Protein Phosphatase 1 Regulatory Subunit 3B Gene Variation Protects Against Hepatic Fat Accumulation and Fibrosis in Individuals at High Risk of Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver damage and has a strong genetic component. The rs4841132 G>A variant, modulating the expression of protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*), which is involved in glycogen synthesis, has been reported to reduce the risk of NAFLD but at the same time may favor liver disease by facilitating glycogen accumulation. The aim of this study was to assess the impact of rs4841132 on development of histologic steatosis and fibrosis in 1,388 European individuals in a liver biopsy cohort, on NAFLD hepatocellular carcinoma in a cross-sectional Italian cohort (n = 132 cases), and on liver disease at the population level in the United Kingdom Biobank cohort. We investigated the underlying mechanism by examining the impact of the variant on gene expression profiles. In the liver biopsy cohort, the rs4841132 minor A allele was associated with protection against steatosis (odds ratio [OR], 0.63; 95% confidence interval [CI], 0.42-0.95; *P* = 0.03) and clinically significant fibrosis (OR, 0.35; 95% CI, 0.14-0.87; *P* = 0.02) and with reduced circulating cholesterol (*P* = 0.02). This translated into protection against hepatocellular carcinoma development (OR, 0.22; 95% CI, 0.07-0.70; *P* = 0.01). At the population level, the rs4841132 variation was not associated with nonalcoholic or nonviral diseases of the liver but was associated with lower cholesterol (*P* = 1.7 × 10^{-8}). In individuals with obesity, the A allele protecting against steatosis was associated with increased *PPP1R3B* messenger RNA expression and activation of lipid oxidation and with down-regulation of pathways related to lipid metabolism, inflammation, and cell cycle. *Conclusion*: The rs4841132 A allele is associated with protection against hepatic steatosis and fibrosis in individuals at high risk of NAFLD but not in the general population and against dyslipidemia. The mechanism may be related to modulation of *PPP1R3B* expression and hepatic lipid metabolism. (*Hepatology Communications* 2018;2:666-675)

Nonalcoholic fatty liver disease (NAFLD) is becoming a leading cause of liver damage worldwide.[1] NAFLD pathogenesis is strongly intertwined with excessive adiposity, insulin resistance, and dyslipidemia.[2] Dietary factors, such as alcohol, fructose, and physical activity, are other major risk factors for this condition.[3] NAFLD has a strong genetic component, and variants in proteins regulating hepatocellular lipid handling, including patatin-like phospholipase domain-containing 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*), membrane bound O-acyltransferase domain-containing 7 (*MBOAT7*), and glucokinase regulator (*GCKR*), predispose to the development and progression to nonalcoholic steatohepatitis (NASH), which is the inflammatory form of NAFLD, and to hepatic fibrosis,[4,5]

which is the major prognostic determinant in patients with NAFLD.[6] By exploiting naturally occurring variation at these loci, we recently highlighted by a Mendelian randomization approach that hepatic fat accumulation is a key driver of liver disease progression and fibrosis development in at-risk individuals.[7] However, whether this is generalizable to other risk factors for NAFLD development and progression and for protective variants remains to be demonstrated.

Protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*) has been identified as another locus associated with hepatic fat content, as estimated by computed tomography in genome-wide association studies.[8] *PPP1R3B* encodes for a protein involved in glycogen synthesis.[9] Subsequent studies have been consistent with the hypothesis that the rs4240624 variant, which is located 175 kilobase (kb) upstream of the *PPP1R3B* coding region, associates with reduced fat as estimated by ultrasonography[10,11] and with increased hepatic glycogen content.[12] However, when liver fat was directly measured by histology, the association with the *PPP1R3B* variation was not confirmed.[8,13] Furthermore, Stender et al.[9] showed that the minor allele of rs4841132, which is in complete linkage disequilibrium with rs4240624, was associated with increased X-ray attenuation but not with decreased hepatic fat content in population studies. On the contrary, the minor A allele was associated with increased aminotransferases and liver disease.[9] Therefore, the overall impact of the *PPP1R3B* variation on hepatic fat and progressive liver damage remains controversial.

Within this context, we evaluated the impact of the rs4841132 G>A variant, located in *LOC157273*, 175 kb upstream of the *PPP1R3B* gene, on a histologic steatosis, metabolic profile and on liver damage in a large cohort of European individuals.[14] In addition, we evaluated the impact of the variant on liver

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TABLE 1. DEMOGRAPHIC, ANTHROPOMETRIC, AND CLINICAL FEATURES OF THE LIVER BIOPSY COHORT STRATIFIED FOR ENROLLMENT CRITERIA (LIVER CLINIC, BARIATRIC SURGERY) AND THE NAFLD-HCC COHORT

	Liver Biopsy Cohort (N = 1,388)				
	Liver Clinic (n = 509)	Bariatric Surgery (n = 879)	NAFLD-HCC (n = 132)	P Value*	P Value†
Sex, M	409 (80)	218 (25)	101 (77)	<0.0001	<0.0001
Age, years	49.9 ± 12.4	44.9 ± 10.3	67.5 ± 1.05	<0.0001	<0.0001
BMI, kg/m²	28.4 ± 4.4	42.3 ± 6.7	29.3 ± 0.8	<0.0001	<0.0001
T2D, yes	111 (22)	227 (26)	76 (58)	0.06	<0.0001
Total cholesterol, mg/dL	201.1 ± 42.5	185.6 ± 46.4	170 ± 7.7	<0.0001	<0.0001
LDL cholesterol, mg/dL	124 ± 38.6	108.2 ± 38.6	100.5 ± 7.7	<0.0001	0.0008
HDL cholesterol, mg/dL	46.4 ± 15.4	50.3 ± 19.3	50.2 ± 3.9	0.41	0.61
Triglycerides, mg/dL	148.7 ± 78.7	139.9 ± 69.9	139.9 ± 017.5	0.03	0.79
ALT, IU/L‡	47 (29-74)	30 (23-41)	45 (28-69)	<0.0001	0.11
AST, IU/L‡	20 (16-27)	27 (18-42)	44 (30-61)	<0.0001	<0.0001
PNPLA3, I148M				<0.0001	<0.0001
I/I	180 (35.4)	464 (52.8)	31 (23.5)		
I/M	227 (44.6)	317 (36)	54 (419		
M/M	101 (19.8)	70 (8)	49 (37)		
TM6SF2, E167K				0.003	0.59
E/E	431 (84.7)	757 (86.1)	113 (85.6)		
E/K	70 (13.8)	87 (9.9)	19 (14.4)		
K/K	8 (1.6)	2 (0.2)	2 (1.5)		
MBOAT7, rs641738 C>T				0.98	0.04
C/C	179 (35)	295 (34)	28 (21.2)		
C/T	225 (44)	367 (42)	69 (52.2)		
T/T	105 (21)	172 (20)	35 (26.5)		
GCKR, P446L				0.0001	0.64
C/C	112 (22)	215 (24)	32 (24.2)		
C/T	235 (46)	368 (42)	65 (49.2)		
T/T	162 (32)	171 (19)	33 (25)		
PPP1R3B, rs4841132 G>A				0.01	0.04
G/G	433 (85)	698 (79)	124 (93.9)		
G/A	72 (14)	173 (20)	8 (6.1)		
A/A	4 (0.8)	8 (0.9)	0		

Values are reported as mean ± SD and number (%), except where noted. *Liver clinic versus bariatric surgery; †Milan liver biopsy cohort versus Italian NAFLD-HCC cohort; ‡median (interquartile range).
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; *GCKR,* glucokinase regulator; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *MBOAT7,* membrane bound O-acyltransferase domain-containing 7; *PNPLA3,* patatin-like phospholipase domain-containing 3; T2D, type 2 diabetes; *TM6SF2,* transmembrane 6 superfamily member 2.

diagnoses in the publicly available United Kingdom Biobank cohort. Finally, we explored the mechanism underlying the epidemiological association by examining the impact of the variant on gene expression profiles.

Patients and Methods

THE LIVER BIOPSY COHORT

The liver biopsy cohort (LBC) has been described.[5,15] A total of 1,388 adult individuals of European descent were consecutively enrolled from the Metabolic Liver Diseases outpatient service and

bariatric surgery center, Fondazione IRCCS Ca' Granda Ospedale Policlinico Milano, Milan, Italy, and from the Northern Savo Hospital District, Kuopio, Finland. Inclusion criteria were availability of a liver biopsy for suspected NASH or severe obesity, DNA samples, and clinical data. Individuals with excessive alcohol intake (men, >30 g/day; women, >20 g/day), viral and autoimmune hepatitis, or other causes of liver disease were excluded. The study conformed to the Declaration of Helsinki and was approved by the Institutional Review Board of the Fondazione Ca' Granda IRCCS of Milan and relevant Institutions. All participants gave written informed consent. The clinical features of individuals evaluated in the study are presented in Table 1.

HISTOLOGIC AND LIVER DAMAGE EVALUATION

Steatosis was graded into the following four categories based on the percentage of affected hepatocytes: 0, 0%-4%; 1, 5%-32%; 2, 33%-65%; and 3, 66%-100%. Disease activity was assessed according to the NAFLD activity score, with systematic evaluation of hepatocellular ballooning and necroinflammation; fibrosis was also staged according to the recommendations of the NAFLD Clinical Research Network.[16] The scoring of liver biopsies was performed by independent pathologists unaware of patient status and genotype.[15,17] NASH was diagnosed in the presence of steatosis, lobular necroinflammation, and hepatocellular ballooning.

NAFLD-HEPATOCELLULAR CARCINOMA COHORT

Part of the NAFLD-hepatocellular carcinoma (NAFLD-HCC) cohort of the exome sequencing for the identification of inherited variants associated with hepatocellular carcinoma in NAFLD (EPIDEMIC-NAFLD) study has been described.[18,19] Briefly, this is a case-control study in which we genotyped Italian patients with NAFLD-HCC and compared the frequency of risk variants with healthy individuals and with controls with advanced fibrosis due to NAFLD. The NAFLD-HCC patients of Italian descent were enrolled between January 2008 and January 2015 at the Milan, Udine, Turin, Rome, and Palermo hospitals. Diagnosis of HCC was based on the European Association for the Study of the Liver –European Organization for Research and Treatment of Cancer Clinical Practice Guidelines.[20] In the absence of liver biopsy, diagnosis of NAFLD required detection of ultrasonographic steatosis plus at least one criterion of the metabolic syndrome. Clinical features of these individuals are presented in Table 1.

GENOTYPING

The LBC cohort was genotyped for the rs738409 C>G (*PNPLA3* I148M), rs58542926 C>T (*TM6SF2* E167K), rs1260326 C>T (*GCKR* P446L), rs641738 C>T *MBOAT7*, and rs4841132 G>A (*LOC157273-PPP1R3B*) variants as described.[5,15] Genotyping of the LBC was performed in duplicate using TaqMan 5′-nuclease assays (Life Technologies, Carlsbad, CA). Genotype frequencies of the four variants were in agreement with Hardy-Weinberg proportions ($P > 0.1$).

UNITED KINGDOM BIOBANK COHORT

The associations of the rs4841132 variant with phenotype related to metabolic disorders and liver disease were evaluated in the United Kingdom Biobank cohort, a prospective population study of half a million subjects not selected for liver diseases. These subjects were identified at 22 centers across the United Kingdom during 2006 to 2010, and almost all were aged 40-69 years old. Freely available basic association data were downloaded from Neale Lab (http://geneatlas.roslin.ed.ac.uk).

TRANSCRIPTOMIC ANALYSIS

Gene expression was measured in a subset of 125 patients with obesity belonging to the Milan cohort, after percutaneous liver biopsy performed during bariatric surgery. Their clinical features are presented in Supporting Table S1. Total RNA was isolated using the miRNeasy mini-kit (Qiagen, Hulsterweg, Germany), according to the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer, and samples with RNA integrity numbers greater than or equal to 7 were used for library preparation. RNA sequencing was performed in paired-end mode with a read length of 150 nucleotides using the Illumina HiSeq 4000 (Novogene, Hong Kong, China). Raw reads were mapped against the Human Genome,[21] using a custom pipeline based on the standard primary analysis procedure. The pipeline performed the primary analysis step, including FASTQ quality check (FastQC software; Babraham Bioinformatics, Cambridge, United Kingdom), low-quality reads trimming with Trimmomatic,[22] and mapping on the GRCh37 reference genome using STAR mapper.[23] RNA sequencing quality control was performed, and samples with <10 million reads uniquely mapped or with <60% uniquely mapped/mapped reads were excluded from the analysis. Gene reads count (Ensemble human transcript reference assembly, version 75) was performed using RSEM software.[24] To quantify gene expression, RSEM per gene counts were normalized using the DESeq2 package.[25]

STATISTICAL ANALYSIS

For descriptive statistics, continuous variables were shown as mean and SD or median and interquartile range for highly skewed biological variables. Variables

TABLE 2. INDEPENDENT PREDICTORS OF LIVER DAMAGE (PRESENCE OF STEATOSIS, NASH, AND FIBROSIS STAGE F2-F4) IN THE LIVER BIOPSY COHORT

	Steatosis			NASH			Fibrosis F2-F4		
	OR	95% CI	P Value	OR	95% CI	P Value	OR	95% CI	P Value
Age, years	1.02	0.99-1.04	0.07	1.01	1.00-1.02	0.09	1.05	1.03-1.08	<0.001
Sex, M	1.49	0.94-2.39	0.09	1.61	1.19-2.17	0.002	0.99	0.54-1.84	0.99
BMI, kg/m^2	1.07	1.03-1.10	<0.001	1.07	1.04-1.09	<0.001	1.03	0.99-1.08	0.17
IFG/T2DM, yes	2.18	1.29-3.67	0.004	2.06	1.49-2.86	<0.001	3.43	1.93-6.11	<0.001
PNPLA3, I148M alleles	1.39	1.02-1.90	0.04	1.51	1.25-1.82	<0.001	2.24	1.54-3.26	<0.001
TM6SF2, E167K	1.62	0.83-3.13	0.16	1.88	1.32-2.68	<0.001	1.58	0.80-3.09	0.19
MBOAT7 rs641738 T	1.06	0.82-1.39	0.65	1.06	0.89-1.27	0.48	0.91	0.61-1.33	0.62
GCKR, L446P	1.06	0.80-1.39	0.69	1.05	0.88-1.25	0.62	0.77	0.53-1.12	0.17
PPP1R3B, rs4841132 A alleles	0.63	0.42-0.95	0.03	0.79	0.57-1.09	0.16	0.35	0.14-0.87	0.02

Logistic regression models were used to test the association of demographic, clinical, and genetic factors with liver damage. In addition to the predictors shown above, models were adjusted for indication for liver biopsy (severe obesity versus increased liver enzymes in NAFLD).
Abbreviations: BMI, body mass index; *GCKR,* glucokinase regulator; IFG, impaired fasting glucose; *MBOAT*7, membrane bound O-acyltransferase domain-containing 7; *PNPLA3,* patatin-like phospholipase domain-containing 3; *TM6SF2,* transmembrane 6 superfamily member 2; T2DM: type 2 diabetes mellitus.

with skewed distributions were log-transformed before analyses. Categorical variables were presented as number and proportion. All genetic analyses were performed under additive models.

Analyses were performed by fitting data into generalized linear regression models. In particular, general linear models were fit to examine continuous traits. Logistic regression models were fit to examine binary traits (NASH, severe fibrosis stage F3-F4), and ordinal regression models were fit for ordinal traits (components of the NAFLD activity score: severity of steatosis, necroinflammation and hepatocellular ballooning, stage of fibrosis). When specified, confounding factors (including recruitment center) were included in a model.

Statistical analyses were carried out using JMP 13.0 (SAS Institute, Cary, NC) and R statistical analysis software, version 3.3.2 (http://www.R-project.org/). *P* < 0.05 (two-tailed) was considered statistically significant.

Results

PPP1R3B GENE VARIATION IS ASSOCIATED WITH PROTECTION AGAINST HISTOLOGIC STEATOSIS

The frequency distribution of the rs4841132 G>A variant was in Hardy-Weinberg equilibrium (*P* = 0.75; Supporting Table S2). The independent predictors of liver damage in the liver biopsy cohort are shown in Table 2. Logistic regression analysis adjusted

for sex, age, body mass index, type 2 diabetes mellitus, indication for liver biopsy, and known genetic risk variants for NAFLD showed the rs4841132 minor A allele was associated with protection against steatosis (OR, 0.63; 95% confidence interval [CI], 0.42-0.95; *P* = 0.03) and against clinically significant fibrosis (OR, 0.35; 95% CI, 0.14-0.87; *P* = 0.02). With ordinal regression analysis adjusted as above, the minor A allele was associated with reduced steatosis grade
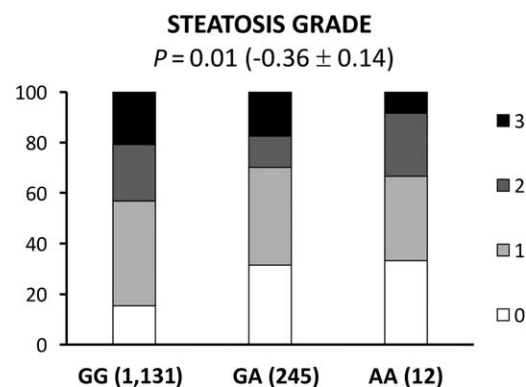
FIG. 1. In the LBC, the *PPP1R3B* rs4841132 variant is associated with protection against steatosis development. The association between the *PPP1R3B* rs4841132 variant and the risk of NAFLD was tested by multivariate ordinal regression analysis adjusted for age, sex, body mass index, presence of impaired fasting glucose/type 2 diabetes mellitus, *PNPLA3* I148M alleles, presence of the *TM6SF2* E167K variant, *MBOAT7/TMC4* T allele, *GCKR* P446L variant, and indication of liver biopsy (severe obesity versus nonalcoholic fatty liver with increased liver enzymes. Abbreviations: AA, homozygotes for the A allele (carriers); GA, heterozygotes; GG, homozygotes for the G allele.

TABLE 3. DEMOGRAPHIC, ANTHROPOMETRIC, AND CLINICAL FEATURES OF THE LIVER BIOPSY COHORT (N = 1,388) STRATIFIED BY PPP1R3B rs4841132 GENOTYPE

	GG (n = 1,131)	GA (n = 245)	AA (n = 12)	P Value*
Sex, M	522 (46)	100 (41)	5 (42)	0.36
Age, years	47 ± 11	47 ± 11	54 ± 6	0.12
BMI, kg/m^2	37 ± 9	38 ± 8	38 ± 7	0.64
IFG/T2D, yes	276 (24)	60 (24)	2 (17)	0.08
HOMA-IR	6.2 ± 15	6.9 ± 13	4.1 ± 1.9	0.92
Insulin, IU/mL	22 ± 33	23 ± 27	17 ± 7.6	0.99
Total cholesterol, mg/dL	193.3 ± 42.5	181.7 ± 42.5	170.1 ± 50.3	0.02
LDL cholesterol, mg/dL	116 ± 38.6	112.1 ± 38.6	96.7 ± 34.8	0.18
HDL cholesterol, mg/dL	50.3 ± 15.5	46.4 ± 11.6	46.4 ± 11.6	0.03
Triglycerides, mg/dL	139.9 ± 78.7	131.2 ± 61.2	113.7 ± 35	0.07
ALT, IU/L[†]	32 (20-54)	36 (24-55)	32 (22-43)	0.78
AST, IU/L[†]	24 (18-34)	26 (20-36)	27 (21-40)	0.87

Values are reported as mean ± SD or number (%), except where indicated. Characteristics of participants were compared across rs236918 genotypes using linear regression model (for continuous characteristics) or logistic regression model (for categorical characteristics). *Models were adjusted for sex, age, BMI, *PNPLA3* I148M alleles, *TM6SF2* E167K alleles, *MBOAT7* rs641738 T, GCKR P446L variant and statin use. [†]median (IQR).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IFG, impaired fasting glucose; LDL, low-density lipoprotein; T2D, type 2 diabetes.

(estimated beta, −0.36; 95% CI, −0.64 to −0.08; *P* = 0.01; Fig. 1).

Findings were generally consistent after stratification of patients for the enrollment criteria. The rs4841132 A allele was associated with protection against fibrosis (OR, 0.26; 95% CI, 0.08-0.80; *P* = 0.02) in the liver clinic cohort (Supporting Table S3). In the bariatric cohort, the A allele was associated with protection against steatosis (OR, 0.61; 95% CI, 0.40-0.93; *P* = 0.02; Supporting Table S3; estimated beta, −0.50; 95% CI, −0.86 to −0.15; *P* = 0.006; Supporting Fig. S1A) and lobular inflammation (estimated beta −0.41; 95% CI, −0.83 to −0.02; *P* = 0.04; Supporting Fig. S1A).

In the overall series of patients at risk of NASH, the A allele was associated with reduced total and high-density lipoprotein cholesterol (*P* < 0.05; Table 3). After stratification for enrollment criteria, the impact of the variant on circulating lipids was larger in the bariatric cohort (Supporting Table S4).

PPP1R3B GENE VARIATION IS ASSOCIATED WITH REDUCED RISK OF NAFLD-HCC

To examine the impact of the variant on specific clinically relevant outcomes, we assessed the association with NAFLD-HCC in the Italian EPIDEMIC case controls (n = 132 cases, n = 995 controls) whose characteristics and prevalence of the *PPP1R3B* variant are shown in Table 1 and Fig. 2. The independent predictors of NAFLD-HCC are reported in Table 4. We detected a reduced frequency of the minor A allele in patients with HCC than in those without HCC (*P* = 0.02; Fig. 2), and the minor A allele was independently associated with protection against HCC development (OR, 0.22; 95% CI, −2.6 to −0.4; *P* = 0.01). Notably, the protective effect of the variant seemed to depend on liver disease severity as adjustment for advanced fibrosis abolished the association of the A allele with NAFLD-HCC (Table 4). These findings
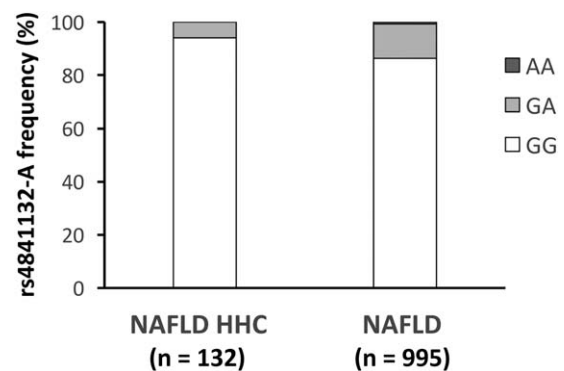
FIG. 2. Frequency distribution of the rs4841132 A allele in 1,127 Italian patients with NAFLD stratified by the presence of HCC. Comparisons were performed by logistic regression setting HCC as the dependent variable, and the association with the *PPP1R3B* variant was analyzed assuming an additive model. Abbreviations: AA, homozygotes for the A allele; GA, heterozygotes; GG, homozygotes for the G allele.

TABLE 4. INDEPENDENT PREDICTORS OF NAFLD-HCC IN 1,127 PATIENTS WITH NAFLD (CASES = 132)

	Model 1			Model 2		
	OR	95% CI	P Value	OR	95% CI	P Value
Age, years	1.18	1.13-1.22	<0.0001	1.18	1.09-1.28	<0.0001
Sex, M	1.21	0.65-2.26	0.54	0.37	0.11-1.30	0.12
BMI, kg/m²	0.92	0.87-0.96	0.002	0.89	0.78-1.02	0.09
IFG/T2DM, yes	4.61	2.56-8.28	<0.0001	4.2	1.07-16.5	0.04
PNPLA3, I148M alleles	1.68	1.14-2.46	0.008	1.14	0.51-2.53	0.75
Advanced fibrosis, F3-F4	NA	NA	NA	91.4	10.6-790	<0.0001
PPPR13B rs4841132, A alleles	0.22	0.07-0.70	0.01	1.76	0.30-10.3	0.53

Comparisons were performed by logistic regression analysis setting HCC as the dependent variable. Model 1 was adjusted for age, sex, BMI and T2DM, PNPLA3, TM6SF2, MBOAT7 rs643718, and PPP1R3B rs4841132 genetic variants. Model 2 was further adjusted for presence of advanced fibrosis.
Abbreviations: BMI, body mass index; IFG, impaired fasting glucose; NA, not addressed; T2DM, type 2 diabetes mellitus.

suggest that the rs4841132 variation does not protect against HCC independently of hepatic fat accumulation and fibrosis development.

PPP1R3B GENE VARIATION IS NOT ASSOCIATED WITH INCREASED LIVER DISEASE RISK IN THE UNITED KINGDOM BIOBANK COHORT

We next examined the impact of the rs4841132 minor A allele on liver disease and metabolic traits in the United Kingdom Biobank cohort, which is representative of the general population (N = 500,000; Table 5). Here, we did not find any significant association between the minor A allele and the risk of liver disease. These findings suggest that carriage of the A allele does not reduce the predisposition to develop liver disease at the population level, despite seeming to protect against the development of NAFLD in high-risk individuals. However, consistent with results observed in the LBC, the A allele was associated with lower circulating cholesterol levels ($P = 8.4 \times 10^{-8}$).

TABLE 5. ASSOCIATION OF THE PPP1R3B VARIANT WITH LIVER-RELATED OUTCOMES IN THE UNITED KINGDOM BIOBANK COHORT (N = 500,000)

Phenotype	n Cases	Beta	P value
K70: Alcoholic liver disease	808	−0.00014799	0.39
K74: Fibrosis and cirrhosis of liver	805	−0.0001773	0.30
K75: Other inflammatory liver diseases	662	−0.00026552	0.09
K76: Other diseases of liver	3,351	0.00035659	0.31
High cholesterol	50,421	−0.0064655	8.4×10^{-8}

PPP1R3B rs4841132 IMPACTS ON *PPP1R3B* AND *LOC157273* EXPRESSION

The rs4841132 variant is located in exon 2 of *LOC157273*, a liver-specific long noncoding RNA, 175 kb upstream of the *PPP1R3B* gene, which encodes a protein that promotes hepatic glycogen synthesis. To determine whether the epidemiological association of rs4841132 with hepatic steatosis is mediated by modulation of transcription at this locus, we examined the hepatic expression of *LOC157273*, *PPP1R3B,* and tankyrase (*TNKS*) in a subset of patients with available liver samples (n = 125; Fig. 3A). The rs4841132 A allele was associated with higher *PPP1R3B* messenger RNA levels ($P = 0.04$) and with reduced *LOC157273* expression ($P = 0.008$), whereas *TNKS* expression was not affected (*P* value not significant).

Next, we assessed whether the *PPP1R3B* variation was associated with altered expression of genes involved in hepatic lipid metabolism. Notably, we found that the minor A allele was associated with up-regulation of peroxisome proliferator-activated receptor alpha ($P = 0.004$), whereas expressions of sterol regulatory element binding protein 1 and 2, microsomal triglyceride transfer protein, and apolipoprotein B were not affected (Fig. 3B).

Gene set enrichment analysis on differentially expressed genes (n = 1,060; nominal $P < 0.05$) revealed down-regulation of several pathways related to cell cycle and inflammation in patients who carried the A allele. Consistently, we found a lower expression of genes involved in inflammation and fibrogenesis, cholesterol homeostasis, oxidative phosphorylation, and glycolysis. We also found a nonsignificant down-regulation of other metabolic pathways, including fatty acid metabolism, phosphoinositide 3-kinase–protein
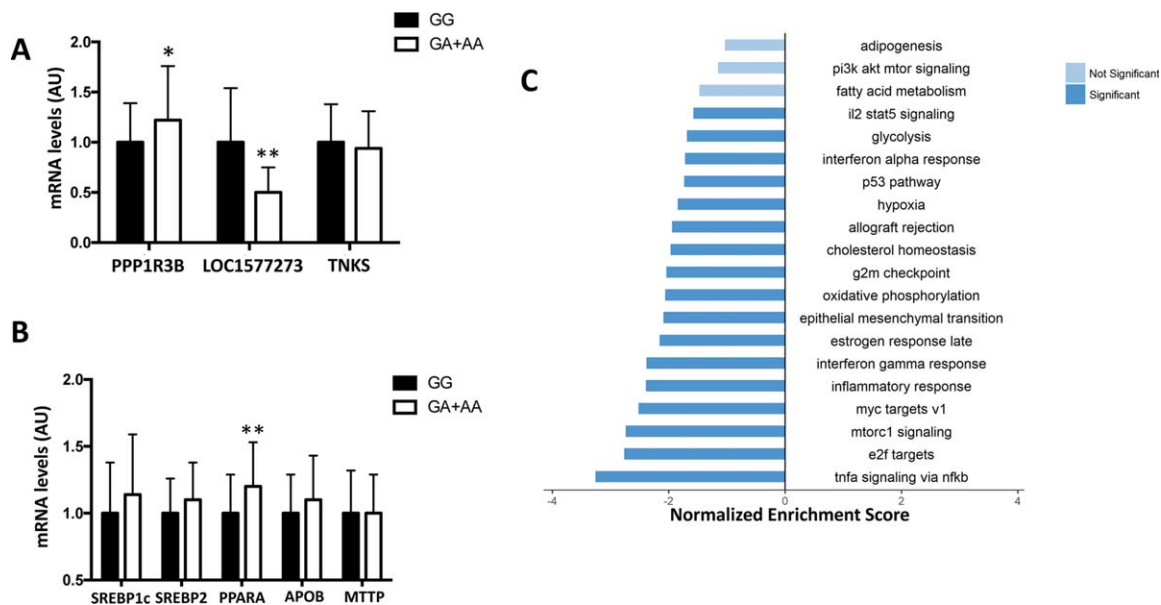
FIG. 3. Gene expression in human liver samples collected from liver biopsies was evaluated by transcriptome analysis. (A) *PPP1R3B, LOC157273, TNKS*; (B) beta-oxidation (*PPARα*), lipogenesis (*SREBP1c*), cholesterol synthesis (*SREBP2*), and lipoprotein secretion (*MTTP, APOB*). *$P < 0.05$, **$P < 0.01$ compared to noncarriers (n = 125). (C) Hallmark molecular functions enriched in patients who carried the rs4841132 A allele, by RNA sequencing analysis. Bar plots show the gene set enrichment analysis hallmark gene sets that were found down-regulated (FDR <0.1). Light blue bars indicate gene sets slightly below the significance (FDR between 0.1 and 0.6). Bars were ordered by normalized enrichment score that indicated the strength of the enrichment. Data are presented as beta coefficient ± SE. Abbreviations: AA, homozygotes for the A allele; APOB apolipoprotein B; AU, arbitrary unit; FDR, false discovery rate; GA, heterozygotes; GG, homozygotes for the G allele; il2 stat5, interleukin 2 signal transducer and activator of transcription 5; mRNA, messenger RNA; mtorc1, mammalian target of rapamycin complex 1; MTTP, microsomal triglyceride transfer protein; nfkb, nuclear factor kappa β; pi3k akt mtor; phosphoinositide 3-kinase–protein kinase B–mammalian target of rapamycin; PPARα, peroxisome proliferator-activated receptor alpha; SREBP, sterol regulatory element binding protein; tnfa, tumor necrosis factor α; TNKS, tankyrase.

kinase B–mammalian target of rapamycin, and lipogenesis (adipogenesis). Gene set enrichment analysis results are summarized in Fig. 3C and Supporting Table S5.

Finally, to further investigate the mechanism whereby the rs4841132 variation protects against hepatic fat accumulation, we performed a gene expression differential analysis of the whole transcriptome in patients carrying the A allele (n = 19) compared to noncarriers (n = 106). The analysis revealed that after adjustment for the false discovery rate the expression of *PLEXIN* A4 (*PLXNA4*) and protein kinase, membrane-associated tyrosine/threonine 1 (*PKMYT1*) was lower in carriers of the A allele ($P = 1.3 \times 10^{-5}$ and $P = 2.1 \times 10^{-5}$, respectively; Supporting Table S6).

Discussion

In this study, we examined whether *PPP1R3B* gene variation influences the susceptibility to liver damage in European individuals at high risk of NASH. Our results suggest that the minor rs4841132 A allele is associated with a reduced risk of histologic steatosis, which also resulted in protection against clinically significant fibrosis and HCC. Although the direction of the association is protective and the variant is rarer, the size effect was marked, making it very similar to that of the *PNPLA3* I148M variant on these three major outcomes. Despite previous contrasting evidence,[8-13] these novel data lend strong support to the hypothesis that the *PPP1R3B* variation does protect against hepatic fat accumulation, at least in at-risk individuals. Furthermore, they support the notion that steatosis is a major driver of liver disease progression to fibrosis[7] and HCC[18] in individuals with NAFLD.

However, in the United Kingdom Biobank cohort, a well-powered population study, the A allele had a neutral impact on liver disease risk. It might be speculated the protective effect of the rs4841132 A allele on steatosis development can be observed only in individuals at high baseline risk for NASH and/or in the presence

of unknown environmental triggers. Alternatively, in individuals at lower risk of NAFLD, predisposition to glycogen accumulation in carriers of the A allele may offset the protective effect on hepatic fat accumulation. Indeed, in two other population studies, namely the Copenhagen City Heart Study and in the Dallas Liver Study, carriage of the A allele was borderline associated with an increased risk of a composite outcome of liver-related outcomes.[9] Therefore, a limitation of this study is that we could not assess hepatic glycogen content.

Concerning the mechanism underpinning the epidemiological association, we confirmed previous findings indicating that rs4841132 variation determines down-regulation of the expression of *LOC157273* as well as and increased levels of *PPP1R3B*.[9] However, we could not demonstrate whether this is mediated by an alteration of the chromatin structure of the whole locus or *LOC157273* controls *PPP1R3B* transcription.

It is tempting to speculate that higher expression of *PPP1R3B* would favor energy storage under the form of glycogen in the liver.[9] This may lead to shunting of glucose to glycogen synthesis from glycolysis and suppression of *de novo* lipogenesis in carriers of the protective variant. Interestingly, although we did not directly measure lipid fluxes, our novel gene expression data from transcriptomic analysis suggest that carriers of the variant had reduced glycolysis and lipid oxidation, which are associated with reduced activation of inflammatory and fibrogenic pathways. However, we cannot rule out that LOC157273 may directly affect the expression of genes involved in steatosis development and lipid handling. The association with down-regulation of cholesterol metabolism genes is consistent with the reduced circulating levels of cholesterol in severely obese individuals carrying the A allele, which was also confirmed at the population level both in the United Kingdom Biobank and in previous studies.[8] Moreover, differential analysis of gene expression revealed that two genes involved in tumor progression and inflammation[26,27] were significantly down-regulated in patients who carried the A allele.

This study has other limitations. These include semiquantitative assessment of hepatic fat accumulation by histology, lack of prospective assessment of the impact of the variant on disease outcomes, and lack of availability of individual patient data in the United Kingdom Biobank cohort, precluding a formal evaluation of the interaction between *PPP1R3B* and adiposity in determining the risk of liver disease.

Furthermore, results are only applicable to European individuals.

In conclusion, rs4841132 variation influencing *PPP1R3B* expression is associated with protection against hepatic steatosis and fibrosis in individuals at high risk of NAFLD and against dyslipidemia. The mechanism seems related to modulation of hepatic lipid metabolism.

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