patient-oriented and epidemiological research

Combined effects of the *PNPLA3* rs738409, *TM6SF2* rs58542926, and *MBOAT7* rs641738 variants on NAFLD severity: a multicenter biopsy-based study[1]

Marcin Krawczyk,[*,†] Monika Rau,[§] Jörn M. Schattenberg,[**] Heike Bantel,[††] Anita Pathil,[§§] Münevver Demir,[***] Johannes Kluwe,[†††] Tobias Boettler,[§§§] Frank Lammert,[*] and Andreas Geier[§,2] for the NAFLD Clinical Study Group

Department of Medicine II,* Saarland University Medical Center, Homburg, Germany; Laboratory of Metabolic Liver Diseases, Department of General, Transplant and Liver Surgery,† Medical University of Warsaw, Warsaw, Poland; Division of Hepatology, Department of Medicine II,§ University Hospital Wuerzburg, Wuerzburg, Germany; I. Department of Medicine,** University Medical Center Mainz, Johannes Gutenberg University, Mainz, Germany; Department of Gastroenterology, Hepatology and Endocrinology,†† Hannover Medical School, Hannover, Germany; Department of Internal Medicine IV, Gastroenterology and Hepatology,§§ University of Heidelberg, Heidelberg, Germany; Clinic for Gastroenterology and Hepatology,*** University Hospital of Cologne, Cologne, Germany; Department of Medicine I,††† Hamburg University Medical Center, Hamburg, Germany; and Department of Medicine II,§§§ Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Germany

Abstract The *PNPLA3* p.I148M, *TM6SF2* p.E167K, and *MBOAT7* rs641738 variants represent genetic risk factors for nonalcoholic fatty liver disease (NAFLD). Here we investigate if these polymorphisms modulate both steatosis and fibrosis in patients with NAFLD. We recruited 515 patients with NAFLD (age 16–88 years, 280 female patients). Liver biopsies were performed in 320 patients. PCR-based assays were used to genotype the *PNPLA3*, *TM6SF2*, and *MBOAT7* variants. Carriers of the *PNPLA3* and *TM6SF2* risk alleles showed increased serum aspartate aminotransferase and alanine transaminase activities ($P < 0.05$). The *PNPLA3* genotype was associated with steatosis grades S2–S3 ($P < 0.001$) and fibrosis stages F2–F4 ($P < 0.001$). The *TM6SF2* genotype was associated with steatosis ($P = 0.003$) but not with fibrosis ($P > 0.05$). The *MBOAT7* variant was solely associated with increased fibrosis ($P = 0.046$). In the multivariate model, variants *PNPLA3* ($P = 0.004$) and *TM6SF2* ($P = 0.038$) were associated with steatosis. Fibrosis stages were affected by the *PNPLA3* ($P = 0.042$) and *MBOAT7* ($P = 0.021$) but not by the *TM6SF2* polymorphism ($P > 0.05$). The *PNPLA3*, *TM6SF2*, and *MBOAT7* variants are associated with increased liver injury. The *TM6SF2* variant seems to modulate predominantly hepatic fat accumulation, whereas the *MBOAT7* polymorphism is linked to fibrosis. The *PNPLA3* polymorphism confers risk of both increased steatosis and fibrosis.—Krawczyk, M., M. Rau, J. M. Schattenberg, H. Bantel, A. Pathil, M. Demir, J. Kluwe, T. Boettler, F. Lammert, A. Geier, NAFLD Clinical Study Group. **Combined effects of the *TM6SF2* rs58542926, *PNPLA3* rs738409, and *MBOAT7* rs641738 variants on NAFLD severity: A multicenter biopsy-based study. *J. Lipid Res.* 2017.** 58: **247–255.**

Supplementary key words adiponutrin • fatty liver • fibrosis • steatosis

Nonalcoholic fatty liver disease (NAFLD) affects more than 30% of adults in developed countries. Given the increasing prevalence of environmental risk factors for this condition (e.g., hypercaloric diets and sedentary lifestyles) (1), the frequency of fatty liver is predicted to further increase in the coming years. In addition to environmental triggers, genetic predisposition is known to modulate the degree of steatosis and liver injury (2). Conceptually, the term "hepatic steatosis" refers to traits that are governed by multiple variants with modest effects. The major part of the genetic predisposition is, according to current knowledge, related to two common missense SNPs: *PNPLA3* p.I148M and *TM6SF2* p.E167K. These two polymorphisms, detected in genome-wide (3) and exome-wide (4) association studies in patients with fatty livers, seem to impose different

This work was supported, in part, by Interdisciplinary Center for Clinical Research (IZKF) Würzburg and by Grant SFB 841 from the Deutsche Forschungsgemeinschaft (J.K.). The authors declare no conflicts of interest.

Manuscript received 24 February 2016 and in revised form 13 October 2016.

Published, JLR Papers in Press, November 11, 2016
DOI 10.1194/jlr.P067454

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HCV, hepatitis C virus; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio.

[1] This study was presented in part at the AASLD Liver Meeting 2015, San Francisco, CA, November 13–17, 2015.

[2] To whom correspondence should be addressed.
e-mail: Geier_A2@ukw.de

The online version of this article (available at http://www.jlr.org) contains a supplement.

risks on their carriers. The *PNPLA3* (patatin-like phospholipase domain containing 3, also known as adiponutrin) p.I148M polymorphism is commonly regarded to be the risk factor for both increased fat accumulation and fibrosis (5, 6). The association with steatosis was demonstrated in several candidate studies, whereas the link between *PNPLA3* and liver scarring was substantiated by meta-analyses in patients with chronic hepatitis C virus (HCV) infection (7) and in alcoholics (8). The data concerning the involvement of *TM6SF2* (transmembrane 6 superfamily member 2) in liver injury are less definitive. So far, only a few studies investigating the *TM6SF2* risk genotype in NAFLD (9) and in HCV (10) have been published. Liu et al. (11) reported that carriers of the minor allele are at risk of increased steatosis and fibrosis. Interestingly, both *PNPLA3* and *TM6SF2* variants have been associated with "metabolically silent" NAFLD; i.e., carriers of the risk genotypes seem to develop NAFLD and its severe forms even in the absence of characteristics commonly associated with fatty liver (5, 12). Indeed, numerous genetic studies failed to detect equivocal evidence for the association between *TM6SF2* and *PNPLA3* variants and traits such as obesity, insulin resistance, or hyperlipidemia. Most recently, the *MBOAT7* polymorphism rs641738 was identified as the new risk factor for NAFLD (13), also associated with severity of fibrosis in alcoholic liver disease (14) and in HCV infection (15).

Liver biopsy represents the gold-standard method of quantifying the degree of NAFLD (16). Although several noninvasive methods have been developed, liver biopsy represents the only reliable tool to distinguish between nonalcoholic fatty liver and nonalcoholic steatohepatitis. Analysis of liver specimens also provides exact data concerning steatosis, fibrosis, and inflammation. Hence, it is a powerful tool for quantifying the role of inherited predisposition in liver injury.

To further elucidate the role of the genetic predisposition in modulation of NAFLD, we performed genetic analyses in a large cohort of patients with fatty liver to analyze the signs of liver injury in combination with the carriage of the *PNPLA3* p.I148M, *TM6SF2* p.E67K, and *MBOAT7* rs641738 variants The frequencies of these variants were related to *i*) results of liver biopsy, *ii*) circulating levels of markers of liver injury, and *iii*) metabolic traits. Analysis of genotype-phenotype interactions performed in this group of patients demonstrated different effects of the *PNPLA3*, *TM6SF2*, and *MBOAT7* variants on hepatic steatosis and fibrosis, underscoring the notion that they play distinct roles in NAFLD progression.

MATERIALS AND METHODS

Patients

Patients for the study were recruited in eight German university centers within the framework of the NAFLD Clinical Study Group (NAFLD CSG) project (17). In brief, the project was started in 2012 as a multicentric study in Germany and was intended to investigate triggers and modulators of NAFLD development, including common genetic variants. All patients gave written informed consent to participate in these studies. The ethical committees at participating centers approved the study protocol. Ethanol intake (>20 g per day for women and >30 g for men) was regarded as exclusion criterion. NAFLD was diagnosed either by imaging techniques (abdominal sonography, MRI, CT) or by liver biopsy. Liver biopsies were performed percutaneously under ultrasound guidance or intraoperatively. Acquired liver samples were evaluated by experienced local pathologists. The presence of acute and chronic liver diseases other than NAFLD was excluded in all patients. All study subjects underwent a standardized clinical examination. Fasted venous blood samples were drawn for routine biochemical analyses, including liver function tests and DNA genotyping. Liver function tests were determined by clinical-chemical assays in the central laboratories of participating centers. In a subgroup of 320 patients with NAFLD with available histology, hepatic steatosis (grades S0–S3) and fibrosis (grades F0–F4) were quantified according to the Kleiner score (18).

Genotyping of the *PNPLA3* (rs738409), *TM6SF2* (rs58542926), and *MBOAT7* (rs641738) variants

Genotyping of the *PNPLA3* (rs738409), *TM6SF2* (rs58542926), and *MBOAT7* (rs641738) variants was performed in a central laboratory (Homburg) by a technician blinded to the phenotype of patients. DNA was extracted from peripheral blood mononuclear cells using the DNeasy Blood and Tissue Kit (Qiagen). DNA concentrations were measured using a NanoDrop spectrophotometer. All variants were genotyped using TaqMan assays (19). The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2.

Statistical analysis

Unless stated otherwise, all statistical analyses were performed with SPSS 20.0 (SPSS, Munich, Germany) or GraphPad Prism 5.0 (GraphPad Software Inc., CA). Quantitative data were expressed as medians and ranges. The association between the *PNPLA3*, *TM6SF2*, and *MBOAT7* variants and markers of liver injury was tested using ANOVA with post hoc tests. Exact tests were performed to check the consistency of genotyping results in with Hardy-Weinberg equilibrium (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Genotype frequencies were compared in contingency tables. Power analysis was performed using PS: Power and Sample Size Calculation v.3.0 (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). Differences in anthropometric and clinical traits between patients with *PNPLA3* and *MBOAT7* genotypes were compared using linear regression analysis under an additive genetic model. Comparisons between carriers of the *TM6SF2* genotypes were performed under a dominant genetic model (due to the low number of homozygotes for the 167K mutant allele) using linear regression analysis. All models were adjusted for confounding factors (age, gender, BMI, diabetes mellitus, and statin use, as appropriate). The effects of the studied variants, as well as additional risk factors, on hepatic steatosis and fibrosis were analyzed in univariate and multivariate models using logistic regression analysis.

RESULTS

Characteristics of the study cohort

A total of 515 German patients with NAFLD (99.9% white) were recruited. **Table 1** summarizes the baseline data of this study cohort, and **Table 2** presents the results of liver biopsies in 320 biopsied patients. More women (54%)

TABLE 1. Baseline characteristics and genotype frequencies in the study cohort

Variables	Entire cohort	Biopsied patients
N (female/male)	515 (280/235)	320 (186/134)
Age (years)	50 (16–88)	49 (16–88)
BMI (kg/m^2)	32 (17–70)	33 (17–69)
ALT (U/l)	52 (12–279)	58 (13–279)[a]
AST (U/l)	38 (5–397)	42 (4–397)[a]
GGT (U/l)	61 (4–1,658)	67 (4–1,463)
Triglycerides (mg/dl)	152 (45–770)	154 (49–770)
Total cholesterol (mg/dl)	204 (72–379)	206 (107–379)
Glucose (mg/dl)	98 (55–367)	99 (63–286)
Incidence of diabetes type 2 (%)	24.7	26.7
Statin use (%)	10.6	10.6
TM6SF2 p.E167K genotypes (n)		
[EE]	409	253
[EK]	97	61
[KK]	9	6
PNPLA3 p.I148M genotypes (n)		
[II]	215	126
[IM]	222	138
[MM]	78	56
MBOAT7 rs641738 genotypes (n)		
[CC]	159	98
[CT]	242	157
[TT]	114	65

E, glutamic acid; I, isoleucine; K, lysine; M, methionine; *MBOAT7*, membrane bound O-acyltransferase domain containing 7; p, protein (amino acid number); *PNPLA3*, patatin-like phospholipase domain-containing protein 3; *TM6SF2*, transmembrane 6 superfamily member 2. Values are given as medians (ranges), unless stated otherwise.

[a]$P < 0.001$ as compared with nonbiopsied individuals.

than men (46%) were included. The median age was 50 years. In 320 patients who underwent liver biopsy, 57% had steatosis grades 2 or 3 (Table 2). Fibrosis stage F2 or higher was present in 30% of patients. Patients undergoing liver biopsy had significantly higher alanine transaminase (ALT) and aspartate aminotransferase (AST) (both $P < 0.001$) but not γ−glutamyl transferase (GGT) activities ($P = 0.26$) (Table 1). We did not detect any differences in serum glucose, triglyceride, and cholesterol concentrations between biopsied and nonbiopsied patients (all $P > 0.05$). Individuals presenting with steatosis grade 2 or 3 had significantly higher serum glucose ($P = 0.002$) and triglyceride ($P = 0.025$) concentrations as compared with individuals with lower grades of steatosis (Fig. 1A, B). There were no differences in terms of serum cholesterol in relation to hepatic steatosis ($P > 0.05$) (Fig. 1C).

TABLE 2. Distribution of steatosis and fibrosis in biopsied individuals with NAFLD

Biopsy results	Distribution
Grade of steatosis[a]	
0/1	48%
2	27%
3	25%
Grade of fibrosis[b]	
0/1	70%
2	16%
3	7%
4	7%

[a]Data available for 320 patients.
[b]Data available for 295 patients.

PNPLA3 p.I148M and *TM6SF2* p.E167K variants are associated with increased serum markers of liver injury

The *PNPLA3* p.I148M, *TM6SF2* p.E167K, and *MBOAT7* rs641738 variants were successfully genotyped in all patients. The genotype frequencies (Table 1) do not differ from frequencies presented in previous publications and are localized on the Hardy-Weinberg equilibrium parabola ($P > 0.05$, exact test), which validates the genotyping quality. Relations of the studied variants to patient baseline characteristics are presented in supplemental Table S1 (for *PNPLA3* p.I148M), supplemental Table S2 (for *TM6SF2* p.E167K), and supplemental Table S3 (for the *MBOAT7* rs641738). As presented in the supplemental materials, the *PNPLA3* and *TM6SF2* variants were significantly associated with BMI (both $P = 0.01$). We did not detect any significant association between clinical characteristics and the *MBOAT7* polymorphism (supplemental Table S3). In the entire cohort (i.e., 515 patients with NAFLD), the *PNPLA3* p.I148M polymorphism was associated with increased serum AST (ANOVA, $P < 0.001$) (**Fig. 2A**) and ALT (ANOVA, $P = 0.002$) (Fig. 2B) but not with GGT activities (ANOVA, $P = 0.74$) (Fig. 2C). Similarly, the *TM6SF2* variant was associated with increased AST ($P < 0.001$) (Fig. 2D) and ALT ($P = 0.011$) (Fig. 2E) but not with GGT activities ($P = 0.14$) (Fig. 2F). We did not detect any significant association between the *MBOAT7* polymorphism and liver function tests (all $P > 0.05$) (Fig. 2G–I). We detected a significant ($P < 0.0001$) increase of serum AST activities with the increment of risk alleles of either of the genotypes (Fig. 3A). We also detected trends for increased ALT ($P = 0.08$) and GGT ($P = 0.07$) levels with increasing risk allele number (Fig. 3B, C).

PNPLA3 p.I148M and *TM6SF2* p.E167K have different effects on hepatic steatosis and fibrosis

We performed separate analysis of the variants' effects on the risk of developing hepatic steatosis and fibrosis in specimens acquired by liver biopsy. Overall, carriers of the *PNPLA3* risk allele ($P = 0.043$), but not *TM6SF2* or *MBOAT7* variants (both $P > 0.05$), were more frequently scheduled for liver biopsy. The *PNPLA3* polymorphism was significantly associated with the risk of developing steatosis grades S2 and S3 [common odds ratio (OR) = 1.896; $P < 0.001$] and fibrosis stages F2–F4 (common OR, 2.348; $P < 0.001$) (**Tables 3** and **4**). Analysis of *TM6SF2* genotype frequencies (**Tables 5** and **6**) reveals that this variant was associated with steatosis (common OR, 1.539; $P = 0.003$) but had no major effects on fibrosis ($P > 0.05$). Based on the frequency of the minor allele among individuals with fibrosis grade <F2 (Table 6), this analysis had a power of 0.81 to detect genetic effects with OR of at least 2.0. Although the *MBOAT7* polymorphism was not associated with hepatic steatosis (all $P > 0.05$), it was significantly associated with the development of liver fibrosis (common OR, 1.446; $P = 0.046$) (**Table 7**). We also detected an increase in the number of risk *PNPLA3*, *TM6SF2*, and *MBOAT7* alleles with increasing hepatic fibrosis (supplemental Fig. S1) and most of all steatosis (supplemental Fig. S2). In the univariate model, *PNPLA3* and *TM6SF2* polymorphisms, but not *MBOAT7*, were associated with increased steatosis

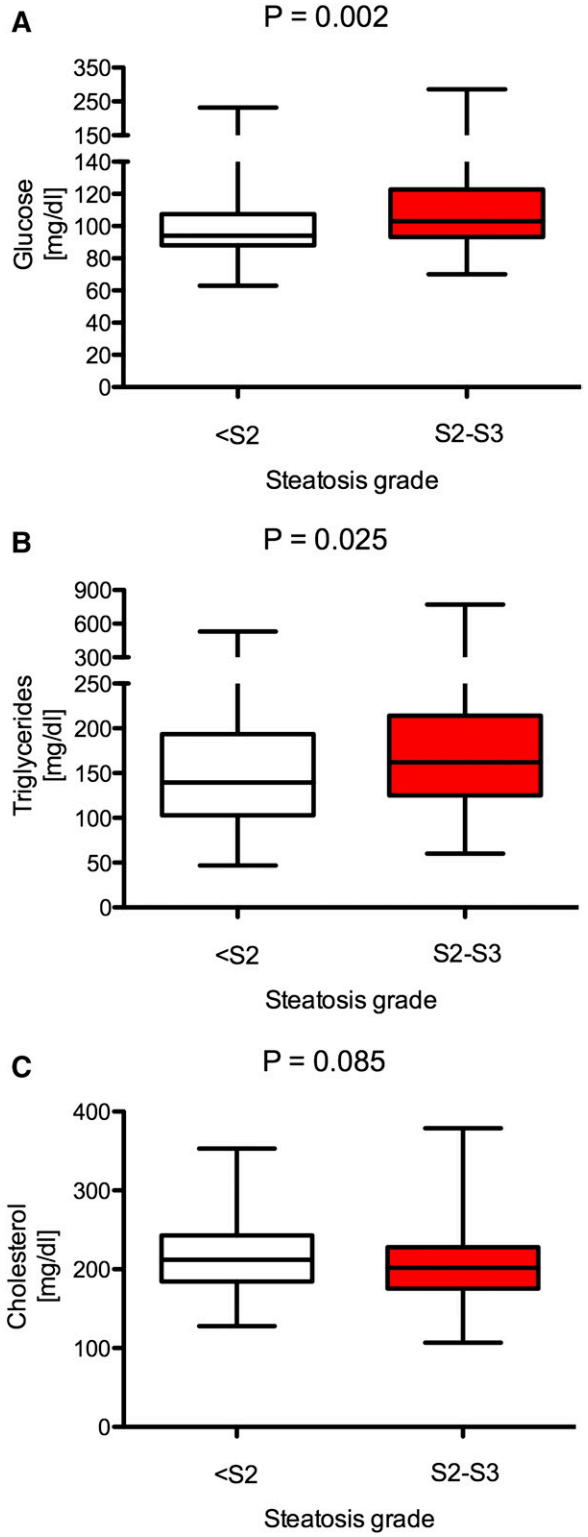

Fig. 1. Relation between steatosis grade at liver biopsy and metabolic traits. Increased steatosis was associated with higher serum glucose (A) and triglyceride (B) levels, but it did not affect total cholesterol (C).

(**Table 8**). The association remained significant for these two genotypes in the multivariate analysis (Table 8). In the analyses of liver function tests restricted to biopsied patients, the *PNPLA3* polymorphism was associated with significantly increased AST ($P = 0.013$) (supplemental Fig. S3A) but not ALT ($P = 0.17$) (supplemental Fig. S3B) or GGT ($P = 0.13$) (supplemental Fig. S3C). Notably, among individuals scheduled for the liver biopsy, the *TM6SF2* polymorphism was associated with increased AST ($P = 0.005$) (supplemental Fig. S3D), ALT ($P = 0.025$) (supplemental Fig. S3E), and GGT ($P = 0.025$) (supplemental Fig. S3F). We did not detect any significant association between liver function test and the *MBOAT7* polymorphism in biopsied patients (supplemental Fig. S3G-I). **Table 9** summarizes the results of regression analyses for factors associated with liver fibrosis in biopsied patients. Of note, in the multivariate model we detect a significant association for *PNPLA3* and *MBOAT7* genotypes (both $P < 0.05$) but not for the *TM6SF2* polymorphism ($P > 0.05$).

DISCUSSION

In the current study we analyzed a thoroughly phenotyped cohort of patients with NAFLD. According to current knowledge (2, 5), the three variants that we chose to genotype might play major roles in the development of hepatic steatosis. We demonstrate that both *PNPLA3* and *TM6SF2* polymorphisms are associated with increased aminotransferase activities, which might mirror enhanced liver injury in NAFLD. However, the analysis of biopsy samples underscored that the deleterious effects conferred by the tested variants are apparently related to distinct mechanisms: whereas the *PNPLA3* genotype modulates the progression of both fibrosis and steatosis, the *TM6SF2* variant seems to be predominantly associated with steatosis. The *MBOAT7* polymorphism is likely to be, in turn, associated with the risk of liver scarring.

Our observations with respect to the *PNPLA3* variant are in line with the majority of previous studies in patients with NAFLD (6). Patients with *PNPLA3*-associated steatohepatitis are known to be at risk of progressive liver fibrosis, cirrhosis, and eventually hepatocellular carcinoma (20). Previously published results concerning the *TM6SF2* variant are less consistent. The association between this variant and fibrosis postulated by Liu et al. (11) was not replicated a biopsy-based study from Argentina (21). In this study, the authors analyzed a total of 361 patients, among them 226 with biopsy-proven mostly mild NAFLD, and found a genetic association with steatosis but not with fibrosis. These results are in line with our study. In contrast, Sookoian et al. (21) did not detect any major effects of the *TM6SF2* polymorphism on liver function tests. Interestingly, the analysis of patients with chronic HCV infection (10) provided hints that the presence of variant *TM6SF2* enhances liver fibrogenesis in this setting. Also, alcoholics carrying the susceptible *TM6SF2* genotype seem to be at risk of liver cirrhosis (14). Recently, Eslam et al. (22) analyzed the effects of this variant on metabolic traits and liver status in a cohort of 3,260 individuals, among which a total of 502 presented with NAFLD. In this study, variant *TM6SF2* was overrepresented in patients with NAFLD, among whom presence of the minor *TM6SF2* allele was associated with increased fibrosis and lower serum triglycerides. It did not

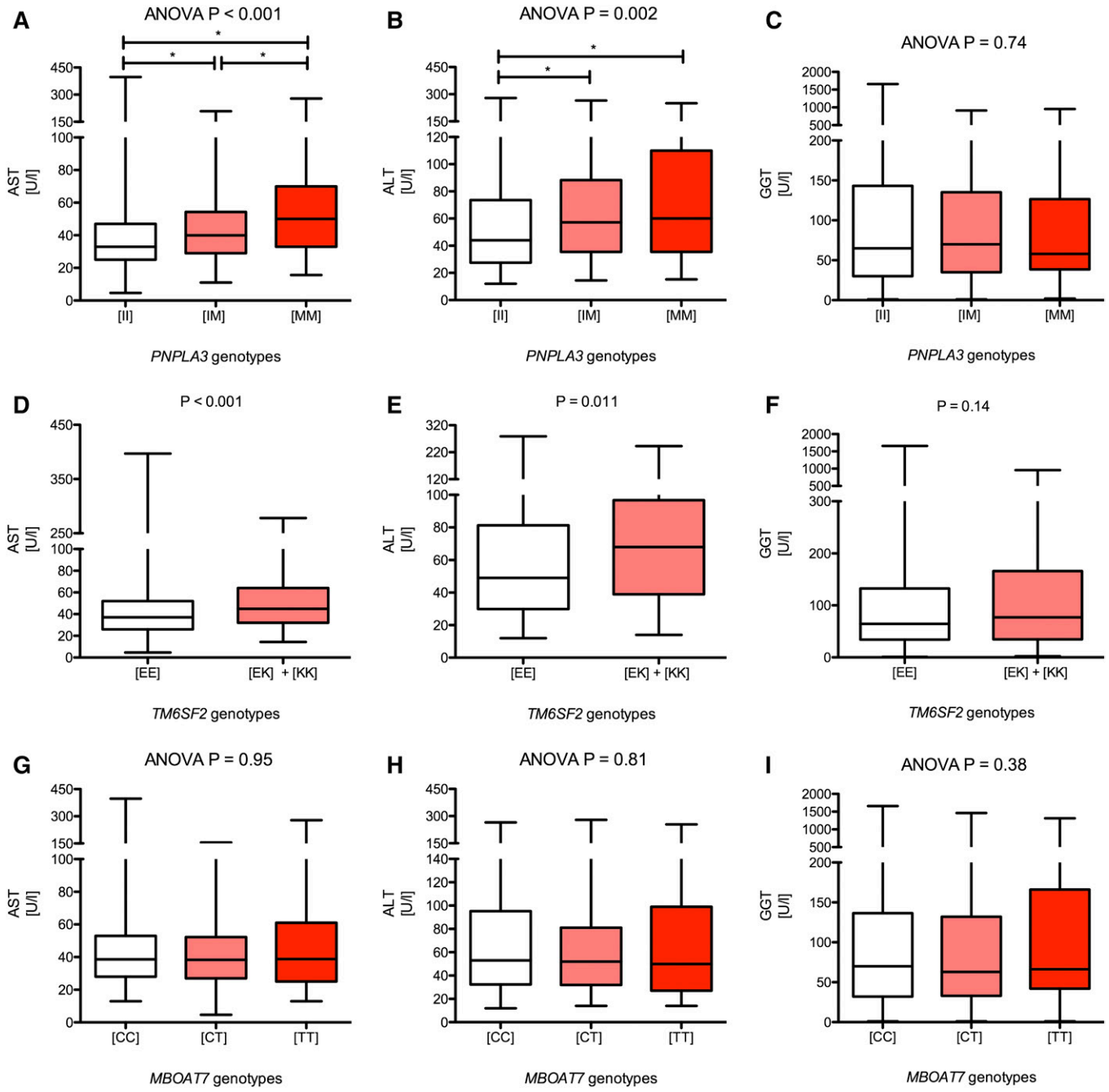

Fig. 2. Box-and-whisker plots illustrating liver function tests in carriers of distinct *PNPLA3*, *TM6SF2*, and *MBOAT7* variants. Carriers of either *PNPLA3* or *TM6SF2* risk alleles present with increased AST and ALT activities (A and B for *PNPLA3*; D and E for *TM6SF2*). We did not detect any major effects of these variants on the GGT activities (C and F). The *MBOAT7* polymorphism did not affect liver function tests. All tests were performed using ANOVA with post hoc tests or with Mann-Whitney *U* as appropriate. $*P < 0.05$ in post hoc tests.

affect other metabolic traits, NAFLD activity score (NAS) or transaminase activities. Overall, these data might suggest that the *TM6SF2* polymorphism is associated with advanced liver fibrosis in the presence of additional nongenetic factors (e.g., alcohol or viral hepatitis). However, these additional factors that might promote fibrogenesis in patients with NAFLD carrying the *TM6SF2* risk genotype are yet to be defined. The *MBOAT7* polymorphism has lately emerged as a new risk factor for severe liver diseases. First detected by Buch et al. (14) as a genetic determinant of an increased cirrhosis risk in alcoholics, it was subsequently associated with severe NAFLD by Mancina et al. (13). Most recently, it was demonstrated that its presence is associated with an increased fibrosis risk in patients with HCV (15). The association between fibrosis and *MBOAT7* in our cohort is, hence, in line with the previous studies.

We did not detect a link between the *MBAOT7* genotype and increased steatosis. This lack of association is in line with our recent results in patients undergoing bariatric surgery (23) but might be also related to an insufficient power of our cohort, which included fewer subjects than the above-mentioned studies (13–15). Furthermore, the

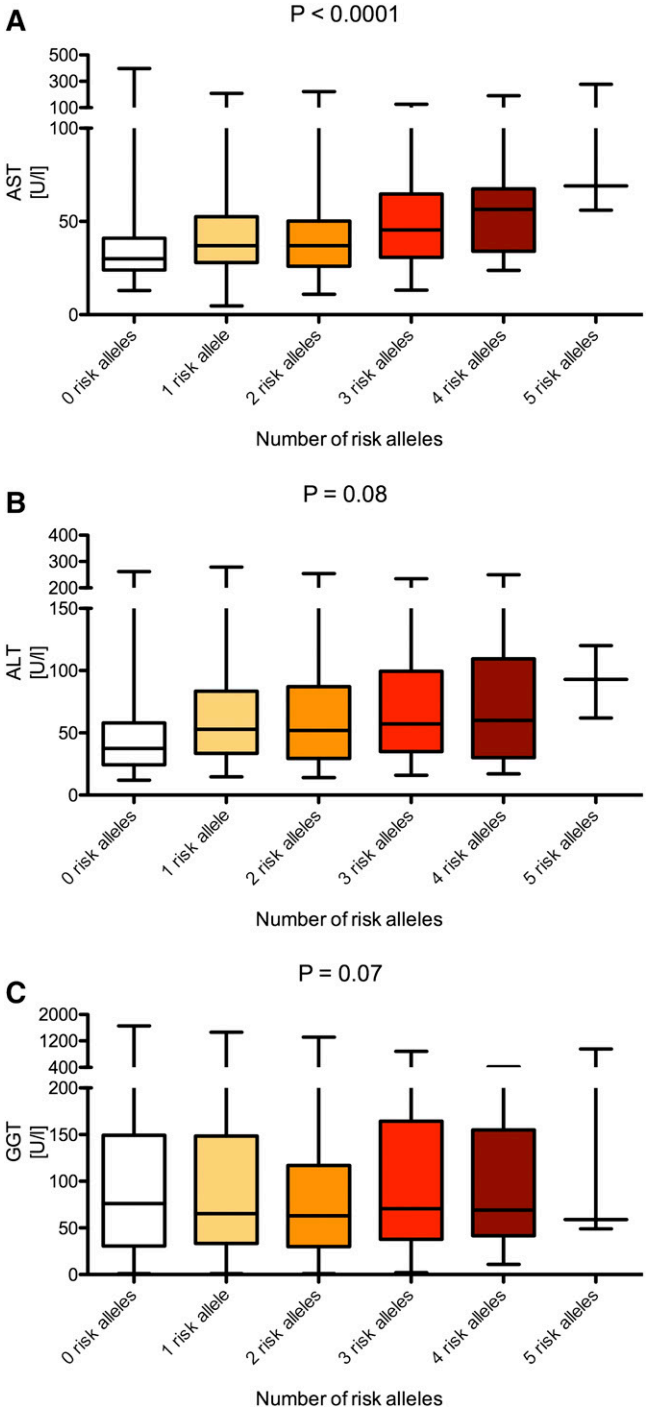

Fig. 3. Combined analysis of the *PNPLA3* p.I148M, *TM6SF2* p.E167K, and *MBOAT7* rs641738 risk alleles on liver function tests. The graphs demonstrate median AST (A), ALT (B), and GGT (C) by the number of risk alleles in either of the tested genes. Analyses were performed using trend test. The following frequencies of carriers of risk alleles were detected: zero risk alleles, n = 56; one risk allele, n = 142; two risk alleles, n = 170; three risk alleles, n = 117; four risk alleles, n = 27; five risk alleles, n = 3.

currently studied cohort encompassed well-characterized patients from eight centers, but each biopsy was evaluated only by local pathologists, so interobserver discrepancies in defining fibrosis and steatosis and their effects of the association tests could not be excluded.

TABLE 3. Distribution of alleles and genotypes for *PNPLA3* p.I148M and association tests in respect to steatosis grade

	Count of alleles/genotypes	
PNPLA3 p.I148M allele/genotype	Steatosis grade <S2 (2N = 306)	Steatosis S2–S3 (2N = 334)
[I]	211 (0.69)	197 (0.54)
[M]	95 (0.31)	155 (0.46)
[II]	73 (0.48)	53 (0.31)
[IM]	65 (0.42)	73 (0.44)
[MM]	15 (0.10)	41 (0.25)
Association test	OR	*P* value
Armitage's trend test	1.896	<0.001
OR statistics	OR (95% CI)	*P* value
[M] ↔ [I]	1.923 (1.391–2.659)	<0.001
[MM] ↔ [II]	3.765 (1.890–7.499)	<0.001
[MM] ↔ [IM + II]	2.994 (1.580–5.671)	<0.001
[MM + IM] ↔ [II]	1.936 (1.246–3.091)	0.003

I, isoleucine; M, methionine; p, protein (amino acid number); *PNPLA3*, adiponutrin. [M] represents the steatosis risk allele. Allele and genotype frequency differences were assessed by chi^2 test or by Armitage's trend test as appropriate (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Metabolic syndrome is believed to be the major trigger of hepatic steatosis. The presence of steatosis was associated with increased serum glucose and triglyceride concentrations in our cohort as well (Fig. 1). No major association between the risk of *PNPLA3*, *TM6SF2*, or *MABOT7* genotypes and distorted metabolic status has been described. Because *PNPLA3*- and *TM6SF2*-driven steatosis might even be "metabolically silent" (12), the inclusion of these two genotypes in the diagnostic work-up of patients with NAFLD could help, together with a detailed analysis of environmental determinants of fatty liver, to identify individuals at increased risk of liver injury even in the absence of the full ensemble of metabolic traits commonly associated with fatty liver disease. By adding the *MBOAT7* polymorphism as the third genetic factor to the clinical work-up of the patients with NAFLD, one could further improve the chance of detecting patients who are at risk of liver fibrosis. According to a recent study (24), fibrosis represents the

TABLE 4. Distribution of alleles and genotypes for *PNPLA3* p.I148M and association tests in respect to fibrosis grade

	Count of alleles/genotypes	
PNPLA3 p.I148M allele/genotype	Fibrosis grade <F2 (2N = 410)	Fibrosis grade F2–F4 (2N = 180)
[I]	211 (0.69)	197 (0.54)
[M]	95 (0.31)	155 (0.46)
[II]	95 (0.46)	18 (0.20)
[IM]	83 (0.41)	44 (0.49)
[MM]	27 (0.13)	28 (0.31)
Association test	OR	*P* value
Armitage's trend test	2.348	<0.001
OR statistics	OR (95% CI)	*P* value
[M] ↔ [I]	2.491 (1.740–3.565)	<0.001
[MM] ↔ [II]	5.473 (2.637–11.361)	<0.001
[MM] ↔ [IM + II]	2.977 (1.630–5.439)	<0.001
[MM + IM] ↔ [II]	3.455 (1.925–6.200)	<0.001

CI, confidence interval; I, isoleucine; M, methionine; p, protein (amino acid number); *PNPLA3*, adiponutrin. [M] represents the steatosis risk allele. Allele and genotype frequency differences were assessed by chi^2 test or by Armitage's trend test as appropriate (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

TABLE 5. Distribution of alleles and genotypes for *TM6SF2* p.E167K and association tests with respect to steatosis grade

	Count of alleles/genotypes	
TM6SF2 p.E167K allele/genotype	Steatosis grade <S2 (2N = 306)	Steatosis S2–S3 (2N = 334)
[E]	280 (0.92)	287 (0.86)
[K]	26 (0.08)	47 (0.14)
[EE]	130 (0.85)	123 (0.74)
[EK]	20 (0.13)	41 (0.25)
[KK]	3 (0.02)	3 (0.01)
Association test	OR	*P* value
Armitage's trend test	1.539	0.003
OR statistics	OR (95% CI)	*P* value
[K] ↔ [E]	1.764 (1.063–2.927)	0.026
[KK] ↔ [EE]	1.057 (0.209–5.336)	0.946
[KK] ↔ [EK + EE]	0.915 (0.182–4.601)	0.913
[KK + EK] ↔ [EE]	2.022 (1.153–3.544)	0.001

CI, confidence interval; E, glutamic acid; K, lysine; p, protein (amino acid number); *TM6SF2*, transmembrane 6 superfamily member 2. [K] represents the steatosis risk allele. Allele and genotype frequency differences were assessed by chi^2 test or by Armitage's trend test as appropriate (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

TABLE 7. Distribution of alleles and genotypes for *MBOAT7* rs641738 and association tests in respect to fibrosis grade

	Count of alleles/genotypes	
MBOAT7 rs641738 allele/genotype	Fibrosis ade F0 (2N = 206)	Fibrosis grade F1–F4 (2N = 384)
[C]	122 (0.59)	194 (0.51)
[T]	44 (0.41)	190 (0.49)
[CC]	34 (0.33)	53 (0.28)
[CT]	54 (0.52)	88 (0.46)
[TT]	15 (0.15)	51 (0.26)
Association test	OR	*P* value
Armitage's trend test	1.446	0.046
OR statistics	OR (95% CI)	*P* value
[T] ↔ [C]	1.422 (1.010–2.003)	0.043
[TT] ↔ [CC]	2.181 (1.063–4.476)	0.031
[TT] ↔ [CT + CC]	2.122 (0.251–9.319)	0.012
[TT + CT] ↔ [CC]	1.292 (0.770–2.170)	0.350

CI, confidence interval; p, protein (amino acid number); *MBOAT7*, membrane bound O-acyltransferase domain containing 7. [T] represents the risk allele. Allele and genotype frequency differences were assessed by chi^2 test or by Armitage's trend test as appropriate (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

most important factor affecting the long-term survival in patients with NAFLD. Currently, liver biopsy is mostly recommended in patients with signs of severe steatohepatitis or fibrosis, which might lead to a selection bias in genetic studies. Hence, combined analyses of invasive and noninvasive markers of liver injury might be required in the future to elucidate the risks conferred by the *PNPLA3*, *TM6SF2*, and *MBOAT7* variants.

Recently, it has been suggested that increased serum aminotransferase activities in patients with NAFLD might indicate metabolic adaptation of the liver to the fat overload rather than hepatic injury (25). Hence, it is not surprising that although both *PNPLA3* and *TM6SF2* polymorphisms were associated in our patients with NAFLD with increased liver functions tests, analyses of liver biopsy results demonstrate their different involvement in steatosis and fibrosis. Although the *PNPLA3* and *MBOAT7* risk variants display a clear association with NAFLD-driven liver fibrosis, in our cohort the *TM6SF2* polymorphism was linked solely to the grade of steatosis. Importantly, the presence of variant *TM6SF2* might even represent a protective factor against metabolic challenges (9, 12, 26). Based on our current results, one can still argue that, in comparison to the *TM6SF2* and *MBOAT7* genotypes, the *PNPLA3* p.I148M variant plays a more important role as the determinant of severe hepatic phenotypes ranging from steatosis to fibrosis and cirrhosis. This is in line with our latest controlled-attenuation, parameter-based study in patients with chronic liver diseases (19). In this analysis, we did not identify any major effects of the *TM6SF2* variant on liver injury, which, however, were detected in carriers of the *PNPLA3* minor allele. These discrepancies might be related to substantially higher frequencies of the *PNPLA3* risk allele in the general population as compared with the

TABLE 6. Distribution of alleles and genotypes for *TM6SF2* p.E167K and association tests with respect to fibrosis grade

	Count of alleles/genotypes	
TM6SF2 p.E167K allele/genotype	Fibrosis grade <F2 (2N = 410)	Fibrosis grade F2–F4 (2N = 180)
[E]	366 (0.89)	156 (0.87)
[K]	44 (0.11)	24 (0.13)
[EE]	164 (0.80)	68 (0.76)
[EK]	38 (0.19)	20 (0.22)
[KK]	3 (0.01)	2 (0.02)
Association test	OR	*P* value
Armitage's trend test	1.269	0.370
OR statistics	OR (95% CI)	*P* value
[K] ↔ [E]	1.280 (0.752–2.177)	0.362
[KK] ↔ [EE]	1.608 (0.269–9.838)	0.608
[KK] ↔ [EK + EE]	1.530 (0.251–9.319)	0.642
[KK + EK] ↔ [EE]	1.294 (0.717–2.335)	0.391

CI, confidence interval; E, glutamic acid; K, lysine; p, protein (amino acid number); *TM6SF2*, transmembrane 6 superfamily member 2. [K] represents the steatosis risk allele. Allele and genotype frequency differences were assessed by chi^2 test or by Armitage's trend test as appropriate (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

TABLE 8. Risk factors for developing hepatic steatosis

Factor	OR	95% CI	*P* value
	Univariate analysis		
PNPLA3 p.I148M	2.418	1.323–4.419	0.004
TM6SF2 p.E167K	4.622	1.077–19.831	0.039
MBOAT7 rs641738	1.260	0.749–2.119	0.384
Glucose	1.015	0.994–1.037	0.168
BMI	0.966	0.933–1.001	0.055
Age (years)	1.005	0.979–1.033	0.692
Sex	2.080	0.933–4.634	0.073
Presence of diabetes	1.224	0.504–2.973	0.656
Triglycerides	1.002	0.996–1.007	0.594
Cholesterol	0.997	0.988–1.007	0.539
	Multivariate analysis		
PNPLA3 p.I148M	2.424	1.326–4.419	0.004
TM6SF2 p.E167K	4.725	1.093–20.429	0.038

CI, confidence interval; E, glutamic acid; I, isoleucine; K, lysine; M, methionine; *MBOAT7*, membrane bound O-acyltransferase domain containing 7; p, protein (amino acid number); *PNPLA3*, adiponutrin; *TM6SF2*, transmembrane 6 superfamily member 2. The relationships between steatosis *PNPLA3*, *TM6SF2*, and *MBOAT7* variants as well as other potentially prosteatotic factors were assessed by univariate and multivariate logistic regression analysis. Genetic analyses were calculated by using either additive (for *PNPLA3* and *MBOAT7*) or dominant (for *TM6SF2*) models.

TABLE 9. Risk factors for developing hepatic fibrosis

Factor	OR	95% CI	*P* value
Univariate analysis			
PNPLA3 p.I148M	1.679	1.192–2.367	0.003
TM6SF2 p.E167K	1.060	0.587–1.914	0.846
MBOAT7 rs641738	1.410	1.003–1.982	0.048
Glucose	1.020	1.008–1.033	0.002
BMI	0.989	0.965–1.015	0.413
Age (years)	1.020	1.002–1.039	0.027
Sex	1.088	0.671–1.763	0.732
Presence of diabetes	2.092	1.136–3.852	0.018
Triglycerides	1.003	1.000–1.007	0.083
Cholesterol	0.997	0.991–1.003	0.314
Multivariate analysis			
PNPLA3 p.I148M	1.676	1.019–2.757	0.042
MBOAT7 rs641738	1.766	1.089–2.864	0.021

CI, confidence interval; E, glutamic acid; I, isoleucine; K, lysine; M, methionine; *MBOAT7*, membrane bound O-acyltransferase domain containing 7; p, protein (amino acid number); *PNPLA3*, adiponutrin; *TM6SF2*, transmembrane 6 superfamily member 2 The relationships between steatosis *PNPLA3*, *TM6SF2*, and *MBOAT7* variants as well as other potentially profibrotic factors were assessed by univariate and multivariate logistic regression analysis. Genetic analyses were calculated using either additive (for *PNPLA3* and *MBOAT7*) and dominant (for *TM6SF2*) models.

TM6SF2 minor allele. Indeed, the first one is carried by ~10% of Europeans in the homozygous form, whereas <1% of individuals are homozygous carriers of the *TM6SF2* p.167K allele. Hence, as in the case of *MBOAT7*, larger cohorts of patients might be required to fully elucidate the involvement of the *TM6SF2* polymorphism in hepatic injury. Indeed, as described by Mancina et al. (13), the *PNPLA3* variant has larger impact on the whole spectrum of liver disease than *TM6SF2*. Therefore, we could not exclude the possibility that the lack of association may be related to insufficient power in our study.

In conclusion, *PNPLA3*, *TM6SF2*, and *MBOAT7* variants might be associated with liver injury in patients with NAFLD. Carriers of variant *PNPLA3* present with progressive disease, but the *TM6SF2* and *MBAOT7* polymorphisms might also have deleterious effects on liver health. Future longitudinal studies are warranted to fully elucidate the involvement of these variants in the modulation of NAFLD.

The authors thank Annika Bohner (Homburg) for technical assistance.

REFERENCES

1. Marchesini, G., S. Petta, and R. Dalle Grave. 2016. Diet, weight loss, and liver health in nonalcoholic fatty liver disease: pathophysiology, evidence, and practice. *Hepatology.* **63:** 2032–2043.
2. Anstee, Q. M., and C. P. Day. 2015. The genetics of nonalcoholic fatty liver disease: spotlight on PNPLA3 and TM6SF2. *Semin. Liver Dis.* **35:** 270–290.
3. Romeo, S., J. Kozlitina, C. Xing, A. Pertsemlidis, D. Cox, L. A. Pennacchio, E. Boerwinkle, J. C. Cohen, and H. H. Hobbs. 2008. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* **40:** 1461–1465.
4. Kozlitina, J., E. Smagris, S. Stender, B. G. Nordestgaard, H. H. Zhou, A. Tybjaerg-Hansen, T. F. Vogt, H. H. Hobbs, and J. C. Cohen. 2014. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* **46:** 352–356.
5. Krawczyk, M., P. Portincasa, and F. Lammert. 2013. PNPLA3-associated steatohepatitis: toward a gene-based classification of fatty liver disease. *Semin. Liver Dis.* **33:** 369–379.
6. Sookoian, S., and C. J. Pirola. 2011. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology.* **53:** 1883–1894.
7. Fan, J. H., M. Q. Xiang, Q. L. Li, H. T. Shi, and J. J. Guo. 2016. PNPLA3 rs738409 polymorphism associated with hepatic steatosis and advanced fibrosis in patients with chronic hepatitis C virus: a meta-analysis. *Gut Liver.* **10:** 456–463.
8. Salameh, H., E. Raff, A. Erwin, D. Seth, H. D. Nischalke, E. Falleti, M. A. Burza, J. Leathert, S. Romeo, A. Molinaro, et al. 2015. PNPLA3 gene polymorphism is associated with predisposition to and severity of alcoholic liver disease. *Am. J. Gastroenterol.* **110:** 846–856.
9. Pirola, C. J., and S. Sookoian. 2015. The dual and opposite role of the TM6SF2-rs58542926 variant in protecting against cardiovascular disease and conferring risk for nonalcoholic fatty liver: a meta-analysis. *Hepatology.* **62:** 1742–1756.
10. Milano, M., A. Aghemo, R. M. Mancina, J. Fischer, P. Dongiovanni, S. De Nicola, A. L. Fracanzani, R. D'Ambrosio, M. Maggioni, R. De Francesco, et al. 2015. Transmembrane 6 superfamily member 2 gene E167K variant impacts on steatosis and liver damage in chronic hepatitis C patients. *Hepatology.* **62:** 111–117.
11. Liu, Y. L., H. L. Reeves, A. D. Burt, D. Tiniakos, S. McPherson, J. B. Leathart, M. E. Allison, G. J. Alexander, A. C. Piguet, R. Anty, et al. 2014. TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. *Nat. Commun.* **5:** 4309.
12. Zhou, Y., G. Llaurado, M. Oresic, T. Hyotylainen, M. Orho-Melander, and H. Yki-Jarvinen. 2015. Circulating triacylglycerol signatures and insulin sensitivity in NAFLD associated with the E167K variant in TM6SF2. *J. Hepatol.* **62:** 657–663.
13. Mancina, R. M., P. Dongiovanni, S. Petta, P. Pingitore, M. Meroni, R. Rametta, J. Boren, T. Montalcini, A. Pujia, O. Wiklund, et al. 2016. The MBOAT7–TMC4 variant rs641738 increases risk of nonalcoholic fatty liver disease in individuals of European descent. *Gastroenterology.* **150:** 1219–1230 e6.
14. Buch, S., F. Stickel, E. Trepo, M. Way, A. Herrmann, H. D. Nischalke, M. Brosch, J. Rosendahl, T. Berg, M. Ridinger, et al. 2015. A genome-wide association study confirms PNPLA3 and identifies TM6SF2 and MBOAT7 as risk loci for alcohol-related cirrhosis. *Nat. Genet.* **47:** 1443–1448.
15. Thabet, K., A. Asimakopoulos, M. Shojaei, M. Romero-Gomez, A. Mangia, W. L. Irving, T. Berg, G. J. Dore, H. Gronbaek, D. Sheridan, et al., International Liver Disease Genetics Consortium. 2016. MBOAT7 rs641738 increases risk of liver inflammation and transition to fibrosis in chronic hepatitis C. *Nat. Commun.* **7:** 12757.
16. Chalasani, N., Z. Younossi, J. E. Lavine, A. M. Diehl, E. M. Brunt, K. Cusi, M. Charlton, A. J. Sanyal. 2012. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology.* **142:** 1592–1609.
17. Weiss, J., M. Rau, H. Bantel, H. Bock, M. Demir, J. Kluwe, M. Krawczyk, A. Pathil-Warth, J. M. Schattenberg, F. Tacke, et al. 2015. First data concerning the medical supply of patients with nonalcoholic fatty liver disease in Germany: a survey in university hospital centers of hepatology. [Article in German] *Z. Gastroenterol.* **53:** 562–567.
18. Kleiner, D.E., E. M. Brunt, M. Van Natta, C. Behling, M. J. Contos, O. W. Cummings, L. D. Ferrell, Y. C. Liu, M. S. Torbenson, A. Unalp-Arida, M. Yeh, A. J. McCullough, and A. J. Sanyal. 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* **41:** 1313–1321.
19. Arslanow, A., C. S. Stokes, S. N. Weber, F. Grünhage, F. Lammert, and M. Krawczyk. 2016. The common PNPLA3 variant p.I148M is associated with liver fat contents as quantified by controlled attenuation parameter (CAP). *Liver Int.* **36:** 418–426.
20. Dongiovanni, P., S. Romeo, and L. Valenti. 2014. Hepatocellular carcinoma in nonalcoholic fatty liver: role of environmental and genetic factors. *World J. Gastroenterol.* **20:** 12945–12955.
21. Sookoian, S., G. O. Castano, R. Scian, P. Mallardi, T. Fernandez Gianotti, A. L. Burgueno, J. San Martino, and C. J. Pirola. 2015. Genetic variation in transmembrane 6 superfamily member 2 and the risk of nonalcoholic fatty liver disease and histological disease severity. *Hepatology.* **61:** 515–525.

22. Eslam, M., A. Mangia, T. Berg, H. L. Chan, W. L. Irving, G. J. Dore, M. L. Abate, E. Bugianesi, L. A. Adams, M. A. Najim, et al., International Liver Disease Genetics Consortium. 2016. Diverse impacts of the rs58542926 E167K variant in TM6SF2 on viral and metabolic liver disease phenotypes. *Hepatology.* **64:** 34–46.
23. Krawczyk, M., R. Jimenez-Aguero, J. M. Alustiza, J. I. Emparanza, M. J. Perugorria, L. Bujanda, F. Lammert, and J. M. Banales. 2016. PNPLA3 p.I148M variant is associated with greater reduction of liver fat content after bariatric surgery. *Surg. Obes. Relat. Dis.* In press.
24. Angulo, P., D. E. Kleiner, S. Dam-Larsen, L. A. Adams, E. S. Bjornsson, P. Charatcharoenwitthaya, P. R. Mills, J. C. Keach, H. D. Lafferty, A. Stahler, et al. 2015. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. *Gastroenterology.* **149:** 389–397.e10.
25. Sookoian, S., G. O. Castano, R. Scian, T. Fernandez Gianotti, H. Dopazo, C. Rohr, G. Gaj, J. San Martino, I. Sevic, D. Flichman, et al. 2016. Serum aminotransferases in nonalcoholic fatty liver disease are a signature of liver metabolic perturbations at the amino acid and Krebs cycle level. *Am. J. Clin. Nutr.* **103:** 422–434.
26. Musso, G., M. Cassader, E. Paschetta, and R. Gambino. 2016. TM6SF2 may drive postprandial lipoprotein cholesterol toxicity away from the vessel walls to the liver in NAFLD. *J. Hepatol.* **64:** 979–981.