like Coronary atherosclerosis (25.3% *vs* 27.7%), previous history of stroke (13.1% *vs* 14.8%), hypercholesterolemia (34.4% *vs* 56.5%) were found to be higher among the non-cirrhotic group (Table 1). Anticoagulation use, which is considered to be associated with a lower risk of ischemic stroke was more prevalent among the non-cirrhotics at 6.8% compared to 4.4% in cirrhotics.

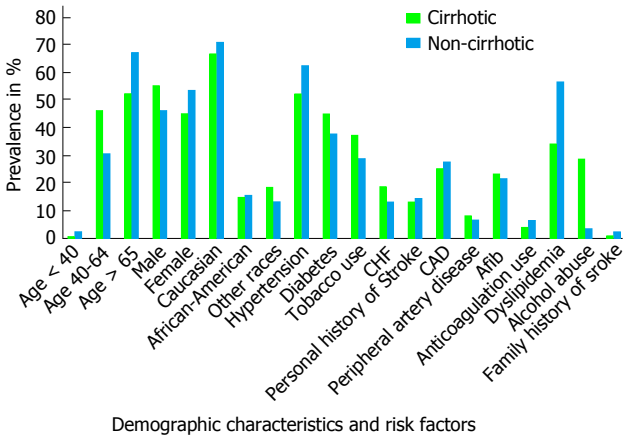

Figure 1 Distribution of demographic characteristics and risk factors in cirrhotic and non-cirrhotic groups with ischemic stroke. CAD: Coronary artery disease; CHF: Congestive heart failure; Afib: Atrial fibrillation.

The overall *P*-value of the logistical regression model was statistically significant at < 0.001. The odds of having an AIS for patients with cirrhosis were 72% lower than patients without cirrhosis (OR = 0.28, 95%CI: 0.26 to 0.29) and was statistically significant with a *P*-value < 0.001. The all cause in-hospital mortality among the cirrhotic group (5%) with AIS was significantly higher than non-cirrhotic group (3.3%) (*P* < 0.001). Even after adjusting (using logistic regression) for various co-morbidities using Charlson comorbidity index (modified to exclude liver disease)[28,29], patient demographics, and hospital characteristics; the mortality remained higher among cirrhotics with stroke (OR = 1.6, 95%CI: 1.22-2.10, *P* = 0.001).

DISCUSSION

The impact of cirrhosis on stroke has been controversial

for a long time. Our study demonstrates that the odds of having an AIS for cirrhotics are significantly lower (72%) than non-cirrhotics. This is consistent with some of the other smaller non-United States based studies done previously. The magnitude of association however, is different[12,13]. The study by Chen *et al*[12] showed that risk of having an AIS was lower in non-alcoholic cirrhosis. But, it was conducted in Taiwan, and has limited generalizability due to predominance of only one kind of ethnic population. Since, ethnicity is itself an independent risk factor of AIS, our results have a more generalized applicability. Our study also had a much larger sample size and adjusted for the most number of risk factors of stroke in any study so far. The reduced likelihood of AIS in patients with cirrhosis represents a very important clinical finding. It may aid a clinician in determining the optimal management of often complicated cirrhotic patients with co-morbidities, that put them at a higher than usual risk of AIS, such as atrial fibrillation. The mechanism of this "protective effect of cirrhosis" is unclear but could be related to the underlying coagulopathy, thrombocytopenia or the altered hemodynamic flow patterns[12,13]. This study demonstrates the need for a prospective study to further explore this "protective effect of cirrhosis" on AIS.

Our study is to date the largest of its kind, and likely represents the true association between cirrhosis and strokes after adjusting for several known risk factors. Despite patients with cirrhosis being less likely to have a stroke, the mortality was significantly higher in them compared to non-cirrhotics. This is likely due to complications arising from the cirrhosis which may interfere with the usual management of stroke, for example, the coagulopathy due to cirrhosis may pose problems for planned interventions, if needed.

Our study findings, although important, need to be interpreted in light of some limitations. Firstly, NIS being an administrative database is not free from coding errors, especially related to liver diseases and acute strokes. However, we have used either the previously validated or commonly used codes for cirrhosis and AIS which have been shown to have good accuracy[3,30-32]. Secondly, the OR provides a close estimate of Relative Risk due to relatively low prevalence of Stroke in our population, but its not a replacement for the true relative risk which can only be obtained from a prospective cohort study.

Our study demonstrates that in a large, nationally representative sample, cirrhosis is associated with a lower likelihood of having an ischemic stroke, after adjusting for known risk factors. Although the odds of having a stroke are lower in cirrhotics, the mortality is significantly higher in them compared to non-cirrhotics. Prospective studies are needed to establish the causal relationship and better define this association in future.

COMMENTS

Background

Cirrhosis is one of the leading causes of morbidity and mortality in the United States. Cirrhotic patients usually suffer from coagulopathy while simultaneously being at an increased risk of deep venous thrombosis. These problems along with the usually encountered thrombocytopenia imply that the risk of an ischemic cerebrovascular accident (CVA) in a cirrhotic would be different from that of general population. This impact of cirrhosis on the risk of ischemic cerebrovascular events (ischemic stroke) has been controversial.

Research frontiers

The relationship between cirrhosis and ischemic CVA has not been studied in detail.

Innovations and breakthroughs

The study is the first study with such a large sample size that controls for so many known risk factors of stroke to explore the true relationship between ischedmic stroke and cirrhosis.

Applications

The reduced likelihood of acute ischemic stroke (AIS) in patients with cirrhosis represents a very important clinical finding. It may aid a clinician in determining the optimal management of often complicated cirrhotic patients with co-morbidities, that put them at a higher than usual risk of AIS, such as atrial fibrillation (Afib).

Terminology

Charlson comorbidity index is a tool to adjust for the impact of co-morbidites developed for use with administrative databases utilizing ICD-9 codes.

Peer-review

This paper is described in detail, which, as valuable information, could help the readers that have better understand the first-hand knowledge of this topic to start novel studies.

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ORIGINAL ARTICLE

Prospective Study

Immune function biomarker QuantiFERON-monitor is associated with infection risk in cirrhotic patients

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Abstract

AIM

To investigate whether a novel immune function biomarker QuantiFERON-Monitor (QFM) can identify cirrhotic patients at greatest risk of infection.

METHODS

Adult cirrhotic patients on the liver transplant waiting list were recruited for this observational cohort study from a tertiary liver transplant referral unit. The immune function biomarker, QFM was performed using the same method as the widely available Quantiferon-gold assay, and measures output in interferon gamma in IU/mL after dual stimulation of the innate and adaptive immune systems. Ninety-one cirrhotic patients were recruited, with 47 (52%) transplanted on the day of their QFM. The remaining 44 (48%) were monitored for infections until transplant, death, or census date of 1st February 2014.

RESULTS

Cirrhotic patients express a median QFM significantly lower than healthy controls (94.5 IU/mL *vs* 423 IU/mL), demonstrating that they are severely immunosuppressed.

Several factors including model for end stage liver disease, presence of hepatocellular carcinoma, bilirubin, international normalized ratio and haemoglobin were associated with QFM on univariate analysis. Disease aetiology did not appear to impact QFM. On multivariate analysis, only Child-Pugh score and urea were significantly associated with a patient's immune function as objectively measured by QFM. In the 44 patients who were not transplanted immediately after their blood test and could be monitored for subsequent infection risk, 13 (29.5%) experienced a pre-transplant infection a median 20 d (range 2-182) post-test. QFM < 214 IU/mL was associated with HR = 4.1 (P = 0.01) for infection. A very low QFM < 30 IU/mL was significantly associated (P = 0.003) with death in three patients who died while awaiting transplantation (HR = 56.6).

CONCLUSION

QFM is lower in cirrhotics, allowing objective determinations of an individual's unique level of immune dysfunction. Low QFM was associated with increased susceptibility to infection.

Key words: Infection; Biomarker; Immune dysfunction; Immune function; Immunosuppression; Liver; Immune system; Cirrhosis; Mortality

Core tip: QuantiFERON-Monitor (QFM) is a net immune function biomarker that measures interferon-γ after stimulation of the innate and adaptive immune systems and is based on a readily available pathology platform. Measuring QFM in cirrhotic patients provides an objective marker of their immune dysfunction, which has otherwise been difficult to quantify. Low QFM is significantly associated with the risk of pre-transplant infection, and very low QFM may be associated with increased risk of mortality.

Sood S, Yu L, Visvanathan K, Angus PW, Gow PJ, Testro AG. Immune function biomarker QuantiFERON-monitor is associated with infection risk in cirrhotic patients. *World J Hepatol* 2016; 8(35): 1569-1575 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i35/1569.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i35.1569

INTRODUCTION

QuantiFERON-Monitor (QFM, Qiagen Ltd, United States) was developed as an immune function monitoring tool in the post-transplant setting and provides a general biomarker of immune function based on stimulation of both the innate and adaptive immune systems[1]. It was developed based on the same diagnostic platform as the widely available QuantiFERON-Gold assay (QFN-gold, Qiagen Ltd, United States) and requires minimal laboratory processing. A high QFM result suggests a robust immune response, whilst a low result implies impaired immunity. Initial pilot data showed low QFM compared with age-sex matched controls not just in patients on immunosuppression post-transplant, but also in cirrhotic patients on the waiting list prior to transplant[1].

Patients with decompensated cirrhosis have inherently impaired immune responses, with bacterial infections occurring in 20%-60% of patients hospitalized for cirrhosis[2] and responsible for up to 25% of deaths in patients with liver disease[3]. The immune dysfunction in cirrhosis involves impairments of both quantity and quality of many immune cells that have been individually studied but are not always appreciated in clinical care.

In this study we present data that represents the first well described clinical cohort of patients to be evaluated with the QFM assay. We describe their immune function and investigate whether low QFM is associated with infection risk in this prospective cohort of pre-transplant cirrhotic patients.

MATERIALS AND METHODS

We performed a prospective observational cohort study on 91 patients with cirrhosis awaiting liver transplantation at a single centre. Patients were recruited between November 2011 to December 2013 and followed until the census date of 1st February 2014. Approximately half the patients had blood taken immediately prior to their transplant surgery, while the remainder had a period of time in between their blood test and transplantation, death or the census date.

The QFM assay was performed on 1 mL of whole blood. As per manufacturer's guidelines, blood was stimulated with the QFM immune ligands anti-CD3 and R848 in the form of a single lyophilized ball within 8 h of being taken. Stimulated blood was incubated overnight at 37 ℃. Following incubation, the blood underwent centrifugation and plasma harvested. An enzyme-linked immunosorbent assay (ELISA) was performed by a separate investigator who was blinded to clinical data. Clinicians caring for the cirrhotic patients were blinded from the QFM assay results. QFM output was measured as interferon-γ (IFN-γ) production measured as IU/mL, in a process similar to that applied to perform a QFN-gold assay: Samples were brought to room temperature and given 60 min to equilibrate. The lyophilized IFN-γ standard was reconstituted with deionized water. This was gently mixed to minimize frothing and ensure complete solubilisation. Dilutions were prepared to validate the standard curve.

The lyophilized conjugate was reconstituted with 0.3 mL of deionized water and mixed gently to minimize frothing and ensure complete solubilisation. Further dilutions were performed with addition of Green Diluent. Fifty microliters of prepared conjugate was added to each ELISA well, after which 50 μL of each sample were added. Plates were covered and mixed using a microplate shaker for 1 min and then incubated at room

Table 1 Baseline characteristics of cirrhotic patients

	Demographics	Median QFM (95%CI) IU/mL
Age (median, yr)	54 (20-72)	94.5 (37.3-158)
Male	62 (68.1%)	124.5 (37.3-223)
Female	29 (31.9%)	73.9 (7.50-158)
Child-Pugh score		
A	7	381 (12.9-1234)
B	29	224 (94.4-506)
C	55	37.3 (19.5-128)
MELD score		
0-10	10	319 (12.9-904)
11-20	42	155.5 (94.5-240)
21-30	34	30.0 (9.16-157)
⩾ 30	5	8.81 (0.63-47.6)
Primary aetiology of cirrhosis, *n* (%)		
HCV	39 (42.9)	130 (47.6-223)
PSC	10 (11.0)	61.6 (1.19-279)
ETOH	10 (11.0)	113.3 (8.81-385)
NASH	9 (9.89)	20.3 (6.20-375)
AIH	5 (5.49)	37.3 (0.04-137)
PBC	4 (4.40)	93.0 (24.1-168)
HBV	3 (3.30)	904 (799-1132)
Retransplant	3 (3.30)	163 (2.06-318)
Other	8 (8.79)	6.59 (0.07-774)
HCC	31 (34.1)	194 (87.9-425)
No HCC	61 (65.9)	73.9 (28.0-154)

QFM: QuantiFERON-Monitor; HCV: Hepatitis C virus; PSC: Primary sclerosing cholangitis; ETOH: Alcohol; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

temperature for 120 min ± 5 min.

Wells were then washed with 400 μL of working strength wash buffer for at least 6 cycles in a microplate washer. Plates were tapped, while facing down on absorbent, low-lint towels to remove residual wash buffer. One hundred microliters of enzyme substrate solution was then added to each well, and plates covered with a lid. These were mixed using a microplate shaker, and then incubated at room temperature for a further 30 min.

Following this further incubation, 50 μL of enzyme stopping solution was added to each well and mixed thoroughly with the microplate shaker. The optical density was then measured within 5 min of stopping the reaction using a microplate reader fitted with a 450 nm filter, as well as a 620 nm-650 nm reference filter. The optical density values were used to calculate the output result of IFN-γ in IU/mL. Low QFM was suggestive of an immunosuppressed state.

Basic clinical data was collected from participants who were recruited as part of a post-transplant research trial. Collected data included age, gender, disease aetiology and blood biochemistry. Patients were also evaluated for commonly used scoring systems for the severity of liver disease, the Child-Pugh Score and model for end stage liver disease (MELD) score. Patients were monitored prospectively for infection occurring after their QFM sample and up to either liver transplant, infection, death or the census date. Infections were per pre-defined criteria of "probable" or "definite" infection adjusted from The International Sepsis Forum Consensus Conference on Definitions of Infection in the Intensive Care Unit[4]. All patients were admitted to hospital for intravenous antimicrobial treatment.

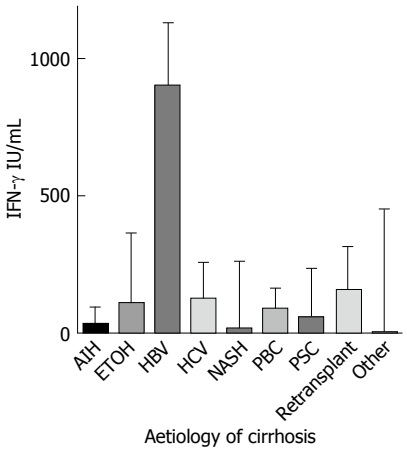

Figure 1 Median QuantiFERON (± IQR) by aetiology of liver disease. IFN-γ: Interferon-γ; AIH: Autoimmune hepatitis; ETOH: Alcohol; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

Logistic regression, Mann-Whitney *U* test and Kaplan-Meier survival curves were analyzed with GraphPad Prism 6.0 for Mac (IBM, United States). All variables that showed potential predictive capacity of 15% ($P < 0.15$) were entered into a multivariate logistic regression mode using STATA/SE version 12.0 for Mac (Statacorp, United States). *P* values under 0.05 were considered significant. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the appropriate institutional review committee. All patients provided written informed consent.

RESULTS

Ninety-one cirrhotic patients were recruited (Table 1). The majority ($n = 62$, 68.1%) were male. The mean age was 51 years (median 54, range 20-72 years). The most common aetiology of liver disease was hepatitis C virus infection (HCV, 43%), followed by Primary Sclerosing Cholangitis (PSC, 11%), alcoholic liver disease (ETOH, 11%), and non-alcoholic steatohepatitis (NASH, 10%) (Figure 1). ETOH was a significant co-factor in 14/24 (58.3%) of patients with HCV. QFM level did not vary significantly by aetiology ($P = 0.08$).

The mean QFM in cirrhotics was 214.3 IU/mL, median 94.5 IU/mL compared to a historical cohort of healthy controls (mean 555.2 IU/mL, median 423 IU/mL)[1]. There was no significant difference between QFM in males and females ($P = 0.11$). Of the patient group as a whole, the median MELD was 20 and Child-Pugh Score was 10. Hepatocellular carcinoma (HCC) was present in

Table 2 Univariate analysis of QuantiFERON-Monitor in cirrhotic patients

	Coefficient	*P* vaule	95%CI
MELD score	-17.3	< 0.001	-25.3:-9.29
Child-Pugh score	-65.6	< 0.001	-91.1:-40.2
Alcohol	-69.3	0.285	-197.4:58.7
HCC	193.9	0.002	71.6:316.1
Age	3.18	0.252	-2.30:8.66
WCC	-9.4	0.351	-29.2:10.5
Neutrophils	-19.0	0.137	-44.1:6.17
HCV	-44.4	0.476	-168.0:79.1
Male	70.1	0.288	-60.1:200.4
Bilirubin	-0.59	0.001	-0.95:-0.24
Urea	-14.5	0.023	-26.9:-2.00
Creatinine	0.17	0.702	-0.71:1.06
Haemoglobin	5.04	< 0.001	2.48:7.59
Platelets	0.59	0.164	-0.24:1.42
Albumin	9.50	0.055	-0.21:19.2
INR	-230.6	< 0.001	-342:-119

HCC: Hepatocellular carcinoma; WCC: White cell count; HCV: Hepatitis C virus; MELD: Model for end stage liver disease; INR: International normalized ratio.

31 patients (34.1%) and associated with a lower median MELD compared with non-HCC patients (15 *vs* 20, $P < 0.0001$). Accordingly, HCC patients who expressed a more robust immune response with a median QFM more than double that of non-HCC patients (194 IU/mL *vs* 73.9 IU/mL, $P = 0.03$).

Several other factors were associated with QFM on univariate analysis. Along with presence of HCC, haemoglobin level was positively associated with QFM. Alternatively, an inverse association was found with advancing MELD score, Child-Pugh score, urea and international normalized ratio (Table 2). On a multivariate regression model, only Child-Pugh score and urea were independently associated with QFM levels in cirrhotic patients (Table 3).

Predicting pre-transplant infection

Of the 91 cirrhotic patients, approximately half (n = 47, 51.6%) were transplanted on the day of their QFM measurement. The remaining 44 (48.4%) had the QFM assay performed a median 46 d (range 2-591) prior to the date of censor. This sub-group were further investigated for rates of infection prior to transplantation. Most were receiving antibiotic prophylaxis (34/44, 77.3%).

At the census date, 33 patients (75%) had been transplanted, 3 patients had died (6.8%) and 8 (18.2%) were still awaiting transplantation. Advanced MELD (r^2 = 0.27, $P = 0.002$) and Child-Pugh score (r^2 = 0.15, P = 0.03) were associated with shorter time to transplant, while QFM was not (r^2 = 0.01, P = 0.64).

Thirteen of 44 patients (29.5%) experienced a pre-transplant infection at a median of 20 d (range 2-182) after their pre-transplant blood test. Three patients had spontaneous bacterial peritonitis (SBP), 4 pneumonia, 3 bacteraemia, 1 fungaemia, 1 urinary tract infection and 1 cholangitis. Most patients (n = 9, 69%) who experienced an infection had Child-Pugh C cirrhosis but Child-Pugh score was not associated with risk of infection (Figure 2A, $P = 0.2$), whereas MELD score ($\geqslant$ 20) was (Figure 2B; HR = 4.7, $P = 0.01$). Urea above the laboratory reference range of 6.7 mmol/L was not associated with infection risk ($P = 0.15$).

Table 3 Multivariate regression analysis

	Coefficent	*P* vaule	95%CI
Child-Pugh score	-51.9	0.013	-92.6:-11.3
MELD score	16.0	0.131	-4.88:36.9
HCC	62.9	0.366	-74.8:201
Bilirubin	-0.39	0.131	-0.91:0.120
Urea	-14.3	0.046	-28.3:-0.261
Haemoglobin	1.77	0.250	-1.27:4.82
Albumin	7.15	0.141	-2.43:16.7
INR	-114	0.168	-278:49.3

HCC: Hepatocellular carcinoma; MELD: Model for end stage liver disease; INR: International normalized ratio.

A QFM under the cohort mean of 214 IU/mL was significantly associated with infection pre-transplant (HR = 4.1, Figure 2C, $P = 0.01$) and the combined outcome of infection or death on the waiting list (Figure 2D, HR = 4.4, $P = 0.006$).

Three patients died in this cohort while awaiting transplantation, two from bleeding (one intracranial, one variceal) and one from sepsis and multiorgan failure. The median MELD of these patients was 24 and Child-Pugh score of 12. Patients who died pre-transplant had a significantly lower QFM (AUROC 0.88, $P = 0.03$), and on survival analysis, a very low QFM (< 30 IU/mL) was most associated with a HR of 56.6 for death (Figure 2E, $P = 0.003$).

DISCUSSION

Infections are implicated in up to 25% of deaths of patients with cirrhosis[3], and are the second leading cause of death in patients with end-stage liver disease awaiting liver transplantation[5,6]. Immune dysfunction in cirrhosis is likely multifactorial, with impaired function identified in neutrophils[7-10], monocytes[11] and lymphocytes[12]. Many of which also show impaired numbers, partly as a result of portal hypertension and splenic sequestration. Advanced cirrhosis is also associated with deficiencies in both structure and function of the reticuloendothelial system[13,14], complement production[15], and a chronic immune activation that appears to result in a systemic immune paralysis[16-20]. Although each individual aspect of immune deficiency has been studied in isolation, estimating a patient's overall level of immune function has been unattainable.

QFM was designed as a net immune function biomarker to manage immunosuppression in the post-transplant setting. Unlike other immune function assays that are predominantly confined to research settings, it has potential clinical utility as it is based on QFN-gold, an assay already in widespread use, and requires

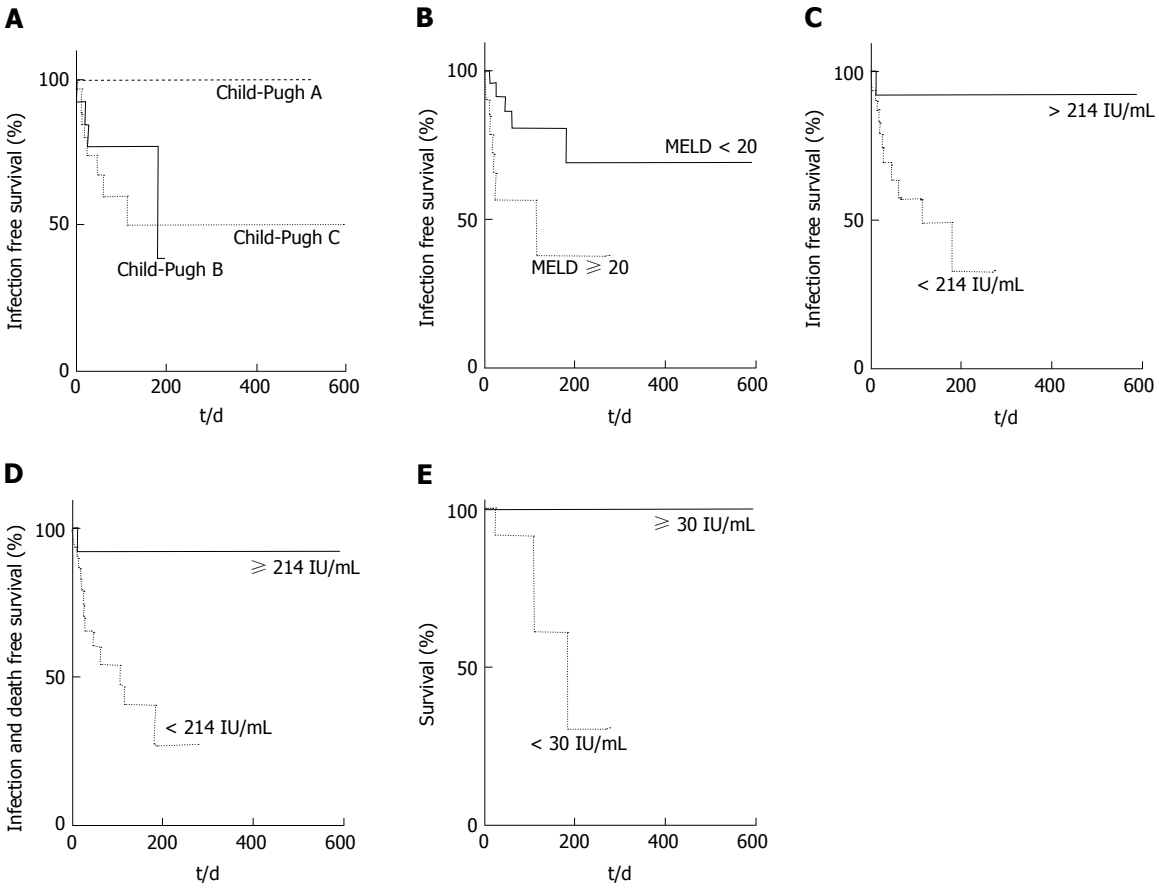

Figure 2 Pretransplant survival based on very low QuantiFERON. A: Infection free survival by Child-Pugh; B: Infection free survival by MELD; C: Infection free survival by QFM; D: Infection and mortality free survival by QFM; E: Pretransplant survival based on very low QFM. QFM: QuantiFERON-Monitor; MELD: Model for end stage liver disease.

only minimal laboratory processing. QFM incorporates both an innate and adaptive stimulant which offers an objective, albeit non-specific overview of a patient's individual immune response. A perhaps not unexpected finding of the original pilot study was that low QFM was identified in patients awaiting, and not only after liver transplantation[1]. In this study, we confirm and are able to quantify the immunosuppressed status of cirrhotic patients, with a median QFM of 94.5 IU/mL less than 25% that of healthy controls (423 IU/mL)[1]. Most importantly, we not only demonstrate a low QFM in cirrhotic patients (indicative of inherent immunosuppression), but that the most severe immune dysfunction is associated with heightened infection risk. Low QFM in cirrhotic patients had a HR of 4.1 for pre-transplant infection risk. A simple blood test that could highlight a patient's individual risk of subsequent infection would be of value to treating clinicians.

There are some limitations when performing a study in a transplant-waitlisted population. Firstly, with the sickest patients (based on MELD) often receiving priority organ selection, there was risk of patients with greatest risk of infection (and lowest QFM) being transplanted earlier. This risks a type Ⅱ error, which potentially underestimates the clinical value of the assay in predicting infections; Secondly, we may have underestimated the infection rate as diagnosing infections in patients with cirrhosis can be difficult, and empirical antibiotics are often used on presentation to hospital with conditions such as variceal bleeding or hepatic encephalopathy; and thirdly, since this data represents the first clinical cohort of non-transplant recipients evaluated with the QFM assay, readily defined set-points for low and very low QFM have not previously been evaluated or described.

Conversely, studying a transplant wait-listed population does offer some advantages since transplant listed patients are more unwell and at greatest susceptibility to infections (reducing the potential sample size and necessary follow-up period). They are closely monitored, with all events being reported to the transplant centre even if occurring at peripheral hospitals, thus allowing all clinical events to be documented.

Early identification and treatment of infections is essential in the management of cirrhotic patients, particularly given the morbidity and mortality often attributed to infections in this vulnerable population. However, infections can be difficult to distinguish from other non-infectious causes of systemic inflammatory response syndrome and symptoms of liver deterioration[21]. Serum biomarkers are therefore being examined, although currently available tests such as C-reactive protein, ferritin and white blood cells lack specificity[21].

To prevent infections, some cirrhotic patients are offered antibiotic prophylaxis, although mainly they have been used to prevent episodes of SBP. SBP has a recurrence rate of 70% within 12 mo[22], and secondary prophylaxis is a part of internationally accepted guidelines[23]. Primary antibiotic prophylaxis has also been recommended in patients with low protein in ascitic fluid as there is an understanding that it improves incidence of infections and short-term survival[24,25]. However, adherence to these guidelines is low, and in part may be due to fears over antimicrobial resistance and reduced effectiveness over time[26,27]. An objective immune function biomarker that could highlight patients with the most severe immune-deficiency could enable the use of more targeted antibiotic prophylaxis to those most at risk of all infections, and not just SBP.

There was no significant difference in QFM based on aetiology of the underlying liver disease. Patients with hepatocellular carcinoma as their primary indication for transplant were often not as unwell as other cirrhotic patients, and therefore had significantly higher QFM results on univariate analysis. This was verified in multivariate analysis where HCC was not independently associated with high QFM. Only Child-Pugh score and urea were individually identified on a logistic multivariate regression model as being associated with low QFM. This suggests that commonly used disease scoring systems such as MELD or biochemical investigations such as white cell count are not truly indicative of a patient's underlying level of immune dysfunction and further highlights the possible value of an objective immune function biomarker. The significance of urea with QFM is interesting and would need further study. It could be a surrogate marker of renal function which is known to impact mortality in cirrhosis, although interestingly creatinine had no relationship to QFM. An alternate hypothesis may be that the urea level reflects an increased catabolic state associated with nutritional deficiencies that may impact immune function.

A very low QFM was significantly associated with pre-transplant mortality in this cohort. Although two patients subsequently died of bleeding rather than sepsis, this may highlight the severely immunosuppressed state of patients with critical illness, and offer QFM as an alternate overall prognostic marker. However, despite reaching statistical significance, it is difficult with low numbers to make any firm conclusions regarding QFM and mortality risk. Further studies in a non-transplant wait-listed cirrhotic population would be needed to further explore and confirm this association.

In conclusion, patients with cirrhosis are at high risk of infection, but quantification of immune dysfunction has been difficult in clinical practice. Immune functional assays are often isolated to one small component of immunity, associated with significant laboratory processing or confined to limited situations and medical research. This study describes the first clinical assessment of the QFM immune function assay in patients not receiving immunosuppressant medications. We show that patients with cirrhosis are not only significantly immunosuppressed, but that a low level of QFM (suggestive of significant immune dysfunction) is associated with a four-fold increased risk for infection. The ability to employ a clinical assay that can objectively provide a biomarker of an individual's innate and adaptive immune function offers obvious benefits to patient care, even outside the transplantation setting. This study also serves as a proof of concept that immune function monitoring may be available and have clinical utility in other fields of medicine where patients are either inherently or iatrogenically immunosuppressed.

COMMENTS

Background

Patients with cirrhosis are known to be immunosuppressed and infections are a significant cause of morbidity and mortality. Predicting patients at greatest risk of infection is difficult. The QuantiFERON-Monitor assay (QFM) is a novel immune function biomarker designed to assess immune function in a transplant setting. In this first study to employ QFM in a non-transplant setting, the authors aim to identify whether QFM can objectively measure a patient's immune dysfunction and whether this correlates with the risk of infection.

Research frontiers

Identifying cirrhotic patients at greatest risk of infection is difficult. Usual biochemical measures such as C-reactive protein and white cell count are not associated with infection in cirrhotic patients.

Innovations and breakthroughs

QFM is a novel immune function biomarker that provides an objective measure of immune function. In particular, it benefits from measuring interferon gamma production after stimulation of both arms of the immune system (innate and adaptive). In this study, the assay is shown to objectively measure immune dysfunction in cirrhotic patients, and that the patients with lower values had the greatest risk of infection.

Applications

QFM may have utility in measuring the level of immune function in patients with cirrhosis. This could then identify patients at greatest risk of infection, and who may benefit from either earlier transplantation or antibiotic prophylaxis. Furthermore, the assay may be useful in other medical conditions where patients are either inherently or iatrogenically immunosuppressed.

Peer-review

This is an questionable topic. First of all, the material method section should be described in a detailed manner. More aspects shous be enlightened. Moreover, discussion part should be enlarged properly and more recent studies should be mentioned and more recent studies should be added to references part.

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SYSTEMATIC REVIEWS

Intrahepatic pancreatic pseudocyst: A review of the world literature

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Abstract

AIM

To investigate and summarize the literature regarding the diagnosis and management of intrahepatic pancreatic pseudocysts (IHPP).

METHODS

A literature search was performed using PubMed (MEDLINE) and Google Scholar databases, followed by a manual review of reference lists to ensure that no articles were missed. All articles, case reports, systematic reviews, letters to editors, and abstracts were analyzed and tabulated. Bivariate analyses were performed, with significance accepted at $P < 0.05$. Articles included were primarily in the English language, and articles in other languages were reviewed with native speakers or, if none available, were translated with electronic software when possible.

RESULTS

We found 41 published articles describing 54 cases since the 1970s, with a fairly steady rate of publication. Patients were predominantly male, with a mean age of 49 years. In 42% of published cases, the IHPP was the only reported pseudocyst, but 58% also had concurrent pseudocysts in other extrapancreatic locations. Average IHPP size was 9.5 cm and they occurred most commonly (48%) in the left hemiliver. Nearly every reported case was managed with an intervention, most with a single intervention, but some required up to three interventions. Percutaneous treatment with either simple aspiration or with an indwelling drain were the most common interventions, frequently performed along with stenting of the pancreatic duct. The size of the IHPP correlated significantly with both the duration of treatment ($P = 0.006$) and with the number of interventions required ($P = 0.031$). The duration of therapy also correlated with the initial white blood cell (WBC) count ($P = 0.048$).

CONCLUSION
Diagnosis of IHPP is difficult and often missed. Initial size and WBC are predictive of the treatment required. With appropriate intervention, most patients achieve resolution.

Key words: Pseudocyst; Intrahepatic; Percutaneous; Pancreatic; Drainage

Core tip: Intrahepatic pancreatic pseudocysts (IHPPs) are rare and the pathophysiology is not entirely clear, but they likely result from proteolytic pancreatic fluid tracking from the pancreas into the surrounding tissue. This fluid may then migrate along planes such as the hepatogastric or hepatoduodenal ligaments, to penetrate the hepatic parenchyma. The initial size of the IHPP and the initial white blood cell are predictive of the number of treatments required and the overall duration of treatment required. Percutaneous approaches have been successful and result in good clinical outcomes.

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INTRODUCTION

A pancreatic pseudocyst is an abnormal collection of pancreatic fluid generally due to pancreatitis, exists for at least 4 wk, have a well-defined wall, and contain essentially no solid material[1,2]. They are more commonly seen in patients with alcohol-associated pancreatitis (20%) than with gallstone pancreatitis (6.6%)[3]. Although most commonly immediately peripancreatic or intrapancreatic, they can occur in truly extrapancreatic locations throughout the peritoneal cavity as well as the mediastinum[4,5].

Extrapancreatic pseudocysts are relatively uncommon, estimated to occur in up to 22% of patients with pancreatic pseudocysts[5]. The location depends on where the pancreatic enzymes are released and the path they travel. One of the least common locations for truly extrapancreatic pseudocysts is within the liver[4,5]. Here we describe such a case of an intrahepatic pancreatic pseudocyst (IHPP), and exhaustively review, and analyze, the world literature on IHPP.

A 56-year-old male with a history of acute alcoholic pancreatitis presented with intermittent chronic abdominal pain. Magnetic resonance imaging revealed a 1.3-cm lesion in the body of the pancreas consistent with a small pancreatic pseudocyst. Computed tomography (CT) 4 mo later revealed a new, 18-cm-long, bilobed fluid collection, wrapped about the hepatoduodenal ligament, not only communicating with the original fluid collection but also insinuating itself deeply into the hepatic parenchyma (Figures 1A), with evidence of communication to the erstwhile intrapancreatic pseudocyst (Figure 1B). Given worsening right upper quadrant abdominal pain, fever, chills, anorexia and significant weight loss, and an unknown age of the new IHPP, percutaneous transhepatic drainage was performed of the more superficial, inferior lobe (Figure 2, fluid was high-amylase and culture-negative), as well as endoscopic pancreatic sphincterotomy, and pancreatic-duct stenting. Follow-up CT one week later revealed a significant reduction in the size of both lobes of the pseudocyst. Three weeks later, however, he developed worsening abdominal fullness, pain and fevers. Repeat CT showed the superficial, inferior lobe to be well drained with the pigtail in place (Figure 3A), but the deeper superior collection was found to be larger containing a small bubble of gas (Figure 3B), with the connecting bridge collapsed. The drain was therefore repositioned into this deeper lobe (Figure 4, culture-positive). Following this procedure, the patient improved clinically and was discharged on 4 more weeks of IV antibiotics. Two weeks later he required aspiration of a small liver abscess (low-amylase, culture-positive), although his pseudocysts remained collapsed. At this point the drain was removed. Interval imaging one month and three months (Figure 5) later revealed no residual fluid collections and he remains drain-free, off antibiotics, gaining weight, and productive at work.

MATERIALS AND METHODS

A PubMed and Google Scholar search using key words "pseudocyst", "pancreatic", and "intrahepatic" followed by extensive cross-reference review revealed 41 published articles on patients with IHPP. All articles, case reports, systematic reviews which also added a case, letters to editors, and abstracts were analyzed and the data tabulated for comprehensive review and statistical analysis. Bivariate analyses were performed in Statistical Package for the Social Sciences (IBM Corporation, New York, NY, United States). Statistical review of the study was performed by a biomedical statistician.

Articles included were primarily in the English language, but also included French, German, Portuguese, Czech, Korean, and Japanese. Foreign-language articles were reviewed with native speakers or, if native speakers were not available, then the articles were translated with electronic software when possible.

RESULTS

Prevalence and patient characteristics

We identified 41 articles containing 54 cases of IHPP in the literature, the earliest identified case being published in 1974 (Table 1). These are primarily single case reports and mini case series but included two relatively thorough review articles which reviewed 26 cases[6] and 23 cases[7]. Two of the cases were notable in that the IHPP formation

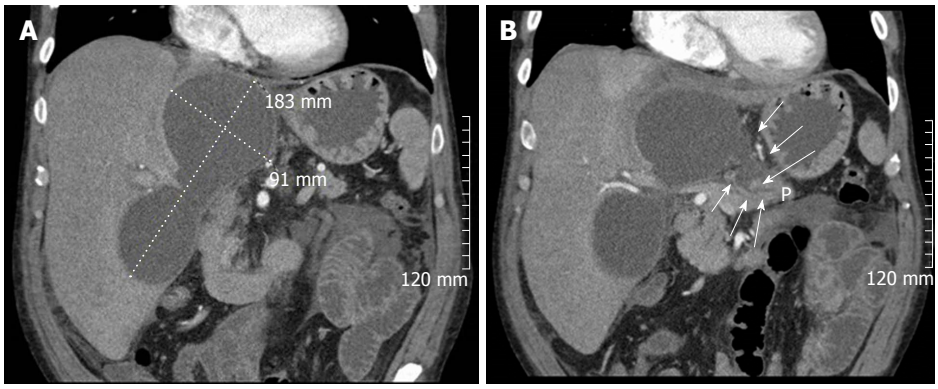

Figure 1 Abdominal computed tomography images showing bilobed intrahepatic pancreatic pseudocysts (A), including connection to main pancreatic duct (B, arrows).

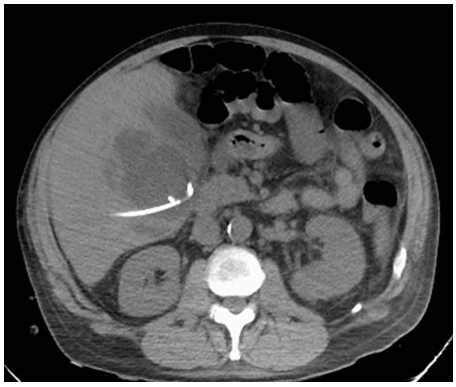

Figure 2 Abdominal computed tomography image showing percutaneous transhepatic drainage of the more superficial, inferior lobe.

was thought to be secondary to ectopic pancreatic tissue and not an inflammatory pancreatic process[8,9]. In many of the cases (42%), the IHPP was the only reported pseudocyst, but a significant number also had other concurrent pseudocysts, the most common of which were intra- or peripancreatic pseudocysts (71%).

Diagnosis

Diagnosis of an IHPP can be difficult as it is uncommon and it is not often part of the initial differential of a patient presenting with abdominal pain. Furthermore, if the presentation is delayed, imaging may reveal the IHPP but without inflammatory changes of the pancreas. Abdominal pain was the primary complaint in 91% of cases, but physical exam was generally nonspecific. Only 17% (n = 9) of patients were noted to have a palpable abdominal mass or hepatomegaly, and 15% (n = 8) had peritoneal signs. Initial diagnosis was often *via* CT (53%) or ultrasound (US) (33%) but nearly every patient in our database (91% of cases where imaging is mentioned) did get a CT scan at some point in the diagnostic or therapeutic process, and CT is generally considered to be the imaging modality of choice for these patients currently. Prior to the widespread availability of the CT scan, however, a significant workup was often done to identify the etiology of a patient's presentation and in some cases would include a gastrointestinal transit studies, endoscopy, venogram, arteriogram, or exploratory laparotomy where the lesions were finally identified[10-12]. Endoscopy has been used effectively in several cases, not only including initial diagnosis[13], but also with therapeutic intervention[14,15], as discussed further below.

The diagnosis of an IHPP was often delayed with the lesions often initially being mistaken for intrahepatic biliary dilatation, hemangioma, hepatic cyst, pyogenic liver abscess, amebic abscess, biloma, malignancy, echinococcal cyst, or even peritoneal tuberculosis[10,13,16-19]. Although IHPP lesions may be clinically suspected in a patient based on the presentation and radiological imaging, definitive diagnosis was rarely made until analysis of the cystic fluid was performed demonstrating a high amylase content[6,7,17,20,21].

Management

Despite advancements in, and the increasing availability of, imaging modalities, especially the CT scan, the number of reported cases and the type of management techniques have not evolved significantly. There are no widely accepted management guidelines for IHPPs and therefore clinicians have tailored the treatment to the individual patient based on judgment, taking into account many factors, such as underlying etiology, location of the pseudocyst, concomitant lesions, and other patient comorbidities.

Most patients reviewed were symptomatic (91% of reported cases) and required either transcutaneous or surgical intervention. Prior to the development of advanced radiological imaging, more patients underwent a laparotomy and open drainage[10,12,22].

In recent years, however, several less invasive methods have been used to manage IHPPs. Unlike the more commonplace peripancreatic or intrapancreatic pseudocysts, for IHPPs the most common method was percutaneous aspiration or drainage (Table 2) which provided a definitive diagnosis, and was usually well tolerated with minimal complications in these patients[6,7,23]. Simple needle aspiration alone with either US or CT

Table 1 Published cases

Ref.	Year	Language	Clinical features
Gautier-Benoit *et al*[12]	1974	French	Abdominal pain, weight loss
Cécile *et al*[10]	1974	French	Same patient as published by Gautier
Quevedo *et al*[16]	1975	Portuguese	Unknown location, died prior to intervention
Siegelman *et al*[4]	1980	English	Edematous pancreas, IHPP aspirated
Epstein *et al*[21]	1982	English	2 patients. Abdominal pain, distension, vomiting, diarrhea, chest pain, ascites
Hospitel *et al*[18]	1983	French	Abdominal pain, jaundice, palpable liver
Atienza *et al*[38]	1987	French	Weight loss, hepatomegaly, splenomegaly
Roche *et al*[11]	1987	French	Abdominal pain, febrile
Shimayama *et al*[39]	1988	Japanese	Abdominal pain
Lantink *et al*[22]	1989	English	Abdominal pain
Schaefer *et al*[8]	1989	German	Abdominal pain, anorexia, DVT/PE
Okuda *et al*[34]	1991	English	2 patients, abdominal pain, anorexia, guarding; 1 resolved spontaneously
Slim *et al*[40]	1992	French	Right epigastric pain
Aiza *et al*[37]	1993	English	Abd pain, fever, weight loss
Hamm *et al*[5]	1993	German	8 patients
Králík *et al*[9]	1993	Czech	Abdominal pain, pruritis, dark urine, light stools
Wang *et al*[27]	1993	English	Abdominal pain, weight loss
Scappaticci *et al*[35]	1995	English	Abdominal pain, blood per rectum
Bayo Poleo *et al*[41]	1997	Spanish	Epigstric pain and tenderness, perotinitis
Lederman *et al*[23]	1997	French	Abdominal pain, palpable liver
Mehler *et al*[30]	1998	French	3 patients, abdominal pain, vomiting, diarrhea, jaundice, guarding
Mofredj *et al*[6]	2000	English	Abdominal pain, tenderness, guarding, diarrhea
Sugiyama *et al*[42]	2000	Japanese	Abdominal pain
Shibaski *et al*[33]	2002	English	Abdominal pain, nausea, vomiting, epigastric tenderness
Bong *et al*[43]	2003	Korean	Abdominal pain
Ancel *et al*[44]	2005	French	Abdominal pain
Balzan *et al*[29]	2005	English	Abdominal pain, cystic dystrophy of duodenal wall
Bhasin *et al*[25]	2005	English	Abdominal pain
Gamanagatti *et al*[20]	2006	English	Abdominal pain, rigid abdomen
Les *et al*[17]	2006	English	Vomiting, melena, tachycardia
Casado *et al*[26]	2007	English	Abdominal pain, nausea
Yi *et al*[45]	2008	Korean	Abdominal pain
Al-Ani *et al*[19]	2009	English	Epigastric pain, fever, diaphoresis, guarding, palpable abdominal mass
Atia *et al*[36]	2009	English	Abdominal pain, nausea, vomiting, hepatomegaly
Chahal *et al*[13]	2009	English	Abdominal pain
Guesmi *et al*[7]	2009	English	Abdominal pain, vomiting, weight loss
Bhasin *et al*[24]	2010	English	2 patients, abdominal pain
Kibria *et al*[14]	2010	English	Abdominal pain
Baydar *et al*[15]	2013	English	Abdominal pain, nausea, vomiting, jaundice
Devangan *et al*[28]	2015	English	Abdominal pain, weight loss, anorexia, palpable epigastric mass
Martínez-Sanz *et al*[46]	2015	English	Abdominal pain
Current case	2016	English	Abdominal pain

DVT: Deep-vein thrombosis; PE: Pulmonary embolism; IHPP: Intrahepatic pancreatic pseudocysts.

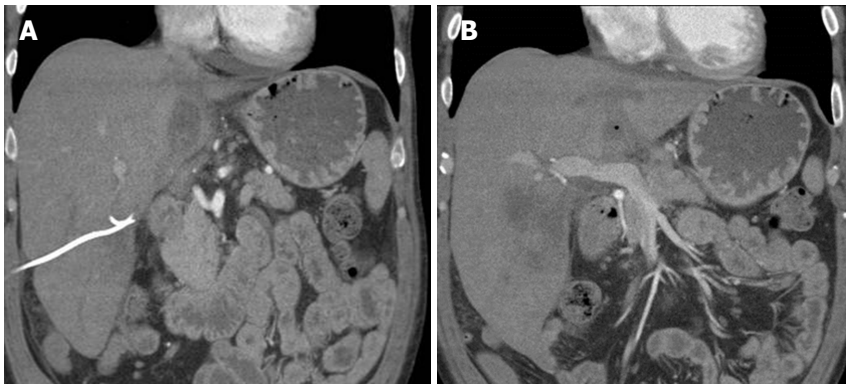

Figure 3 Abdominal computed tomography images showing the superficial, inferior lobe to be well drained with the pigtail in place (A), but the deeper superior collection containing a small bubble of gas (B).

guidance was performed as often as drainage (Table 2). While it aided in the definitive diagnosis by providing an amylase value of the fluid, it was often not completely therapeutic, with 38% of the aspiration-only cases in

Table 2 Summary of cases (n = 54)

Mean age (range)	Gender (%)	No. of IHPP (% of cases)	Size (range)	Location (%, n)	No. of interventions (%, n)[1]	Intervention (%, n)[2]	Infection (%, n)[3]
49 (15-76) yr	Male (80%)	1 (67)	9.5 (3-18) cm	Right lobe (11%, 6)	0 (9%, 4)	Operative (25%, 15)	Culture
		2 (15)		Left lobe (48%, 26)	1 (60%, 27)	Simple aspiration (28%, 17)	positive (16%, 5)
	Female (20%)	3 (13)		Right and left lobes (17%, 9)	2 (24%, 11)	Percutaneous drainage (28%, 17)	Culture negative
		4 (4)		Unavailable (24%, 13)	3 (7%, 3)	Endoscopic (8%, 5)	(84%, 27)

[1]Excludes three cases lacking mention of an intervention, and two cases with non-IHPP interventions; [2]Accounts for total number of interventions performed on patient population; some patients underwent several interventions. Does not include those patients who underwent nasopancreatic drainage (5%, 3) or medical intervention (5%, 3); [3]Excludes 15 reports which did not make mention of culture status. IHPP: Intrahepatic pancreatic pseudocysts.

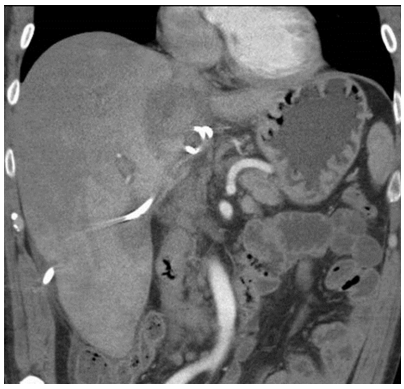

Figure 4 Abdominal computed tomography image showing drain was repositioned into the deeper lobe seen in Figure 3B.

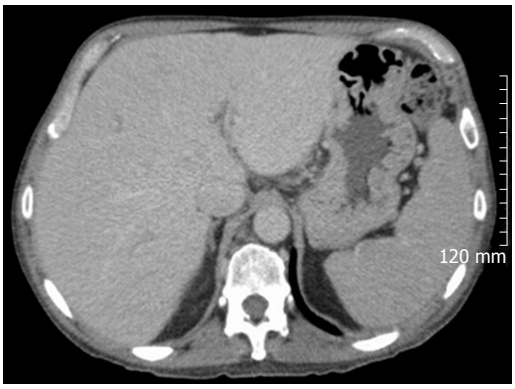

Figure 5 Abdominal computed tomography image showing resolution of the intrahepatic pancreatic pseudocysts at 3 mo following the initial intervention.

the literature requiring additional interventions.

In addition to either percutaneous drainage or aspiration, there were several other approaches or adjunctive procedures which have been utilized to manage an IHPP. Although most cases are managed percutaneously or operatively, there is an increasing experience with endoscopic approaches. These have included endoscopic retrograde pancreatography (ERCP) with pancreatic duct stenting, endoscopic transpapillary nasopancreatic drainage, pancreatic duct balloon dilatation, and ERCP-guided aspiration (Table 2)[13-15,24,25]. Bhasin *et al*[24,25] for example, reviewed 11 patients with atypically located pseudocysts, treated with ERCP and transpapillary nasopancreatic drainage. Placement of a nasopancreatic drain across the disruption was successful in 10 of the 11 patients (90.9%), with resolution of the extrapancreatic pseudocysts in 4-8 wk, with a follow-up period of 3-70 mo.

Operative interventions on patients with IHPPs have been generally reserved for those refractory to, or inappropriate for, nonoperative treatment, such as cases of diagnostic uncertainty[26], rupture[22], or severe infection[27]. All 15 operative interventions (Table 2) to manage these IHPPs were open operations and included partial resection with drainage of the cavity into a Roux limb[8,9,22] and complete resection/excision of the lesion[26,28]. In 10 reports the operation was the first intervention, in 4 reports it was the second intervention, and in one report it was the third intervention (likely, see below). The four second-intervention operations followed percutaneous aspiration in two cases[8,22] and percutaneous drainage in two cases[12,29]. The one third-intervention report[5], however, included 19 extrapancreatic pseudcysts, eight of which were intrahepatic, but it is not clearly reported which if any of those eight IHPP patients underwent which operation. We found no report of postoperative pancreatic fistula development complicating operation.

Outcomes/complications

Although spontaneous resolution of pseudocysts with conservative (noninterventional) management has been reported, complications in these cases included persistent nausea and vomiting, rupture, fistula tract formation, abscess formation if not sterile, or obstruction of the venous or biliary system due to mass effect.

Outcomes were generally very good for patients presenting with these IHPP, with 45% of patients achieved complete resolution of both the cyst and symptoms. In addition, 21% of patients experienced partial resolution of the cyst but total resolution of their symptoms by the time of the follow-up. In our analysis, we noted a statistically significant correlation between the size of the IHPP and both the duration of treatment (P = 0.006) and the number of interventions required (P = 0.031).

Infection of these pseudocysts was reported in

16% of the cases (Table 2), but an organism is not always reported and it is usually unknown whether organisms were part of the original process, or later infected the pseudocyst. Many cases were associated with leukocytosis [mean reported white blood cell (WBC) count of 15000] but without correlation to positive cultures on pseudocyst aspiration. Although there is no correlation between infection and final outcome, we did note a statistically significant positive correlation between the initial WBC count and the duration of treatment ($P = 0.048$).

There are four reported deaths in the IHPP literature, three of which had undergone a percutaneous drainage procedure[7,16,20,30]. Of note, two of these cases had an infectious component either of the intrahepatic pseudocyst or another concomitant pseudocyst[20,30].

DISCUSSION

IHPPs frequently present with abdominal pain and are diagnosed with either US or CT imaging. Although the mechanism by which IHPPs develop is not entirely clear, the time to presentation varies tremendously with reports ranging from 6 d to 2 mo[26,29]. It is understood, however, that although a collection of pancreatic fluid is not called a "pseudocyst" until it has been present for at least 4 wk, according to the 2012 revision of the Atlanta classification and definitions by international consensus[1], many of the IHPP reports reviewed here predate that nomenclature. Therefore, we have retained the term "pseudocyst" in these cases.

The process of IHPP formation begins of course with an inflammatory or traumatic episode during which pancreatic duct disruption occurs, resulting in the leakage of pancreatic fluid into the surrounding tissue. Then, once the pancreatic proteolytic enzymes are found outside the pancreatic parenchyma, they may migrate along planes (*e.g.*, hepatogastric, hepatoduodenal) or, by digesting tissue, across planes into the hepatic parenchyma. The end result of this is often observable by imaging and on anatomical-pathological findings, evidencing rupture of the main pancreatic duct and active communication with the intrahepatic collection, as shown in several reported cases[5,21,31-34], and in our case (Figure 1). However, communication does not always persist and in these select cases, may actually be more amenable to conservative management or observation.

The most common extrapancreatic location for pancreatic pseudocyst development is within the lesser sac and may be seen alone or along with an IHPP[4]. An IHPP may be either subcapsular or intraparynchymal with CT imaging of the former characterized by peripheral location and a biconvex appearance[20,29]. They are further characterized by their spatial location in ether the right lobe, left lobe, or involving both lobes. It has been hypothesized that the location of the pancreatic inflammation (*e.g.*, head *vs* tail) is correlated with the tract the fluid takes and eventual location in the liver of the IHPP with several different paths described[4,5,13,15,16,19,33-37]. However, we did not find this to be a statistically significant correlation. The left lobe was by far the most common location for an IHPP (Table 2) with fluid that likely traveled through the hepatogastric ligament.

Although IHPPs may resolve spontaneously, this is uncommon. As in our case, symptoms, or occasionally diagnostic uncertainly, generally require intervention to prevent complications such as infection, fistula, rupture, and mass-effect obstruction of the biliary or portal systems. Our experience certainly echoes that in the literature, *viz.*, that percutaneous or surgical drainage is usually well tolerated and results in resolution of the pseudocyst and improvement in associated symptoms. Treatment of course depends on the location, size, and effects of the pseudocyst, patient stability, and whether or not the lesion remains in persistent communication with the pancreas. In addition to the primary drainage methods to address the IHPP, several adjunctive procedures have been done, some of which were reportedly novel for this indication. Examples include placement of pancreatic duct stent, endoscopic placement of a nasopancreatic drain, or FNA during endoscopy[13,24,25]. Recurrence of these pseudocysts has not been described in the literature although is certainly possible, and indeed likely, that there were recurrences, the absence of which may be due to lack of longitudinal follow-up, lack of publication, or the rarity of the condition.

Our case was particularly interesting in that the pseudocyst was very large and bilobed, originating around the hepatoduodenal ligament and extending into the liver. The interval presentation between his pancreatitis flair and initial presentation allowed the pancreas to return to fairly normal appearance. This supports the idea that the hepatoduodenal ligament may be a critical structure in the formation of IHPPs.

In conclusion, although IHPPs are often not included in the differential diagnosis of a patient presenting with an intrahepatic lesion, in the right setting and population of patients, it should be considered as an important differential diagnosis. Analysis of this sparse literature has been instructive in revealing a significant correlation between the size of the IHPP and both the duration of treatment and the number of interventions required. The duration of therapy was also correlated with the initial WBC count. These observations may help with prediction of the clinical course in future cases.

COMMENTS

Background

The authors have summarized and analyzed the literature on intrahepatic pancreatic pseudocysts (IHPP), to facilitate an appreciation for this study's relevance and to help understand its significance for the field as a whole.

Research frontiers

Current important areas in the research field as related this study include the establishment of a registry.

Innovations and breakthroughs

The key advances in the current study is the recognition that size of the IHPP correlates with both the duration of treatment and the number of interventions required. The duration of therapy was also correlated with the initial white blood cell count.

Applications

These observations may help with prediction of the clinical course in future cases.

Peer-review

This is an interesting paper on intrahepatic pseudocyst. Conclusions are interesting. Please elaborate if possible more on the role of endoscopic treatment in such cases.

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META-ANALYSIS

PNPLA3 polymorphism increases risk for and severity of chronic hepatitis C liver disease

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Abstract

AIM

To examine the association of *PNPLA3* polymorphisms in chronic hepatitis C patients and development of liver disease spectrum.

METHODS

Literature was searched systematically from PubMed/MEDLINE, EMBASE, and Cochrane search engines for full-length articles written in English that examined *PNPLA3* polymorphism in chronic hepatitis C (CHC) patients. Studies evaluating the association of *PNPLA3* polymorphism spectrum (fatty liver, steatohepatitis, cirrhosis, and hepatocellular carcinoma) of CHC were included. Pooled data are reported as OR with 95%CI. Our study endpoint was the risk of the entire liver disease spectrum including: Steatosis/fatty liver, cirrhosis, and hepatocellular carcinoma in CHC patients with *PNPLA3* polymorphisms.

RESULTS

Of 380 studies identified, a total of 53 studies were included for full-text review. Nineteen on chronic he-

patitis C were eligible for analysis. Pooled ORs for rs738409 GG compared to CC and CG among patients with fatty liver was 2.214 (95%CI: 1.719-2.853). ORs among advanced fibrosis/cirrhosis were 1.762 (95%CI: 1.258-2.468). Similar odds ratios among hepatocellular carcinoma patients were 2.002 (95%CI: 1.519-2.639). Pooled ORs for rs738409 GG and CG compared to CC among patients with fatty liver were 1.750 (95%CI: 1.542-1.986). Pooled ORs for advanced fibrosis/cirrhosis patients were 1.613 (95%CI: 1.211-2.147). All analyses were homogenous and without publication bias except one. The associations were maintained after adjusting for publication bias and heterogeneity.

CONCLUSION

PNPLA3 polymorphisms have strong association with increased risk and severity of the liver disease spectrum in CHC patients.

Key words: *PNPLA3* polymorphism; Cirrhosis; rs738409; Hepatitis C virus; Hepatocellular carcinoma

Core tip: *PNPLA3* polymorphisms (rs738409 CG and GG) are associated with increased risk of steatosis, advanced fibrosis, cirrhosis, and hepatocellular carcinoma in chronic hepatitis C patients.

Salameh H, Masadeh M, Al Hanayneh M, Petros V, Maslonka M, Nanda A, Singal AK. *PNPLA3* polymorphism increases risk for and severity of chronic hepatitis C liver disease. *World J Hepatol* 2016; 8(35): 1584-1592 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i35/1584.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i35.1584

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease in the United States[1]. About 27% of cases of cirrhosis and 25% of hepatocellular carcinoma (HCC) worldwide are secondary to HCV infection[2]. Multiple genetic factors identified within the past few years have been shown to be associated with the predisposition to chronic liver disease and the progression to cirrhosis and HCC[3,4]. The single nucleotide polymorphism (SNP) rs738409 C>G (isoleucine to methionine substitution at position 148, I148M) in the *PNPLA3* gene has been strongly linked to progression of liver disease in multiple studies, and this association was confirmed in meta-analyses of the spectrum of alcoholic liver disease (ALD)[5] as well as non-alcoholic fatty liver disease (NAFLD)[6-9].

The frequency of hepatic steatosis varies with ethnicity where it was reported as 45%, 33% and 24% in Hispanics, Whites and Blacks respectively[10]. At the same time the frequencies of the PNPLA3 rs738409[G] allele were 0.49, 0.23, and 0.17 in Hispanics, European Americans and African Americans[11]. In addition, the prevalence of the GG genotype in different races in fact correlates with the rate of NAFLD in each respective population, with nearly half of all Hispanics possessing the allele, who in turn are also most likely to have NAFLD. The same is true of the inverse, with less than one quarter of African Americans having the PNPLA3 rs738409[G] allele, and they are least likely to develop NAFLD compared to Hispanics and Caucasians[10,11].

Given that the association between *PNPLA3* polymorphism and liver disease spectrum in chronic hepatitis C (CHC) patients has not been consistent, especially for HCC[12,13] and cirrhosis[14,15], we performed this meta-analysis to further examine the association of *PNPLA3* polymorphisms with the predisposition to the entire spectrum of liver disease among patients with CHC.

MATERIALS AND METHODS

Literature search

Utilizing the Meta-analysis of Observational Studies in Epidemiology guidelines, literature was searched from PubMed/Medline, Embase, and Cochrane search engines for full-length articles written in English that examined *PNPLA3* polymorphism in CHC patients[16]. The initial medical subject headings search terms were: "Hepatitis C, Chronic" and "adiponutrin, human". The search was then expanded using the terms: "rs738409" and "patatin-like phospholipase domain-containing 3 protein". All databases were searched from their inception date through March 2015. Meeting abstracts from major gastroenterology conferences over the past 3 years were also searched to identify studies that were potentially overlooked in our database search. Articles were selected for full text review based on title and abstract.

Study selection

Three independent investigators (Masadeh M, Al Hanayneh M and Maslonka M) manually search the retrieved publications to ensure all appropriate articles were discovered and included. Two authors (HS and AKS) reviewed articles in question for possible inclusion. The following inclusion criteria were set for inclusion in this meta-analysis: (1) studies published as full-length articles which reported association of the *PNPLA3* variant (rs738409 C>G) among CHC patients; and (2) studies which analyzed patients with other liver diseases and reported separate data on *PNPLA3* polymorphisms for CHC.

The following exclusion criteria were set: (1) studies without available gene frequency data for analysis; and (2) studies including subjects with other liver diseases without separate data on CHC patients.

Definitions

HCV infection was diagnosed with both positive serum

anti-HCV antibodies and serum HCV ribonucleic acid (RNA). The disease spectrum was defined as the following: steatosis = fatty liver (FL) on imaging without evidence of cirrhosis or HCC; advanced fibrosis and cirrhosis = biopsy-proven bridging fibrosis, or clinical evaluation supported by hematological, biochemical, and radiologic imaging findings; and HCC = diagnostic imaging findings on triple phase magnetic resonance imaging or computed tomography, or using histological confirmation from liver tissue. Healthy controls were defined as subjects without liver disease and without HCV infection.

Data extraction

After determining eligibility for inclusion, two reviewers (Masadeh M and Al Hanayneh M) independently extracted data for (1) study characteristics: Author and year of publication, and study design (population based or not, using controls or not); (2) study population: Liver disease spectrum and sample size; (3) frequencies of *PNPLA3* polymorphism genotypes (rs738409 CC, CG, and GG); and (4) OR: For association of *PNPLA3* polymorphism and the spectrum of liver disease and for severity of liver disease. Any discrepancies amongst the reviewers were resolved by jointly reviewing the study in question. Among studies comparing diseased population with healthy controls, similar data were also extracted on healthy controls.

Endpoints and outcomes

Our study endpoint was the risk of the entire liver disease spectrum including: Steatosis/fatty liver, cirrhosis, and HCC in CHC patients with *PNPLA 3* polymorphisms.

Quality assessment

The quality of included studies was assessed independently by three authors (Masadeh M, Al Hanayneh M and Maslonka M) using the Newcastle-Ottawa Quality Assessment Scale for case-control studies[17]. This scale has one instrument for assessing case-control studies and another one for cohort studies. Each of these instruments includes measures of quality in selection, comparability, and exposure domains. While one point is granted for each of the areas measured within the selection and exposure domains, a maximum of two points can be assigned within the comparability domain with highest possible total score of nine. Previous studies have reported that a score of seven or greater denotes a high-quality study[18]. Any discrepancies between the three coauthors were addressed by a joint reevaluation of the original article.

Statistical analysis

The strength of the association between rs738409 and CHC liver disease spectrum prevalence was expressed by OR and their corresponding 95%CI. The Random effects model was used for analyzing pooled data for all the analyses[19]. Heterogeneity was measured using I^2 statistics for inter-study variance, with the χ^2 test used for statistical analysis. Heterogeneity was defined with $I^2 \geqslant 50\%$ or χ^2 $P < 0.10$[20]. At least two studies are needed to examine and report heterogeneity. To examine the heterogeneous data and source of heterogeneity, sensitivity analyses were performed in a stepwise fashion for (1) study quality; and (2) excluding studies with the highest and lowest OR. Publication bias was assessed using Egger regression and the Begg-Mazumdar rank correlation tests[21-23]. Egger test is a regression method checking for association between effect sizes and standard error and uses actual effect size for each study[23]. Begg-Mazumdar is a rank correlation test examining the potential association between effect estimates (taken as a rank and not exact effect size) and sampling variance (or standard error)[22]. At least three studies are needed for examining and reporting publication bias. For analyses with publication bias, the analyses were repeated either by performing sensitivity analysis or using the Duval and Tweedie Trim and Fill method, a nonparametric (rank-based) data augmentation technique[24]. The method can be used to estimate the number of studies missing from a meta-analysis resulting in a skew of the data due to the suppression of the most extreme results on one side of the funnel plot. The method then amplifies the observed data so that the funnel plot is more symmetric and re-computes the summary estimate based on the comprehensive data. The method should not be regarded as a way of yielding a more "valid" estimate of the overall effect or outcome, but as a means of examining the sensitivity of the results to one particular selection mechanism[25]. All statistical analyses were performed using R (Foundation for Statistical Computing) utilizing the metaphor package, or Comprehensive Meta-analysis (Biostat, Englewood, NJ). Singal AK from University of Alabama, Birmingham, reviewed the statistical methods of this study.

RESULTS

Baseline study characteristics

A total of 380 citations were retrieved on initial search. After reviewing article titles and abstracts, a total of 53 studies were included for full-text review (Figure 1). Of these, twenty articles were excluded because they did not have sufficient data for our analysis. Nine studies were excluded for including subjects with liver disease not caused by HCV[11,26-33], and three studies were excluded for including subjects co-infected with human immunodeficiency virus and/or hepatitis B virus infection[34-36]. One duplicate study[37] and one study that looked at treatment response[38] were excluded. Nineteen studies evaluating 9093 patients (57.6% males, mean body mass index 25.1 kg/m^2) on association of *PNPLA3* polymorphisms in CHC[12-15,39-53] were included for the analysis. Data on study design, ethnicity and genotype frequency are summarized in Table 1.

Table 1 Baseline characteristics of patients from studies included in the analysis

Ref.	Study design	Controls (*n*)	Cases								
			n	Ethnicity	M%	Mean age	Mean BMI	rs 738409 genotype count (CC:CG:GG)[4]			
								FL	Hepatitis	Cirrhosis	HCC
Cai *et al*[39] (2011)	R	-	626	C	61.8	44.7	23.7	62:28[1]	-	-	-
Valenti *et al*[40] (2011)	R	179	819	C + NA	56.4	57.4	24.8	269:219:73	-	119:172:229	17:21:12
Trépo *et al*[41] (2011)	R	-	537	C	63	49.4	25.5	136:106:31	-	108:85:23	-
Corradini *et al*[42] (2011)	P[6]	-	221	C	63	58	-	-	-	-	-
Nischalke *et al*[13] (2011)	P	190	162	C	57	56	28.4	-	-	45:31:05	40:33:08
Valenti *et al*[43] (2012)	P	-	567	NS	-	-	-	-	-	-	-
Valenti *et al*[15] (2012)	P[6]	-	602	NS	51	51	25.1	364:42[2]	-	158:21[2]	-
Guyot *et al*[12] (2013)	P	-	253	NS	54.2	56.7	27.3	-	-	140:75:38	54:26:13
Ezzikouri *et al*[44] (2013)	P	132	230	NA	45.2	63.63	-	-	47:71:11	-	43:35:23
Stättermayer *et al*[45] (2014)	R	-	478	NS	65.7	44.9	25.6	190:23[2]	-	101:57[2]	-
Ampuero *et al*[46] (2014)	P[6]	-	474	M	64.8	43.4	25.7	94:126[3]	-	-	-
Sato *et al*[47] (2014)	R	-	358	A	55.9	69.76	-	41:20[2]	-	112:37[2]	100:176:82
Yasui *et al*[48] (2014)	P[6]	-	276	A	40.6	58.2	23	23:75:39	45:66:38	20:31:21	-
Petta *et al*[14] (2015)	P	-	434	C	53.9	51.7	-	-	40:35:12	71:36:13	-
Nakaoka *et al*[49] (2015)	P	-	231	A	44.6	62.9	22.5	-	-	90:27[2]	12:22:14
Tamaki *et al*[50] (2015)	R	-	176	A	39.8	56.5	22.9	-	-	52:87:37	-
Huang *et al*[51] (2015)	R	-	1018	A	56.6	51.8	24.9	175:205:75	-	-	-
Petta *et al*[52] (2016)	P[6]	-	694	C	53	54	26.5	151:151:45[5]	-	-	-
Ali *et al*[53] (2016)	P	-	937	M	70.1	49.5	-	-	-	172:212[3]	-
Summary		501	9093		57.6	52.7	25.1				

[1]C allele: G allele; [2]CC + CG: GG; [3]CC: CG + GG; [4]Genotype counts were reported as ratios (CC wild genotype: CG heterozygote genotype: GG homozygote genotype) unless indicated by star(s); [5]Calculated from percentages in the original article; [6]Population based studies. A: Asian; BMI: Body mass index; C: Caucasian; FL: Fatty liver; HCC: Hepatocellular carcinoma; M: Mixed (Caucasians and non-Caucasians[46] and White/Black/Hispanic[53]); P: Prospective; R: Retrospective; M%: Males percentage; N: Number of cases; NA: North African; NS: Not specified in the original manuscripts (although all 4 studies included European referral centers only).

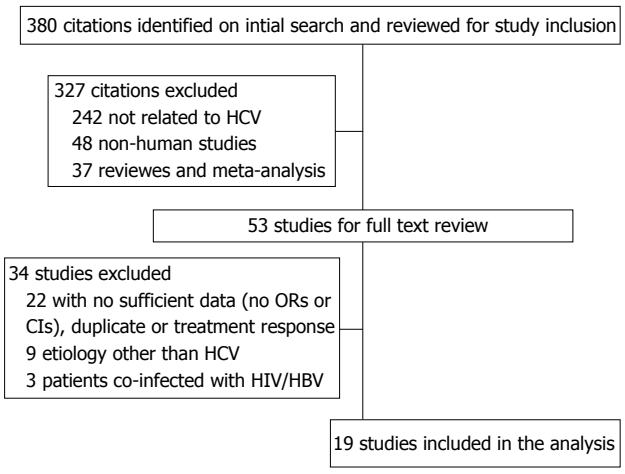

Figure 1 Attrition on literature search and study inclusion. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

Study quality assessment

Based on the Newcastle-Ottawa Scale, nine studies were of "high quality" with a score of seven or more, and the remaining ten studies had a score of six or below (Table 2).

Association between PNPLA3 polymorphism and liver disease spectrum (GG vs CG and CC analysis)

Association of *PNPLA3* polymorphisms with FL in CHC patients: Among six studies on 3310 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 2.214 (95%CI: 1.719-2.853) (Figure 2A). The data was homogeneous (I^2 = 9.4%, P = 0.36) and without publication bias as assessed by Egger test (P = 0.08) and Begg-Mazumdar test (P = 0.14).

Association of *PNPLA3* polymorphisms with advanced fibrosis and cirrhosis in CHC patients: Among seven studies on 3377 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 1.762 (CI: 1.258-2.469) (Figure 2B). The data was heterogeneous (I^2 = 65.9%, P = 0.081), with evidence of publication bias as assessed by Begg-Mazumdar test (P = 0.036) and tendency for publication bias as assessed by Egger test (P = 0.059). Sensitivity analysis after excluding studies with lowest[14] and highest[49] OR revealed similar effect size: 1.82 (95%CI: 1.41-2.34) with I^2 = 18.8%, P = 0.30. Additionally, when Dual and Tweedie trim and fall test was used to assess publication bias, 3 studies were trimmed with no change in effect size (OR = 1.39, 95%CI: 1.01-1.92).

Association of PNPLA3 polymorphisms and HCC in CHC patients: Among seven studies on 2274 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 2.002 (95%CI: 1.519-2.639) (Figure 2C). The data was homogenous (I^2 = 30%, P = 0.19), without publication bias as assessed by Egger test (P = 0.91) and Begg-Mazumdar test (P = 0.99).

Table 2 Newcastle - Ottawa Scale on quality score of the included studies

Ref.	Selection				Comparability	Exposure			Total
	Independent validation	Case representation	Controls selection	Controls definition	Case and control design/analysis	Ascertainment of exposure	Same method of ascertainment	Same response rate	
Cai *et al*[39] (2011)	1	1			2	1	1	1	7
Valenti *et al*[40] (2011)	1	1	1	1	2	1	1	1	9
Trépo *et al*[41] (2011)	1	1			2	1			5
Corradini *et al*[42] (2011)	1	1			2	1			5
Nischalke *et al*[13] (2011)	1	1	1	1	2	1	1	1	9
Valenti *et al*[43] (2012)	1	1			2	1	1		6
Valenti *et al*[15] (2012)	1	1			2	1	1		6
Guyot *et al*[12] (2013)	1	1			2	1			5
Ezzikouri *et al*[44] (2013)	1	1	1	1	2	1	1	1	9
Stättermayer *et al*[45] (2014)	1	1			2	1			5
Ampuero *et al*[46] (2014)	1	1			2	1	1	1	7
Sato *et al*[47] (2014)	1	1			2	1			5
Yasui *et al*[48] (2014)	1	1			2	1			5
Petta *et al*[14] (2015)	1	1			2	1			5
Nakaoka *et al*[49] (2015)	1	1			2	1			5
Tamaki *et al*[50] (2015)	1	1			2	1	1	1	7
Huang *et al*[51] (2015)	1	1			2	1	1	1	7
Petta *et al*[52] (2016)	1	1			2	1	1	1	7
Ali *et al*[53] (2016)	1	1			2	1	1	1	7

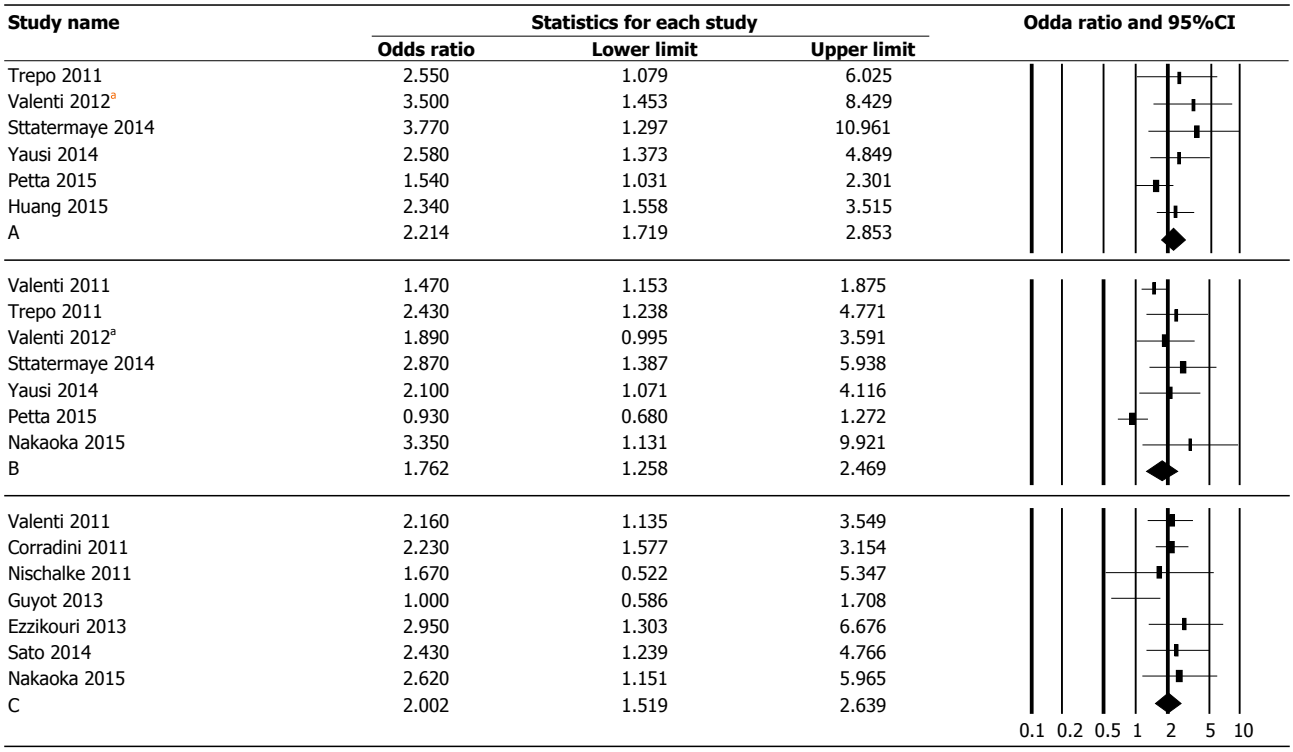

Figure 2 Forest plots for analysis of chronic hepatitis C studies on the association of *PNPLA3* polymorphisms GG *vs* CG and CC with fatty liver in (A), cirrhosis in (B), and hepatocellular carcinoma in (C). The effect size is reported as odds ratio with 95%CI. The bottom line in "the statistics for each study" heading is the pooled effect size analyzed using the random effects model. OR greater than 1 denotes risk for the respective outcome or positive association, and OR less than 1 indicates a protective effect or negative association. The 95%CI not crossing 1 indicates a significant association. Valenti 2012[a] in panel (A) refers to reference[43], and in panel (B) refers to reference[14].

Association between PNPLA3 polymorphism and liver disease spectrum (GG and CG vs CC analysis)

Association of *PNPLA3* polymorphisms with FL in CHC patients: Among three studies on 1794 patients, the pooled OR for rs738409 GG and CG genotypes compared to CC genotype in CHC was 1.750 (95%CI: 1.542-1.986) (Figure 3A). The data was homogeneous (I^2 = 0.0%, P = 0.84), without publication bias as assessed by Egger test (P = 0.57) and Begg-Mazumdar test (P = 0.99).

Association of *PNPLA3* polymorphisms with advanced fibrosis, and cirrhosis in CHC patients: Among three studies on 2131 patients, the pooled OR

Study name	Statistics for each study			Odda ratio and 95%CI
	Odds ratio	Lower limit	Upper limit	
Cai 2011	1.701	1.452	1.993	
Ampuero 2014	1.840	1.057	3.202	
Petta 2015	1.840	1.466	2.309	
A	1.750	1.542	1.986	
Huang 2015	1.340	1.006	1.785	
Tamaki 2015	3.100	1.055	9.110	
Ali 2016	1.760	1.343	2.306	
B	1.613	1.211	2.147	

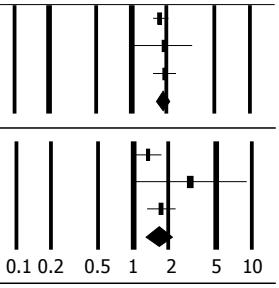

Figure 3 Forest plots for analysis of chronic hepatitis C studies on the association of *PNPLA3* polymorphisms GG and CG *vs* CC with fatty liver in (A), and cirrhosis in (B). The effect size is reported as odds ratio with 95%CI. The bottom line in the "statistics for each study" heading is the pooled effect size analyzed using the random effects model. OR greater than 1 denotes risk for the respective outcome or positive association, and OR less than 1 indicates a protective effect or negative association. The 95%CI not crossing 1 indicates a significant association.

for rs738409 GG and CG genotypes compared to CC genotype in CHC was 1.613 (95%CI: 1.211-2.147) (Figure 3B). The data was homogeneous (I^2 = 41%, P = 0.18), without publication bias as assessed by Egger test (P = 0.056) and Begg-Mazumdar test (P = 0.99).

DISCUSSION

We have previously described that *PNPLA3* polymorphism is a modifier in the natural history of ALD[5] and NAFLD[6-8]. In this meta-analysis, we found a clear association between *PNPLA3* polymorphisms and the entire spectrum (steatosis/fatty liver, cirrhosis, and HCC) of liver disease in CHC patients.

It was previously reported that *PNPLA3* polymorphisms were an independent predictor of more rapid fibrosis progression in patients with chronic hepatitis C[50]. The mechanism whereby rs738409 influences the development of fatty liver likely involves a decreased ability of the 148M *PNPLA3* variant to regulate hepatic lipid metabolism[54]. It is not known whether the rs738409 SNP influences the steatogenic effect of HCV and the progression of CHC. However, if steatosis causes fibrosis progression in CHC, then it may be assumed the rs738409 SNP should also be associated with advanced fibrosis and HCC[40].

Like any other meta-analysis, our study had to face the possibility of publication bias. In order to minimize this possibility, and the subsequent overestimation of the true effect size due to negative study identification failure[55], we combined searches from PubMed/Medline, Embase and Cochrane with manual searches. Although we used procedures in agreement with current guidelines, we cannot formally rule out the possibility that we overlooked studies that were not accessible[55]. Another limitation of this meta-analysis is the inclusion of case-control studies in which the potential for biases (*e.g.,* selection and reporting) is higher when compared to randomized trials, and they are more inherent to confounding factors. In contrary to the previous meta-analysis on *PNPLA3* polymorphisms in alcoholic and non-alcoholic liver diseases that compared different genotypes[5-8], our current analysis used the recessive model when comparing GG genotype *vs* CC and CG genotypes, and the dominant model when comparing GG and CG genotypes related to the CC genotype. Finally, no pooled data were provided on steatohepatitis in chronic HCV patients as only one study had reported such an association[14], while the studies by Ezzikouri *et al*[44] and Yasui *et al*[48] either did not have biopsies performed or reported "necroinflammatory changes". Lack of standard definition amongst these studies prevented pooling them together.

The *PNPLA3* GG genotype was negatively associated with sustained virological response and early viral kinetics in patients receiving peginterferon and ribavirin[15]. Also, in patients with chronic hepatitis C who failed to achieve sustained virologic response following interferon-based therapy, IL28B and *PNPLA3* were independent predictors of rapid fibrosis progression[50]. Tamaki *et al*[50] developed a fibrosis progression-score by combining IL28B and *PNPLA3* genotypes and ALT values, which stratified patients into low, intermediate, and high-risk groups for fibrosis progression. However, this fibrosis progression score needs external validation. In the era of direct-acting antiviral therapy, the question that remains unanswered is whether or not *PNPLA3* polymorphisms identify high-risk CHC patients that are responsive to new treatment regimens. In summary, this meta-analysis provides strong evidence for the association of *PNPLA3* polymorphisms and the spectrum of liver disease in patients with CHC, beginning with fatty liver disease and extending as far as cirrhosis and even HCC in patients with CHC. Further studies on treatment response are needed in this group of patients who carry a higher risk for more rapidly progressive liver disease.

COMMENTS

Background

Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease in the United States. About 27% of cases of cirrhosis and 25% of hepatocellular carcinoma (HCC) worldwide are secondary to HCV infection.

Research frontiers

Given that the association between *PNPLA3* polymorphism and liver disease

spectrum in chronic hepatitis C (CHC) patients has not been consistent, especially for HCC and cirrhosis, the authors performed this meta-analysis to further examine the association of *PNPLA3* polymorphisms with the predisposition to the entire spectrum of liver disease among patients with CHC.

Innovations and breakthroughs

In this meta-analysis, they found a clear association between *PNPLA3* polymorphisms and the entire spectrum (steatosis/fatty liver, cirrhosis, and HCC) of liver disease in CHC patients.

Peer-review

This manuscript is very well designed; the authors did a great effort in selecting the articles to be included in the meta-analysis with a proper quality scoring of selected articles.

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