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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).

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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

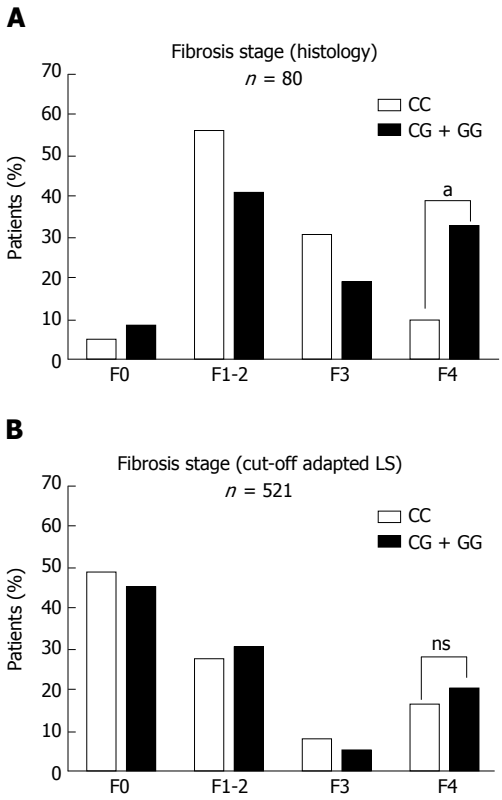

Figure 1 Distribution of fibrosis stages using (A) histology (Kleiner fibrosis score F0-4) or (B) non-invasive liver stiffness measurement (aspartate transaminase-adapted cut-off values). [a]*P* < 0.05. ns: Not significantly; LS: Liver stiffness.

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

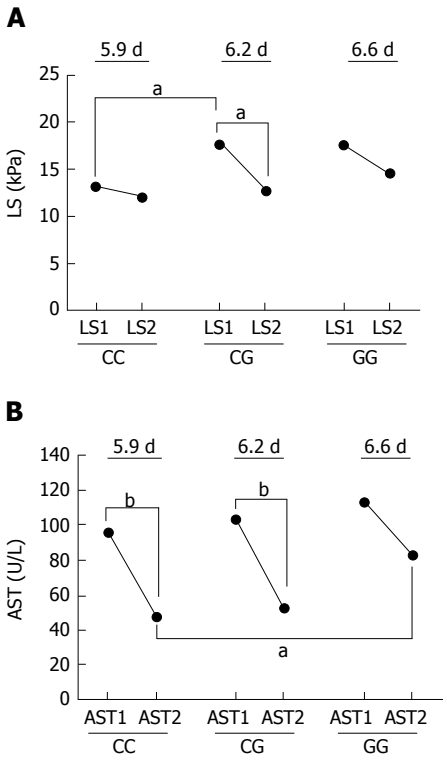

Figure 2 PNPLA3 carrier status and its effect on liver stiffness (A) and aspartate transaminase (B) levels prior and after alcohol detoxification (1 and 2). Mean observation of detoxification periods in days are indicated for each genotype. $^{a}P < 0.05$, $^{b}P < 0.01$. AST: Aspartate transaminase;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