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

Vanessa Rausch, Teresa Peccerella, Carolin Lackner, Eray Yagmur, Helmut-Karl Seitz, Thomas Longerich, Sebastian Mueller

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 (Fibroscanner, Echosens SA, Paris, France). LS and serum markers were prospectively studied in these patients with all stages of the disease during a period of alcohol withdrawal. The protocol was approved by the Ethical Committee of the University of Heidelberg.

Abstract

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Author contributions: Rausch V and Mueller S contributed to the conception and design of the study, analyzed and interpreted the data and wrote the manuscript; Rausch V, Peccerella T, Lackner C, Yagmur E and Longerich T contributed to the acquisition and analysis of data; Lackner C, Seitz HK and Longerich T critically revised the manuscript and approved the final version.

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Data sharing statement: Patients gave informed consent before inclusion in the study; the presented data are anonymized and the risk of identification is very low.

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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), steatohetapitis (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%). Non-invasive fibrosis assessment by LS in all patients showed a significantly higher F0 rate as compared to the biopsy cohort (47% vs 6%).

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

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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).
### 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/m²)                         | 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 ± 0.69                 |                           |
| 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 (/µL)                     | 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.

### 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 Chevalier 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[35]. 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.[25]. In addition, liver size, spleen size, ascites formation and semiquan-
Table 2  Characteristics of alcoholic liver disease sub­cohorts (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/m²) | 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) | 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 ± 23.0² | 17.2 ± 22.2 | 17.5 ± 22.9² |
| 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 ± 89.5³ | 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 ± 55.0⁴ | 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 ± 280.9 | 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 | 111.6 ± 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 ± 20.2² | 20.1 ± 9.9 | 24.0 ± 19.2² |
| 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 (×10⁹/L) | 216.7 ± 92.7 | 201.1 ± 80.0⁵ | 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 | 596.0 ± 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 (*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.

PNPLA3 genotyping

Genomic DNA was isolated from EDTA anti-coagulated blood using standard protocols. The PNPLA3 coding SNP 1148M 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 software⁴³, 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 MgCl₂, 0.2 mMol/L dNTPs, 2 mMol/L of outer primer and 20 mMol/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 χ² 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
Notably, fibrosis distribution differed markedly in the non-cirrhosis as compared to CC carriers as shown in Figure 1B). In both approaches, CG + GG carriers had more F4 invasively assessed cohort by LS (47%, 52.6% and 8.2% vs 20.0%). In contrast, the F0 fraction was much higher 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.

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 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.

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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.

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 assessed cohort (Figure 1A) compared to the histologically (Table 3). In contrast, the F0 fraction was much higher in the non-invasively assessed cohort (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 CC + 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.

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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 \( r = 0.096, \text{ns} \). 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

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

| Parameter | 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<sup>a</sup> | 0.391<sup>b</sup> |
| Microgranulomas (score 0-1) | -0.095 | -0.139 | 0.357<sup>a</sup> | 0.387<sup>b</sup> |
| Ballooning (score 0-2) | -0.221<sup>a</sup> | 0.020 | 0.319<sup>a</sup> | 0.516<sup>c</sup> |
| Glycogenated nuclei (score 0-1) | -0.124 | -0.080 | 0.316<sup>a</sup> | 0.335<sup>c</sup> |
| Steatosis (score 0-3) | -0.125 | -0.045 | 0.264<sup>a</sup> | 0.096<sup>c</sup> |
| Lobular inflammation (score 0-3) | -0.142 | -0.003 | 0.227<sup>a</sup> | 0.428<sup>c</sup> |
| Megamitochondria (score 0-1) | -0.121 | -0.005 | 0.198 | 0.278<sup>c</sup> |
| Large lipogranulomas (score 0-1) | 0.134 | -0.238<sup>a</sup> | 0.145 | 0.144<sup>c</sup> |
| Acidophil bodies (score 0-1) | -0.016 | -0.072 | 0.133 | 0.285<sup>c</sup> |
| Pericellular fibrosis (score 0-3) | -0.224 | 0.141 | 0.131 | 0.567<sup>c</sup> |
| Chevallier fibrosis score (SSS) | -0.189 | 0.112 | 0.131 | 0.828<sup>c</sup> |
| Ballooning k8/18 stain (score 0-2) | -0.537<sup>a</sup> | 0.490<sup>a</sup> | 0.089 | 0.692<sup>c</sup> |
| Kleiner fibrosis score (score 0-4) | -0.163 | 0.148 | 0.035 | 0.745<sup>c</sup> |
| Mallory Denk Bodies (score 0-1) | -0.121 | 0.110 | 0.026 | 0.530<sup>c</sup> |
| Apoptosis M30 stain (score 0-3) | -0.039 | 0.031 | 0.014 | 0.490<sup>c</sup> |
| 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<sup>c</sup> |
| Liver stiffness (kPa) | -0.045 | 0.017 | 0.037 | 1.000<sup>c</sup> |

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. \( P < 0.05 \) vs \( P < 0.01 \). PNPLA3: Adiponutrin.
Mean observation of detoxification periods in days are indicated for AST1 and AST2. LS1 and LS2. 6.6 d mean observation of detoxification periods in days are indicated for AST1 and AST2. LS1 and LS2.

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. *P < 0.05, **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[40], 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[43]. 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[26]. 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.

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