patient-oriented and epidemiological research

PCSK7 gene variation bridges atherogenic dyslipidemia with hepatic inflammation in NAFLD patients[S]

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Abstract Dyslipidemia and altered iron metabolism are typical features of nonalcoholic fatty liver disease (NAFLD). Proprotein convertase subtilisin/kexin type 7 (*PCSK7*) gene variation has been associated with circulating lipids and liver damage during iron overload. The aim of this study was to examine the impact of the *PCSK7* rs236918 variant on NAFLD-related traits in 1,801 individuals from the Liver Biopsy Cohort (LBC), 500,000 from the UK Biobank Cohort (UKBBC), and 4,580 from the Dallas Heart Study (DHS). The minor *PCSK7* rs236918 C allele was associated with higher triglycerides, aminotransferases, and hepatic inflammation in the LBC ($P < 0.05$) and with hypercholesterolemia and liver disease in the UKBBC. In the DHS, *PCSK7* missense variants were associated with circulating lipids. *PCSK7* was expressed in hepatocytes and its hepatic expression correlated with that of lipogenic genes ($P < 0.05$). The rs236918 C allele was associated with upregulation of a new "intra-PCSK7" long noncoding RNA predicted to interact with the protein, higher hepatic and circulating PCSK7 protein ($P < 0.01$), which correlated with triglycerides ($P = 0.04$). In HepG2 cells, *PCSK7* deletion reduced lipogenesis, fat accumulation, inflammation, transforming growth factor β pathway activation, and fibrogenesis. In conclusion, *PCSK7* gene variation is associated with dyslipidemia and more severe liver disease in high risk individuals, likely by modulating *PCSK7* expression/activity.—Dongiovanni, P., M. Meroni, G. Baselli, R. M. Mancina, M. Ruscica, M. Longo, R. Rametta, A. Cespiati, S. Pelusi, N. Ferri, V. Ranzani, V. Nobili, J. Pihlajamaki, A. L. Fracanzani, S. Badiali, S. Petta, S. Fargion, S. Romeo, J. Kozlitina, and L. Valenti. ***PCSK7* gene variation bridges atherogenic dyslipidemia with hepatic inflammation in NAFLD patients.** ***J. Lipid Res.*** **2019.** 60: **1144–1153.**

Supplementary key words genes in lipid dysfunction • genetics • metabolic disease • nonalcoholic fatty liver disease • proprotein convertase subtilisin/kexin type 7

Nonalcoholic fatty liver disease (NAFLD) is emerging as a leading cause of liver damage worldwide (1). From both epidemiological and pathophysiological points of view, NAFLD is strongly intertwined with insulin resistance and

This work was supported by Associazione Italiana per la Ricerca sul Cancro Grant 16888 [for the EPIDEMIC-NAFLD project (L.V.)], Ricerca Finalizzata 2016 Ministero della Salute Grant RF-2016-02364358 (L.V.), Ricerca Corrente Fondazione IRCCS Cà Granda, and National Institutes of Health Grant UL1TR001105 (from the National Center for Advancing Translational Sciences). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors report no financial conflicts of interest.

Manuscript received 14 October 2018 and in revised form 22 March 2019.

Published, JLR Papers in Press, March 27, 2019
DOI https://doi.org/10.1194/jlr.P090449

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPT1, carnitine palmitoyltransferase 1; DHS, Dallas Heart Study; HDL-C, HDL cholesterol; LBC, Liver Biopsy Cohort; LDL-C, LDL cholesterol; lncRNA, long noncoding RNA; MAF, minor allele frequency; MBOAT7, membrane bound *O*-acyltransferase domain containing 7; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PCSK7, proprotein convertase subtilisin/kexin type 7; PNPLA3, patatin-like phospholipase domain-containing protein 3; TGFβ, transforming growth factor β; TM6SF2, transmembrane 6 superfamily member 2; TS, transferrin saturation; UKBBC, UK Biobank Cohort.

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

its clinical correlates, including visceral obesity, hyperglycemia, and atherogenic dyslipidemia (2). NAFLD has a strong genetic component and variants in proteins regulating hepatocellular lipid handling, including patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*), and membrane bound *O*-acyltransferase domain containing 7 (*MBOAT7*), predispose to hepatic fat accumulation and the development of progressive nonalcoholic steatohepatitis (NASH) (3, 4).

Dysregulation of iron metabolism represents another typical feature of NAFLD associated with more severe hepatic and cardio-metabolic damage (5). This so called "dysmetabolic hyperferritinemia" is most frequently observed in patients with risk factors for iron accumulation (6–8), and tracks with increased expression of iron transporters induced by excess fatty acids (9, 10), but the underlying mechanism remains largely unexplained.

Recently, genome-wide association studies revealed an association of missense variants in proprotein convertase subtilisin/kexin type 7 (*PCSK7*) with circulating lipids (11–14). PCSK7, also known as PC7 (15, 16), is a widely expressed transmembrane protease (17). The noncoding rs236918 *PCSK7* variant (NC_000011.10:g.117220893 G>C) has been associated with shedding of membrane transferrin receptor (18, 19) and with liver damage during iron overload (20, 21), but the mechanism underlying the clinical phenotype remains unclear. Moreover, the impact of *PCSK7* variation on hepatic fat accumulation and liver damage in patients with NAFLD has not been evaluated so far, and the underlying mechanism remained elusive.

We hypothesized that *PCSK7* variation bridges dyslipidemia with liver damage and dysregulation of iron metabolism. The aim of this study was, therefore, to examine the impact of *PCSK7* variation on liver damage and metabolic traits in individuals at risk of NAFLD and in the general population. To elucidate the mechanisms underlying the association, we examined the impact of rs236918 and other functional *PCSK7* variants on hepatic expression of the protein, inflammation, and lipogenesis, as well as the phenotype induced by *PCSK7* deletion in hepatocytes in vitro.

METHODS

The Liver Biopsy Cohort

Part of the cross-sectional Liver Biopsy Cohort (LBC) has previously been described (4, 22). Briefly, a total of 1,801 adult individuals of European descent were consecutively enrolled from Italian and Finnish referral centers. Inclusion criteria were liver biopsy for suspected NASH or severe obesity and availability of DNA samples and clinical data. Individuals with increased alcohol intake (>30/20 g/day in males/females) and 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 of Milan. All subjects gave written informed consent. The clinical features of individuals evaluated in the study are presented in supplemental Table S1. For the Milan outpatient service subcohort (n = 332), systematic evaluation of circulating iron parameters [iron, transferrin, transferrin saturation (TS), ferritin] and histological hepatic iron staining were available.

Histological evaluation

In individuals included in the LBC, steatosis was graded based on the percentage of affected hepatocytes as: 0, 0–5%; 1, 6–33%; 2, 34–66%; and 3, 67–100%. Disease activity was assessed according to the NAFLD activity score with systematic evaluation of hepatocellular ballooning and lobular inflammation; fibrosis was also staged according to the recommendations of the NAFLD clinical research network (23). The scoring of liver biopsies was performed by independent expert pathologists unaware of the patients' status and genotype, who trained on a common slide set (22, 24). In the LBC, 87% of patients had NAFLD and 55% had NASH.

UK Biobank Cohort

The association of the rs236918 variant with 98 phenotypes related to metabolic disorders and liver disease was evaluated in the UK Biobank Cohort (UKBBC; supplemental Table S2). Publicly available association data were downloaded from Neale Lab (http://www.nealelab.is/) and *P*-values were corrected for multiple testing using the false discovery rate method (25). Adjusted *P*-values <0.1 were considered statistically significant.

The Dallas Heart Study

The Dallas Heart Study (DHS) is a multiethnic population-based probability sample of Dallas County residents. The study design and recruitment procedures have been previously described (26). The study conformed to the Declaration of Helsinki principles and was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center and all individuals provided written informed consent. Plasma lipids were measured by standard enzymatic assays. Hepatic triglyceride content was measured by proton MRS (^{1}H-MRS) as previously described (27, 28). The present analysis includes up to 4,580 individuals (52% African-Americans, 29.5% European-Americans, 16% Hispanics, and 3% of other ethnicities), with available DNA samples and laboratory data. Liver fat measurements were available in a subset of individuals (n = 2,736). Clinical characteristics of DHS participants are shown in supplemental Table S1.

Genotyping

The LBC was genotyped for rs738409 C>G (*PNPLA3* I148M), rs58542926 C>T (*TM6SF2* E167K), rs641738 C>T near *MBOAT7*, and *PCSK7* rs236918 C>T by TaqMan 5′-nuclease assays (Life Technologies, Carlsbad, CA), as previously described (4, 22). The genotype frequencies of rs236918 were in Hardy-Weinberg proportion ($P = 0.31$; supplemental Table S3). In the DHS, genotyping was performed using Illumina Human Exome BeadChip (29). In addition to the rs236918 variant, seven rare [minor allele frequency (MAF) <1%] missense variants in *PCSK7* were present on the Exome chip, and were examined as part of the current analysis (supplemental Table S4).

Gene expression in human primary cells

RNA was extracted using Trizol reagent (Life Technologies). One microgram of total RNA was retro-transcribed by using the VILO random hexamers synthesis system (Life Technologies). Gene expression was evaluated by quantitative real-time PCR, performed by the 7500 Fast thermocycler (Life Technologies) using the TaqMan Universal PCR Master Mix (Life Technologies) and TaqMan probes for human *PCSK7*. All reactions were performed in triplicate and data were normalized to β-actin gene expression.

Gene and protein expression analysis in liver biopsies

PCSK7 gene expression was assessed in percutaneous liver biopsies of a subset of 125 severely obese patients from the Milan cohort (bariatric surgery cohort), whose clinical features are shown in supplemental Table S5 (*PCSK7* rs236918 GG, n = 100;

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PCSK7 rs236918 GC, n = 22; *PCSK7* rs236918 CC, n = 3). RNA was extracted from liver biopsies using an RNeasy mini-kit (Qiagen Hulsterweg). RNA quality was assessed through an Agilent 2100 bioanalyzer and samples with RNA integrity numbers greater than or equal to 7 were used for library preparation (Ribo-reduction libraries). RNA sequencing was performed in paired-end mode with a read length of 150 nt using the Illumina HiSeq 4000 (Novogene, Hong Kong, China). The RNA sequencing detailed protocol and data analysis approach are described in the supplemental Materials and Methods.

For protein analysis, 10 samples with different rs236918 genotypes [GG or GC (n = 6) + CC (n = 4)] were analyzed, and all reactions were performed in duplicate. The following antibodies were used: rabbit monoclonal anti-PCSK7 (Cell Signaling, Beverly, MA) and goat polyclonal anti β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Linkage disequilibrium analysis

The detailed protocol and data analysis approach are described in the supplemental Materials and Methods.

Histone modification analysis

In order to assess the association of genetic variation with histone modifications, E066 Adult Liver Chip-Seq data (*P*-value signal tracks) and core (15-state) Hidden Markov model chromatin state prediction data were obtained from the Roadmap project (30). The Gviz package was employed for data visualization (31).

De novo assembly and specific transcript evaluation

The detailed protocol and data analysis approach are described in the supplemental Materials and Methods.

PCSK7 gene deletion in HepG2 cells

We generated a *PCSK7* knockout model by exploiting clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology in HepG2 hepatoma cells. We first generated an inducible stable Cas9 cell line by lentiviral transfection and clonal selection by blasticidin. We then transfected pGL3-U6-sgRNA-PGK-puromycin plasmid vector and a small guide RNA targeting within the first exon of the *PCSK7* gene to induce *PCSK7* knockout. We screened transfected clones through a digestion reaction using T7 endonuclease enzyme, and identified a clone carrying a large deletion in heterozygous state ($PCSK7^{-/+}$), which was confirmed by Sanger sequencing (supplemental Fig. S1).

Statistical analysis

For descriptive statistics, continuous variables were presented as mean and SD (for approximately normally distributed variables) or median and interquartile range (for highly skewed variables). Categorical variables were presented as number and percent. Genetic association was tested assuming an additive model. Analyses were performed using generalized linear models: linear regression models were fit to analyze continuous traits, logistic regression for binary traits (NASH, severe fibrosis stage F3-F4), and ordinal regression for ordinal traits (components of the NAFLD activity score: severity of steatosis, lobular inflammation and hepatocellular ballooning, stage of fibrosis). Models were adjusted for confounding factors (including recruitment center), as specified. Variables with skewed distributions were logarithmically transformed before entering the models.

Statistical analyses were carried out using the JMP 12.0 (SAS Institute, Cary, NC) and R statistical analysis software version 3.3.2 (http://www.R-project.org/). *P*-values <0.05 were considered statistically significant.

RESULTS

PCSK7 rs236918 affects circulating lipids, iron, and hepatic inflammation in the LBC

The clinical features of the LBC patients stratified by rs236918 genotype are shown in **Table 1**. No differences in

TABLE 1. Demographic, anthropometric, and clinical features of the LBC (n = 1,801) stratified by *PCSK7* rs236918 genotype

	PCSK7 rs236918				
	GG (n = 1,370)	GC (n = 396)	CC (n = 35)	*P* Additive[a]	*P* Recessive[b]
Sex, female	692 (50)	185 (47)	18 (51)	0.39	0.28
Age, years	43.9 ± 15.2	43.2 ± 14.3	40.7 ± 19.1	0.32	0.55
BMI, kg/m^2	34.8 ± 8.9	34.7 ± 9.0	33.7 ± 11.7	0.88	0.32
Obesity, yes	837 (61)	242 (61)	19 (54)	0.61	0.95
IFG/T2D, yes	358 (26)	107 (27)	11 (31)	0.60	0.08
NASH, yes	749 (55)	222 (56)	18 (51)	0.56	0.67
Total cholesterol, mmol/l	4.9 ± 1.1	5.0 ± 1.2	5.0 ± 1.1	0.44	0.81
Triglycerides, mmol/l	1.5 ± 0.8	1.6 ± 0.9	1.7 ± 1.0	0.033	0.04
LDL cholesterol, mmol/l	3.0 ± 1.0	3.0 ± 1.1	3.1 ± 1.0	0.36	0.90
HDL cholesterol, mmol/l	1.2 ± 0.4	1.2 ± 0.3	1.1 ± 0.3	0.47	0.03
ALT, IU/l	40 {23–68}	42 {23–69}	56 {31–73}	0.06	0.0048
AST, IU/l	28 {20–42}	29 {21–42}	37 {24–51}	0.0008	0.0002
Iron, μg/dl[c]	4.6 {4.4–4.9}	4.7 {4.5–4.9}	4.8 {4.4–4.9}	0.20	0.92
Ferritin, ng/ml[c]	5.7 {5.0–6.5}	5.8 {5.0–6.4}	5.7 {4.7–6.7}	0.56	0.98
Transferrin, mg/dl[c]	265.3 ± 44.7	262.3 ± 42.2	257.1 ± 16.1	0.38	0.96
TS[c]	30 {24–39}	31 {25–42}	36 {22–39}	0.11	0.79

Values are reported as mean ± SD, median {interquartile range}, or number (percent), as appropriate. Characteristics of participants were compared across rs236918 genotypes using linear regression model (for continuous characteristics) or logistic regression model (for categorical characteristics). IFG, impaired fasting glucose.

[a]Additive model was adjusted for gender, age, BMI, IFG/T2D, *PNPLA3* I148M, *TM6SF2* E167K, and *MBOAT7* rs641738 variants.

[b]Recessive model was adjusted for gender, age, BMI, IFG/T2D, *PNPLA3* I148M, *TM6SF2* E167K, and *MBOAT7* rs641738 variants.

[c]Iron parameters were available in a subset of patients (n = 332) from the Milan outpatient service cohort (*PCSK7* rs236918 GG, n = 252; CG, n = 72; CC, n = 8).

demographic and anthropometric features were found across rs236918 genotypes. In multivariable-adjusted models, the minor C allele was associated with increased circulating triglycerides after adjustment for sex, age, BMI, T2D, and *TM6SF2* E167K variant (β 0.09, 95% CI 0.01–0.17; P = 0.033). We did not find any association between rs236918 genotype and LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), insulin resistance, or T2D.

We next evaluated the impact of the *PCSK7* rs236918 C allele on iron metabolism in a subset of patients (n = 332). The C allele was associated with increased TS after adjustment for sex, age, BMI, T2D, and the hemochromatosis gene (*HFE*) p.C282Y and p.H63D variants (β 4.8, 95% CI 0.3–9.3; P = 0.035).

Notably, the C allele was nearly associated with increased alanine aminotransferase (ALT) levels (P = 0.06) and with higher aspartate aminotransferase (AST) (P = 0.0008), and the effect was even more strong by using a recessive model (ALT, P = 0.0048; AST, P = 0.0002) (Table 1). However, under a genotypic model, we have now reported that the rs236918 GC genotype was associated with ALT and AST levels as compared with the reference GG genotype (P = 0.054 and P = 0.0042, respectively), suggesting that the effect was not completely explained by recessive inheritance models, but that heterozygous carriers also may have a slightly increased the risk of liver damage (supplemental Table S6).

The association of the *PCSK7* genotype with the spectrum of histological liver damage related to NAFLD in the LBC is shown in **Table 2**. In the overall cohort, the minor C allele was not associated with steatosis grade; it was independently associated with lobular inflammation (β 0.22, 95% CI 0.02–0.42; P = 0.028; Table 2, supplemental Fig. S2), but not with ballooning and fibrosis. After stratification of patients for enrollment criteria, rs236918 was associated with steatosis grade (β 0.34, 95% CI 0.03–0.64; P = 0.03) and lobular inflammation (β 0.36, 95% CI 0.05–0.66; P = 0.02) in the Liver Clinic cohort, which is made up of individuals at high risk of progressive NAFLD, but not in bariatric subjects (Table 2). In addition, after stratification of patients for the presence of the I148M *PNPLA3* variant, the rs236918 C allele was associated with steatosis grade (β 0.26, 95% CI 0.003–0.51; P = 0.04) and lobular inflammation (β 0.37, 95% CI 0.10–0.62; P = 0.007) in carriers of the *PNPLA3* I148M risk variant, but not in noncarriers (Table 2). These data suggest that the *PCSK7* variation influences circulating triglycerides and hepatic inflammation, but the effect is at least partly independent of modulation of hepatic fat content.

PCSK7 rs236918 affects circulating cholesterol and possibly liver disease in UKBBC

The clinical phenotypes related to the rs236918 variant in the UKBBC are shown in **Table 3** and supplemental Table S2. We observed a strong positive association between the *PCSK7* rs236918 C allele and circulating total cholesterol levels ($P = 8.6 \times 10^{-9}$), use of cholesterol lowering medications ($P = 1.5 \times 10^{-7}$), and the presence of cardiovascular complications (P = 0.003). Notably, the *PCSK7* rs236918 variant was also nominally associated with an increased risk of "liver failure/cirrhosis" phenotype (P = 0.013).

PCSK7 variants impact on lipid metabolism, but not steatosis, in the DHS

To examine whether *PCSK7* influences circulating lipids by altering hepatic lipid accumulation, we evaluated the impact of the *PCSK7* variation on hepatic triglyceride content, metabolic traits, and liver damage in DHS participants (**Table 4**). The minor C allele of rs236918 was not associated with hepatic triglyceride content (β 0.007 ± 0.034; P = 0.83) or circulating triglycerides (β −0.007 ± 0.027; P = 0.79). However, carriers of a low-frequency missense mutation in *PCSK7* [c.1511 G>A, p.Arg504His (R504H), rs142953140, MAF = 0.2% in African Americans, not present in other ancestry groups] had higher HDL-C compared with noncarriers (mean difference 41.8 mg/dl, β 1.8 ± 0.3; $P = 1.4 \times 10^{-9}$), lower triglycerides (mean difference −75.8 mg/dl, β −1.3 ± 0.3; $P = 1.2 \times 10^{-5}$), and lower LDL-C (mean difference −25.5 mg/dl, β −0.7 ± 0.3; P = 0.022). In addition, another low-frequency variant [c.304G>C, p.Ala102Thr (A102T), rs11542139, MAF = 0.1% in African Americans, 0.2% in European Americans, and 2% in Hispanics] was associated with increased circulating triglycerides (mean difference +58.8 mg/dl, β 0.3 ± 0.1; P = 0.035). Neither of these two variants was associated with hepatic fat content.

We did not observe an association between *PCSK7* variants and serum iron levels in the whole DHS population. However, rs236918 was marginally associated with higher serum iron in a subgroup of at-risk individuals, carrying iron-increasing variants in *HFE* (P = 0.049).

Taken together, these data suggest that the impact of *PCSK7* gene variation on circulating lipids is not secondary

TABLE 2. Association of *PCSK7* rs236918 with liver damage in multivariable models in the LBC overall (n = 1,801) and after stratification for enrollment criteria in Liver Clinic (n = 851) and Bariatric Surgery (n = 950) cohorts

PCSK7 rs236918	Steatosis (β ± SE, P)*	Lobular Inflammation (β ± SE, P)*	Ballooning (β ± SE, P)*	Fibrosis (β ± SE, P)*	NASH (β ± SE, P)*
LBC (overall)	+0.11 ± 0.10, 0.26	+0.22 ± 0.10, 0.028	+0.15 ± 0.11, 0.17	+0.17 ± 0.10, 0.10	+0.06 ± 0.11, 0.57
Liver clinic	+0.34 ± 0.16, 0.03	+0.36 ± 0.16, 0.02	+0.13 ± 0.16, 0.40	+0.21 ± 0.15, 0.17	+0.31 ± 0.18, 0.09
Bariatric surgery	−0.03 ± 0.13, 0.84	+0.15 ± 0.13, 0.28	+0.12 ± 0.15, 0.42	+0.13 ± 0.13, 0.34	−0.11 ± 0.15, 0.48
PNPLA3 I148M, yes	+0.26 ± 0.13, 0.04	+0.37 ± 0.13, 0.007	+0.24 ± 0.14, 0.09	+0.23 ± 0.13, 0.08	+032 ± 0.16, 0.04
PNPLA3 I148M, no	−0.06 ± 0.15, 0.69	−0.005 ± 0.15, 0.97	+0.01 ± 0.18, 0.94	+0.06 ± 0.16, 0.70	−0.24 ± 0.17, 0.16

Multivariable-adjusted logistic regression models were used to test the association (*) adjusted for gender, age, BMI, impaired fasting glucose (IFG)/T2D, *PNPLA3* I148M, *TM6SF2* E167K, and *MBOAT7* rs641738 variants. LBC (n = 1,801): GG = 1,370, GC = 396, CC = 35; Liver Clinic (n = 851): GG = 644, GC = 193, CC = 14; Bariatric surgery (n = 950): GG = 726, GC = 203, CC = 21; *PNPLA3* I148M, yes (n = 983): GG = 742, GC = 221, CC = 20; *PNPLA3* I148M, no (n = 788): GG = 606, GC = 169, CC = 13.

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TABLE 3. Nominally significant associations of *PCSK7* rs236918 with phenotypes related to cardio-metabolic disorders and liver disease in the UKBBC

Phenotype	Number of Cases	Number of Controls	β	SE	*P*
Non-cancer illness code, self-reported: high cholesterol	41,296	295,863	0.008	0.001	8.6×10^{-9}
Medication for cholesterol, blood pressure, diabetes, or taking exogenous hormones: cholesterol-lowering medication	22,705	157,498	0.01	0.002	1.5×10^{-7}
Vascular/heart problems diagnosed by doctor: high blood pressure	91,033	245,650	0.005	0.002	0.003
Non-cancer illness code, self-reported: anemia	599	336,600	0.0005	0.0002	0.006
Non-cancer illness code, self-reported: heart valve problem/heart murmur	2,274	334,885	0.001	0.0003	0.007
Non-cancer illness code, self-reported: liver failure/cirrhosis	240	336,919	0.0003	0.0001	0.013

to modulation of hepatic fat content. Two low-frequency variants (R504H and A102T) were identified with opposite impact on metabolic traits, with A102T having a larger, but directionally concordant, effect compared with rs236918 G>C in the LBC.

PCSK7 is highly expressed in hepatocytes and correlates with de novo lipogenesis

As a first step to elucidate the role of *PCSK7* in lipid handling and liver disease, we examined *PCSK7* mRNA expression in primary human hepatocytes and hepatic stellate cells, in peripheral lymphocytes, monocytes/macrophages, and in an endothelial cell line (HUVEC). *PCSK7* expression was highest in primary hepatocytes, whereas it was low in the other cell types ($P < 0.05$; **Fig. 1A**). In a candidate gene approach conducted in severely obese individuals, hepatic expression of *PCSK7* correlated with *FAS* (β = 0.2,1 95% CI 0.09–0.31; $P = 0.0003$) and was borderline associated with *SREBP1c* (β 0.15, 95% CI 0.00–0.30; $P = 0.06$), two genes involved in hepatic de novo lipogenesis (Fig. 1B, C). Hepatic *PCSK7* expression was also correlated with transforming growth factor β (*TGFβ*; $P = 0.04$), α2-actin (*ACTA2*; $P = 0.0005$), collagen type I α1 chain (*COL1A1*; $P = 0.002$), and carnitine palmitoyltransferase 1B (*CPT1b*; $P < 0.0001$) (supplemental Fig. S3).

To further investigate the correlation between *PCSK7* expression and metabolic pathways, a coregulation analysis was performed in the whole transcriptome. Gene set enrichment analysis highlighted an enrichment in *PCSK7* coexpressed genes within pathways related to inflammation [e.g., *TNFα* signaling, interleukin 6 (*IL6*) response], epithelial mesenchymal transition, and mitosis. Conversely, pathways involved in adipogenesis, fatty acid metabolism, and oxidative phosphorylation were inversely correlated with *PCSK7* expression (supplemental Fig. S4A).

Finally, to dissect the transcriptome variability associated with the presence of the *PCSK7* rs236918 variant, differential expression analysis was performed. Collectively, we found a significant modulation of inflammatory pathways and of genes involved in oxidative phosphorylation associated with the minor allele at risk (supplemental Fig. S4B).

Mechanism of association between rs236918 and *PCSK7* function

To investigate whether the observed associations of rs236918 with metabolic traits were due to linkage with other common (MAF ⩾0.01) missense *PCSK7* variants, we examined linkage disequilibrium patterns at the *PCSK7* locus. The rs236918 was not in linkage disequilibrium with common *PCSK7* coding variants (supplemental Fig. S5A,

TABLE 4. Association of selected *PCSK7* variants with hepatic fat content, circulating levels of lipid, iron, and aminotransferases in the DHS

	Number	rs236918 (Non-coding) (β ± SE, *P*)	rs142953140 (R504H) (β ± SE, *P*)	rs11542139 (A102T) (β ± SE, *P*)
MAF				
Overall	4,580	14.9%	0.1%	0.4%
African American	2,365	14.7%	0.2%	0.1%
European American	1,353	10.3%	—	0.2%
Hispanic	743	20.9%	—	1.7%
Hepatic TG (%)	2,736	+0.007 ± 0.034, —	−0.506 ± 0.361, —	+0.012 ± 0.175, —
Hepatic TG (%), nonobese (BMI <30 kg/m^2)	1,530	0.024 ± 0.047, 0.61	−0.497 ± 0.412, 0.23	−0.130 ± 0.265, 0.62
Hepatic TG (%), obese (BMI ⩾30 kg/m^2)	1,206	−0.005 ± 0.054, 0.92	NA	+0.064 ± 0.253, 0.80
Hepatic TG (%)	1,073	−0.032 ± 0.051, 0.529	NA	0.079 ± 0.217, 0.714
TG, (mg/dl)	4,580	−0.007 ± 0.027, —	$-1.338 \pm 0.305, 1.2 \times 10^{-5}$	+0.311 ± 0.148, 0.035
Total cholesterol, (mg/dl)	4,580	+0.021 ± 0.029, 0.48	+0.09 ± 0.327, 0.78	+0.131 ± 0.158, 0.41
LDL-C (mg/dl)	4,580	+0.029 ± 0.029, 0.33	−0.753 ± 0.329, 0.022	−0.087 ± 0.159, 0.58
HDL-C (mg/dl)	4,580	+0.004 ± 0.027, 0.89	$+1.821 \pm 0.3, 1.4 \times 10^{-9}$	+0.053 ± 0.145, 0.71
ALT (IU/l)	4,565	−0.040 ± 0.026, 0.13	+0.225 ± 0.294, 0.44	+0.029 ± 0.142, 0.84
AST (IU/l)	4,565	−0.049 ± 0.028, 0.083	+0.218 ± 0.314, 0.49	−0.002 ± 0.152, 0.99
Serum iron	4,468	+0.008 ± 0.028, 0.77	−0.18 ± 0.311, 0.56	−0.198 ± 0.150, 0.19
Serum iron, *HFE* H63D carriers	800	+0.164 ± 0.083, 0.049	NA	−0.333 ± 0.349, 0.340

Betas are shown in SD units of the outcome per each additional allele. Probabilities were determined using linear regression models adjusted for age, sex, four principal components or ancestry, BMI, and study visit. Analysis of triglyceride levels was in addition adjusted for T2D. The probability for rs142953140 in subgroup analyses could not be calculated because there were fewer than two carriers of R504H in the corresponding subgroup. TG, triglyceride; NA, not available.

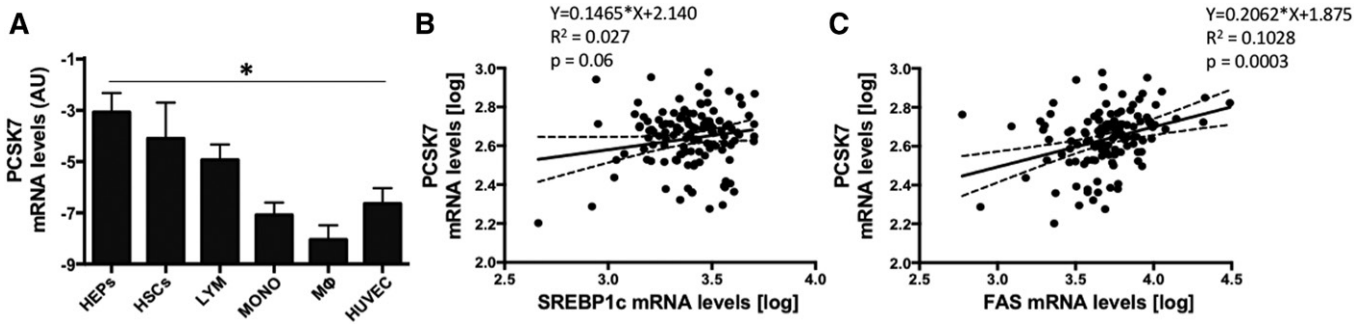

Fig. 1. *PCSK7* is highly expressed in hepatocytes and correlates with de novo lipogenesis. *PCSK7* expression was evaluated by quantitative RT-PCR in different human primary cell types; hepatocytes (HEPs), hepatic stellate cells (HSCs), peripheral lymphocytes (LYM), monocytes (MONO), macrophages (MΦ), and in an endothelial cell line (HUVEC). mRNA levels were normalized for β-actin and log transformed. $^*P < 0.05$ (A). *PCSK7*, *SREBP1c*, and *FAS* gene expression in a human sample collected from liver biopsies were evaluated by transcriptome analysis. Correlation between hepatic *PCSK7* and *SREBP1c* (B) and *PCSK7* and *FAS* (C) gene expression.

supplemental Table S7), suggesting that the impact of rs236918 on the observed phenotypes is not mediated by linkage with other variants influencing PCSK7 protein sequence and activity.

Next, we investigated whether the rs236918 variant impacts on hepatic *PCSK7* mRNA levels. The rs236918 variant was not associated with overall *PCSK7* expression, even if it was marginally associated with the relative abundance of some alternative mRNA transcripts (supplemental Fig. S5B). However, evaluation of epigenomic data obtained by the Roadmap consortium from adult liver highlighted high levels of H3K36 trimethylation (H3K36me3) and H3K27 acetylation (H3K27ac) at the rs236918 region (**Fig. 2A**). Consistently, chromatin state prediction data revealed the presence of two regulatory regions near the rs236918 locus. De novo assembly of the *PCSK7* locus from transcriptomic data revealed the presence of a 1,609 bp transcript mapping in the rs236918 genomic region (chromosome 11, start: 117220122 GRCh38, 110790837 GRCh37; Fig. 2A). This transcript did not overlap with other products of the *PCSK7* gene and was predicted with good confidence (score 0.9, optimal cutoff 0.18) as the product of a nested

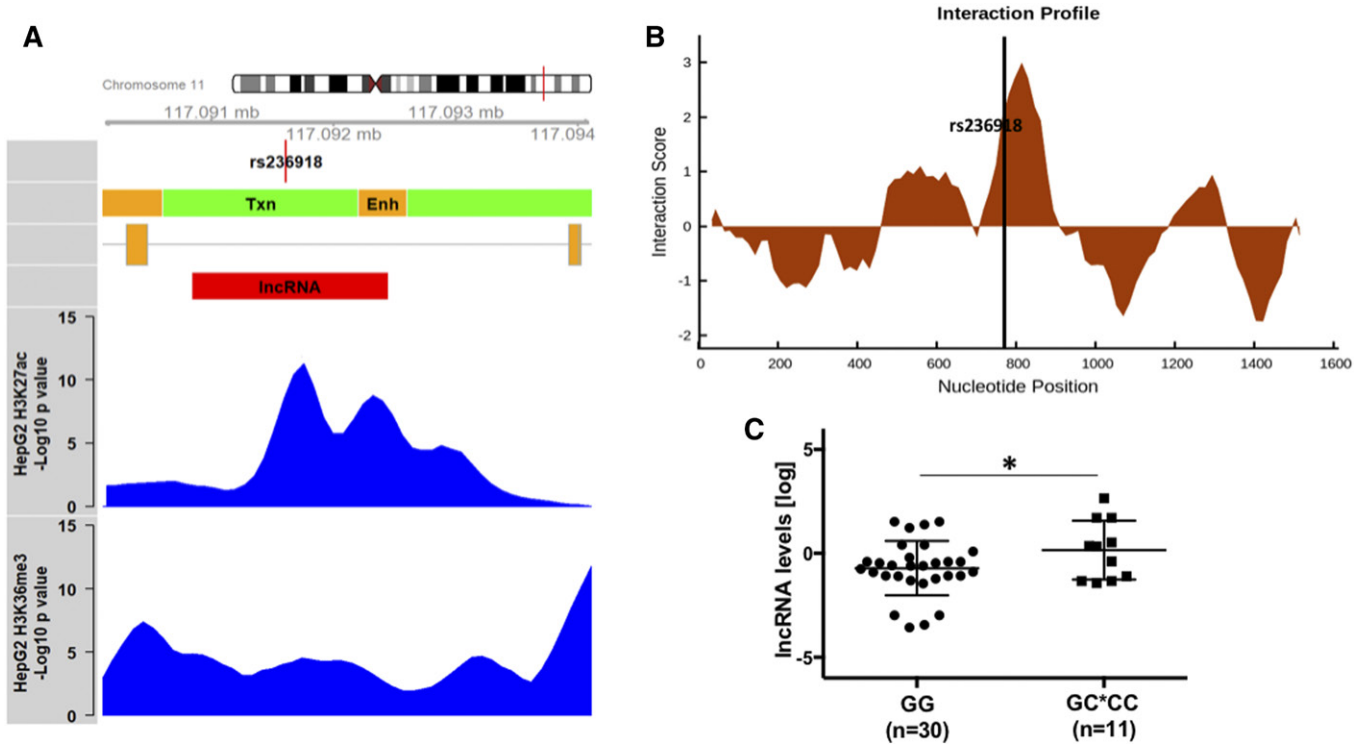

Fig. 2. Epigenomic modification in the rs236918 region might impact on *PCSK7* expression and stability. Genomic region evaluation and de novo assembly analysis. All tracks are aligned on GRCh37 for compatibility with the Roadmap project; ENSEMBL V75 isoforms were used to view the gene model. A: First track from above, rs236918 position; second track, E066 adult liver chromatin state prediction according to 15-state core model from the Roadmap project; third track, *PCSK7* exons (protein coding isoforms); fourth track, de novo identified lncRNA; fifth and sixth tracks, E066 adult liver H3K27Ac and H3K4me3 *P* value signal tracks. B: lncRNA interaction profile. De novo assembly analysis identified lncRNA interaction profile with the 60-111 fragment of PCSK7. C: Hepatic expression levels of intra-PCSK7 lncRNA in 41 severely obese patients stratified by rs236918 genotype. lncRNA levels were log transformed. $^*P < 0.05$.

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gene. As the longest open reading frame in the novel transcript was 83 bp, not consistent with a protein coding mRNA, we characterized this transcript as a long noncoding RNA (lncRNA). This "intra-PCSK7" lncRNA was not predicted to interact with the *PCSK7* main transcript due to lack of sequence similarity. However, the intra-PCSK7 lncRNA was predicted to locate in the cytoplasm and to bind PCSK7 protein by two independent bioinformatic tools: lncPro (score 72.73) and catRAPID. The strongest interaction was predicted by the catRAPID fragments analysis to be located between the initial portion (amino acids 60-111) of PCSK7 protein and a RNA region ranging approximately from position 700 to 900 of the intra-PCSK7 lncRNA (Fig. 2B, discriminative power 59%), with rs236918 located at position 772. Remarkably, we could directly confirm the expression of this intra-PCSK7 lncRNA in the liver of severely obese individuals. The hepatic expression of the intra-PCSK7 lncRNA was higher in carriers of the *PCSK7* rs236918 variant (n = 11), as compared with noncarriers (n = 30) (P = 0.017; Fig. 2C). Therefore, we hypothesized that the modulation of the intra-PCSK7 lncRNA sequence by the rs236918 variant could affect protein synthesis or stability. Indeed, the presence of the risk C allele was associated with higher hepatic protein levels ($P < 0.01$; **Fig. 3A**). Furthermore, in a subset of patients at risk of NAFLD (n = 72), carriers of the C allele showed increased circulating PCSK7 protein concentration as compared with noncarriers (P = 0.007). Importantly, circulating PCSK7 was correlated with triglycerides (β 0.26, 95% CI 0.02–0.50; P = 0.04; Fig. 3B). These data suggest that the rs236918 C allele may predispose to liver damage and influence lipid handling by upregulating PCSK7 protein synthesis and secretion, as in a gain-of-function variant, via a new mechanism encompassing the induction of a novel PCSK7-interacting lncRNA.

PCSK7 deletion in HepG2 cells reduced lipogenesis and *TGFβ* expression

As expected, *PCSK7* mRNA and protein levels were reduced by about 50% in *PCSK7*$^{-/+}$ cells (**Fig. 4A**; $P < 0.05$). In a candidate gene approach, and consistently with the human liver transcriptome data, the expression of *SREBP1c* and *FAS* was reduced in *PCSK7*$^{-/+}$ compared with wild-type cells (Fig. 4B; $P < 0.01$). Similarly, the mRNA levels of *PPARα* and *CPT1*, involved in β-oxidation, were reduced in *PCSK7*$^{-/+}$ cells, although the difference in *CPT1* expression was not significant (P = 0.01 and P = 0.22, respectively; Fig. 4B). As a result, *PCSK7*$^{-/+}$ cells showed lower fat accumulation after exposure to fatty acids (Fig. 4C). Notably, secretion of PCSK7 protein was reduced in *PCSK7*$^{-/+}$ compared with wild-type cells, independently of the exposure to fatty acids ($P < 0.05$, Fig. 4D). Finally, *PCSK7* haploinsufficiency led to reduced expression of genes involved in inflammation (*TNFα*; P = 0.04), but in particular of fibrogenesis in untreated cells (*TGFβ*, P = 0.008; Fig. 4B). FFA exposure induced *TGFβ* expression in wild-type cells, whereas the effect was abrogated in *PCSK7*$^{-/+}$ cells (Fig. 4B, supplemental Fig. S6A). Consistently, p(Thr276) SMAD4/SMAD4 and p(Thr8) SMAD2-3/SMAD2-3 ratios were increased in wild-type cells treated with FFAs compared with *PCSK7*$^{-/+}$ cells. These data are suggestive of hampered TGFβ signaling activation in haploinsufficient cells (supplemental Fig. S6B).

DISCUSSION

In this study, we examined the impact of the *PCSK7* rs236918 noncoding variant on metabolic phenotypes and liver damage associated with NAFLD in individuals at risk and from the general population and compared its effect with that of other *PCSK7* missense variants.

Previous studies revealed an association of low-frequency *PCSK7* missense variants with circulating lipids (11–13), suggesting that altered PCSK7 function accounts for this phenotype, and the noncoding variant, rs236918, has also been associated with liver damage in patients with hereditary hemochromatosis (20, 21). Here, we found that the rs236918 C minor allele was associated with increased circulating triglycerides in NAFLD patients and with dyslipidemia in the UKBBC (12, 13). In the LBC, although we could not

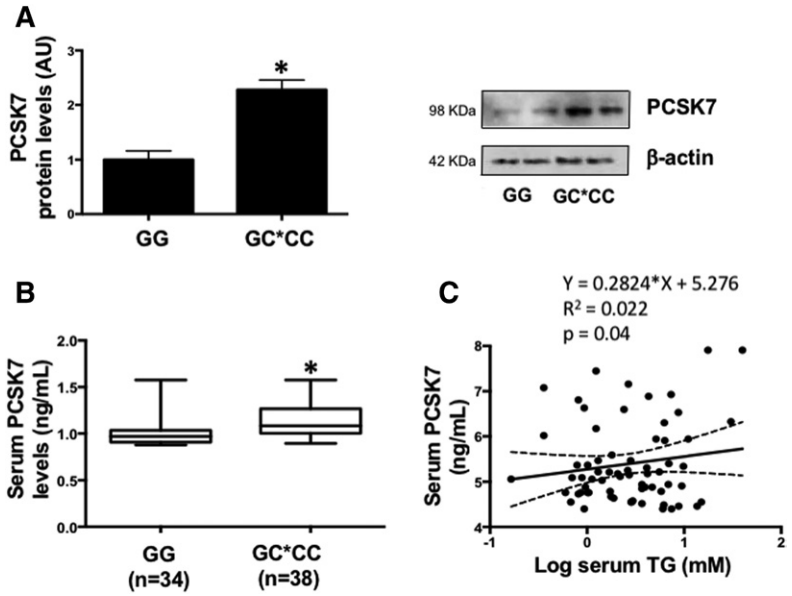

Fig. 3. Mechanism of association between rs236918 and *PCSK7* function. A: PCSK7 protein levels in a human sample collected from liver biopsies were evaluated by Western blotting. Protein levels were normalized for β-actin and expressed as fold increase (arbitrary units, AU). *$P < 0.01$. B: Serum PCSK7 levels were evaluated by ELISA. Boxes span from the 25th to the 75th percentile, while whiskers indicate the 10th and 90th percentiles. *$P < 0.01$ versus rs236918 GG patients. C: Correlation between circulating PCSK7 and serum triglycerides.

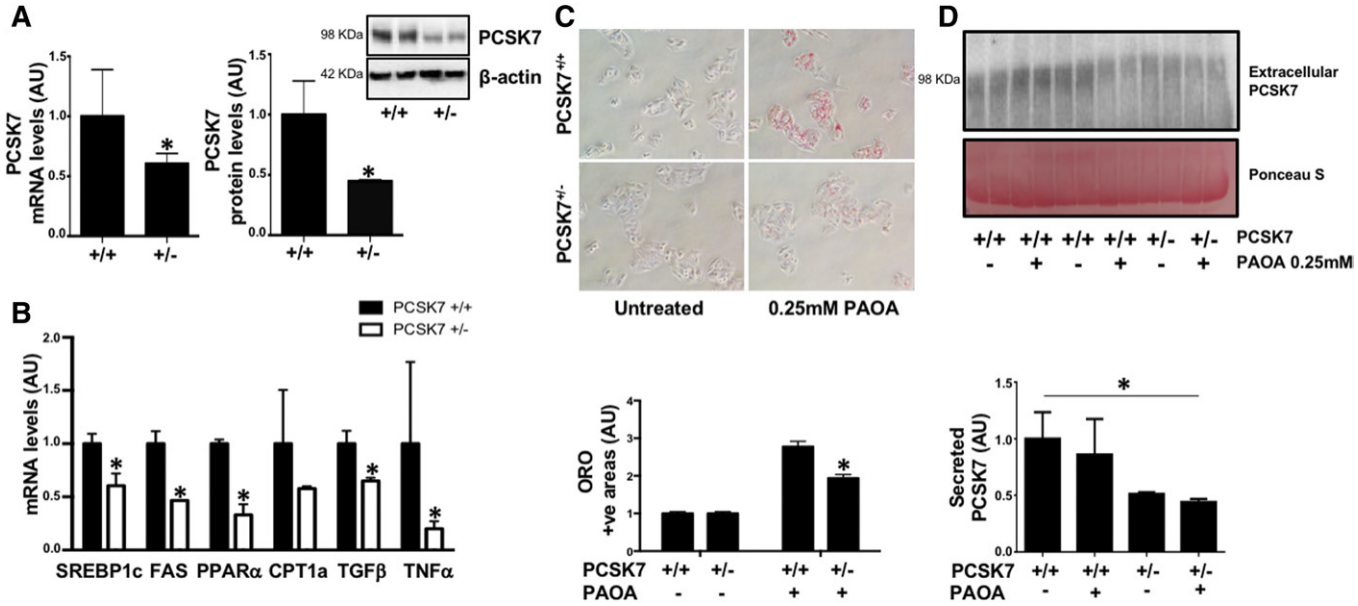

Fig. 4. *PCSK7* deletion in HepG2 cells reduced lipogenesis and *TGFβ* expression. A: *PCSK7* mRNA and protein levels were evaluated by quantitative RT-PCR and Western blot, respectively, normalized for β-actin and expressed as fold increase. *$P < 0.05$ compared with $PCSK7^{+/+}$ cells. B: *SREBP1c*, *FAS*, *PPARα*, *CPT1a*, *TGFβ*, and *TNFα* expression was evaluated in untreated cells by quantitative RT-PCR, normalized for β-actin and expressed as fold increase. *$P < 0.01$ compared with $PCSK7^{+/+}$ cells. C: $PCSK7^{+/+}$ and $PCSK7^{+/-}$ cells were treated with a combination of palmitic and oleic acid (PAOA) (0.25 mM) and lipid accumulation was evaluated by Red Oil staining (200× magnification). *$P < 0.05$ compared with untreated cells. D: Extracellular PCSK7 levels were evaluated by Western blot in the supernatants, normalized for Ponceau staining, and expressed as fold increase. *$P < 0.05$ compared with $PCSK7^{+/+}$ cells.

test the association with circulating transferrin receptor levels, the rs236918 variant was related to increased TS, therefore confirming a possible role of *PCSK7* in dysregulation of iron metabolism (20). The rs236918 variant was also associated with increased aminotransferases, especially with AST levels, a marker of hepatic damage, and with more severe lobular inflammation. However, in the overall LBC, the variant did not have a significant impact on hepatic fat accumulation, suggesting that the mechanism underlying the association with hepatic inflammation is at least partially independent of the total amount of hepatic fat. Notably, in NAFLD patients who carry the *PNPLA3* I148M risk variant, the rs236918 variant was also associated with steatosis grade. It could be speculated that the *PCSK7* variant causes an increase in circulating lipids by affecting lipogenesis, and in patients who carry the *PNPLA3* I148M variant, the effect translates into the development of a more severe steatosis.

The lack of association between *PCSK7* genotype and fibrosis in the LBC, despite more severe inflammation, may be related to a lack of power. Indeed, in the well-powered UKBBC, we detected a nominally significant association between the variant and the risk of advanced liver disease. On the other hand, in the DHS, the minor rs236918 C allele was not associated with lipids, hepatic fat content, and liver enzymes. This discrepancy may be accounted for by the smaller sample size and more limited power of the DHS as compared with the UKBBC, or by a smaller effect of the variant in the general population versus high-risk patients (32). However, the inclusion of individuals of non-European background in the DHS allowed us to evaluate the impact of two low-frequency missense variants in *PCSK7* (A102T and R504H), associated with circulating lipid levels in opposite directions. As we now provide evidence that increased PCSK7 is associated with dyslipidemia, we could therefore speculate that the A107T variant similarly acts by increasing, while the other R504H variant acts by decreasing PCSK7 protein activity (7). However, the impact of these mutations on specific PCSK7 substrates remains to be demonstrated, and neither of these variants was associated with hepatic triglycerides, in line with the notion that the effect of *PCSK7* gene variation on circulating lipids is not secondary to modulation of hepatic fat content.

In the attempt to decipher the role of PCSK7 in lipid handling and liver disease, we showed that *PCSK7* is more expressed in hepatocytes compared with other liver cells. Furthermore, hepatic expression of *PCSK7* correlated with that of *SREBP1c* and *FAS*, involved in de novo lipogenesis, and with *TGFβ*. In line with these results, *TGFβ* expression and activation of the dependent signaling pathway were hampered in *PCSK7* haploinsufficient cells as compared with wild-type cells following exposure to FFAs. It has been demonstrated that the TGFβ-SMAD2/3 signaling pathway promotes lipid accumulation with induction of lipogenesis-related genes and suppression of β-oxidation in hepatocytes (33). These results, obtained in *PCSK7*-haploinsufficient cells, are also in line with previous data obtained in zebrafish, showing that *PCSK7* increases both the mRNA expression and proteolytic cleavage of TGFβ, the premier profibrogenic cytokine and activator of hepatic fibrogenesis (34). It has also been speculated that transferrin caused activation and upregulation of procollagen-type gene expression in hepatic stellate cells, indicating a significant role of the transferrin receptor in iron-mediated liver fibrogenesis

(35). Further studies are required to clarify whether the association of rs236918 with regulation of iron metabolism (36) and liver damage progression (37) is mediated by modulation of *TGFβ* signaling.

Concerning the mechanism linking rs236918 with PCSK7 overall function, we showed that this variant is not in linkage with other protein-altering sequence variants that may affect PCSK7 activity. Furthermore, it may only marginally affect the relative abundance of alternative mRNA transcripts. Epigenomic analysis revealed high levels of H3K36me3 and H3K27ac at the rs236918 region, which may indicate active gene transcription (38), as this histone modification is often found in the core regions of actively transcribed regions or, alternatively, may play a role in maintaining gene expression stability. In addition, high levels of H3K27ac are often found around active enhancers (39), suggesting that rs236918 may fall near or within a regulatory element active in the liver. Consistently, de novo assembly analysis revealed a putative lncRNA in the rs236918 locus, which was predicted to locate into the cytoplasm and to interact with the PCSK7 protein. Remarkably, we could directly detect the expression of this intra-PCSK7 lncRNA in the liver of severely obese individuals, thereby confirming the in silico prediction. We found that the hepatic expression of the intra-PCSK7 lncRNA was higher in carriers of the rs236918 variant compared with noncarriers. Although the causal relationship between the intra-PCSK7 lncRNA and PCSK7 proteins' expression levels and the impact of the rs236918 variant on this process remain to be demonstrated, it could be speculated that the rs236918 C allele increases the PCSK7-intra-PCSK7 lncRNA interaction by enhancing PCSK7 stability and, possibly, by hampering its degradation.

Consistent with this scenario, the presence of the C risk allele was associated with higher hepatic levels and secretion of PCSK7, despite the lack of a change in *PCSK7* mRNA levels. Even though we could not rule out the possibility that rs236918 impacts the expression of nearby genes in the region, several clues suggest that modulation of *PCSK7* expression is the underlying mechanism. Indeed, circulating levels of PCSK7 correlated with serum triglycerides. This is also consistent with the fact that the R504H loss-of-function variant protects from dyslipidemia (7), while the rs236918 variant, which we showed here is associated with increased PCSK7 levels, worsens the lipid profile. By analogy, the A102T variant, which is also associated with increased lipid levels, could be regarded as a gain-of-function mutation. A possible mechanism is represented by PCSK7-mediated cleavage of angiopoietin-like protein 4 (AGPTL4), which then acquires the ability to inhibit lipoprotein lipase, thus impairing triglyceride clearance. Unfortunately, we could not assess activated ANGPTL4 and peripheral LPL expression in our cohort. On the other hand, we provided evidence suggesting that PCSK7 may also be involved in the regulation of de novo lipogenesis and lipid oxidation in a cell-autonomous fashion in hepatocytes. Indeed, human transcriptome data showed a correlation of PCSK7 levels with lipid metabolism in the liver of severely obese individuals. Furthermore, the expression of lipogenic and lipid oxidation genes was reduced in *PCSK7*$^{+/-}$ HepG2 cells as compared with the wild-type counterpart. Consistent with the human genetic data, in *PNPLA3* 148M/M HepG2 cells, *PCKS7* haploinsufficiency protected against fat accumulation following exposure to fatty acids. These data suggest that the reduced *PCSK7* expression alters lipid metabolism in hepatocytes and support the notion that the lipid phenotype associated with *PCSK7* variation is mediated by modulation of PCSK7 expression/activity. The impact of PCSK7 on the regulation of both lipogenic and lipolytic pathways may explain why carriage of the *PCSK7* rs236918 C allele did not result in a uniform increase in the risk of fatty liver, but specifically in individuals at higher risk, e.g., in those carrying the *PNPLA3* I148M risk variant, which impairs the lipid catabolism (3). *PCSK7* deletion was also associated with reduced *TNFα* and *TGFβ* expression. The latter suggests the existence of an interaction between hepatic fat accumulation and PCSK7 in determining hepatic inflammation and activation of fibrogenesis, favored by the presence of the I148M *PNPLA3* variant. The in vitro model also confirmed that decreased protein synthesis leads to reduced detectability of the PCSK7 protein in the supernatants, although further studies are necessary to prove that the PCSK7 protein is actively shed and secreted by hepatocytes.

In conclusion, the present results suggest that the rs236918 *PCSK7* variant links dyslipidemia with a tendency toward more liver damage in NAFLD patients at higher risk. Several clues suggest that the mechanism may involve increased intracellular PCSK7 protein and its secretion from hepatocytes. Furthermore, hemizygous *PCSK7* deletion decreases lipogenesis in hepatocytes and protects from fat accumulation. These data suggest that inhibition of PCSK7 synthesis may decrease circulating lipids, leading also to improvement of liver damage in high risk individuals.

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