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Variants in *PCSK7*, *PNPLA3* and *TM6SF2* are risk factors for the development of cirrhosis in hereditary haemochromatosis

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Summary

Background: Cirrhosis develops in <10% of individuals homozygous for the C282Y variant in the homeostatic iron regulator (*HFE*) gene. Carriage of *PCSK7*:rs236918 is associated with an increased risk of cirrhosis in this population.

Aim: To determine if genetic variants significantly associated with the risk of alcohol- and NAFLD-related cirrhosis also modulate the cirrhosis risk in C282Y homozygotes.

Methods: Variants in *PCSK7*, *PNPLA3*, *TM6SF2*, *MBOAT7* and *HSD17B13* were genotyped in 1319 C282Y homozygotes, from six European countries, of whom 171 (13.0%) had cirrhosis. Genotypic and allelic associations with the risk for developing cirrhosis were assessed, adjusting for age and sex. Fixed effects meta-analyses of the adjusted summary data for each country were performed. *Post hoc* association testing was undertaken in the 131 (76.6%) cases and 299 (26.0%) controls with available liver histology.

Results: Significant associations were observed between *PCSK7*:rs236918 (OR = 1.52 [95% CI 1.06-2.19]; $P = 0.022$; $I^2 = 0\%$); *PNPLA3*:rs738409 (OR = 1.60 [95% CI 1.22-2.11]; $P = 7.37 \times 10^{-4}$; $I^2 = 45.5\%$) and *TM6SF2*:rs58542926 (OR = 1.94 [95% CI 1.28-2.95]; $P = 1.86 \times 10^{-3}$; $I^2 = 0\%$) and the cirrhosis risk in C282Y homozygotes. These findings remained significant in the subpopulation with available liver histology. The population-attributable fractions were 5.6% for *PCSK7*:rs236918, 13.8% for *PNPLA3*:rs738409, 6.5% for *TM6SF2*:rs58542926 and 24.0% for carriage of all three variants combined.

Conclusions: The risk of cirrhosis associated with carriage of *PCSK7*:rs236918 was confirmed in this much larger population of C282Y homozygotes. In addition, *PNPLA3*:rs738409 and *TM6SF2*:rs58542926 were established as significant additional risk factors. More detailed genetic testing of C282Y homozygotes would allow risk stratification and help guide future management.

Stephan Buch, Aneesh Sharma and Eleanor Ryan contributed equally to the presented work and share premier authorship.

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1 | INTRODUCTION

Hereditary haemochromatosis (HH) is an autosomal recessive disorder of dysregulated iron metabolism characterised by elevation of serum ferritin concentrations and transferrin saturations and the accumulation of iron in the liver and other organs.[1]

HH is predominantly attributable to a cysteine to tyrosine substitution at position 282 (p.Cys282Tyr) within the *homeostatic iron regulator* (*HFE*) gene and is the most common autosomal recessive disorder in adults of northern European origin.[2,3] It has an allele frequency of 6.2% and a homozygosity frequency of 0.38% or 1:260, but there is considerable variation across Europe with a decreasing gradient from North-West to South-East. Thus, the allele frequency ranges from 10% to 12.5% in Ireland to 0% to 3% in Southern Europe; homozygosity rates range, along the same divide, from 1:150 to <1:3000.[1,3-6]

Although common, this genetic polymorphism is not always phenotypically expressed. Thus, there is considerable variability in the proportion of C282Y homozygotes who develop iron overload.[7] In a large individual patient meta-analysis of C282Y homozygotes, increased serum ferritin concentrations were found in 32% of men and 26% of women while excess liver iron concentrations were found in 42% of men and 19% of women.[3] Equally, there is considerable variability in the proportion of C282Y homozygotes, with iron overload, who develop a clinical phenotype; it is estimated that approximately 10% to 33% will eventually develop HH-associated morbidity.[7] Penetrance is generally higher in men than in women. Menstruation and pregnancy may counter excess iron absorption, at least until the menopause, but are not entirely protective.[8] Overall, the clinical expressivity of C282Y homozygosity, using strict definitions of both iron overload and iron-related organ damage, ranges from 22% to 28% in men and from 1% to 10% in women.[4,9,10]

Thus, C282Y homozygosity is a necessary but not sufficient condition for the development of HH-associated phenotypes, which strongly suggests that constitutional, environmental and genetic modifiers may play a role in gene expression.[11] Daily consumption of >60 g of alcohol, for example, increases the risk of people with HH developing cirrhosis by a factor of nine.[12,13] Likewise, the presence of obesity, diabetes and chronic viral hepatitis all augment the risk for developing cirrhosis in this population.[14-17]

The role played by additional genetic risk modulators is now attracting considerable attention. In 2012, Valenti and coworkers[18] identified a significant association between carriage of rs738409 in the *patatin-like phospholipase domain containing-3* (*PNPLA3*) gene and the development of severe hepatic fibrosis/cirrhosis in Italian C282Y homozygotes, which was independent of the degree of iron overload. This *PNPLA3* variant had previously been identified as a significant risk factor for the development of cirrhosis in individuals misusing alcohol or with non-alcoholic fatty liver disease (NAFLD).[19,20]

In 2014, Stickel and colleagues[21] reported a significant association between rs236918 in the *proprotein convertase subtilisin/kexin type 7* (*PCSK7*) gene and the risk for developing cirrhosis in C282Y homozygotes in a German-Austrian-Swiss cohort. These findings were later confirmed by Pelucchi and coworkers in an Italian cohort.[22] No significant association was observed between *PCSK7*:rs236918 and the risk for developing alcohol-related cirrhosis.[21]

Several genome-wide association studies (GWAS) have been undertaken in patients with liver disease, almost exclusively European, which have identified rs58542926 in *transmembrane 6 superfamily member 2* (*TM6SF2*) and rs641738 in *membrane bound O-acyltransferase domain containing protein 7* (*MBOAT7*) as risk loci for alcohol-related and NAFLD-related cirrhosis.[19,23-26] Additionally, Abul-Husn and colleagues[27] have identified a splice variant rs72613567 in *hydroxysteroid 17-beta dehydrogenase 13* (*HSD17B13*), which appears to protect against the development of alcohol- and NAFLD-related chronic liver injury in people of European ancestry, and to attenuate the risk associated with carriage of *PNPLA3*:rs738409. The findings in relation to alcohol-related liver disease have been confirmed by others.[28]

Thus, the aim of this study was to further evaluate the role of modifier genes in determining the risk for developing cirrhosis in homozygous carriers of C282Y in *HFE*.

2 | METHODS

2.1 | Study population

Unrelated individuals who were homozygous for C282Y in *HFE* were recruited from centres across Europe (Table S1). All were Caucasian and of self-reported German/Austrian/Swiss/Italian/British or Irish ancestry.

Potential participants were excluded if they had a history of prolonged, sustained alcohol intake of a minimum of 40 g/day in women and 60 g/day in men, or if they had any potential cause of liver injury other than iron overload, specifically if they were positive for hepatitis B surface antigen, anti-hepatitis C immunoglobulin G, anti-nuclear antibodies (titre >1:80) or anti-mitochondrial antibodies (titre >1:40). Patients with a serum caeruloplasmin of <20 mg/dL (0.2 g/dL) or a serum alpha-1 antitrypsin of <70 mg/dL (13 μmol/L) were further investigated and excluded if diagnosed with Wilson's disease or alpha-1 antitrypsin deficiency. Patients were also excluded if they were morbidly obese, ie BMI >40. The presence of diabetes was not an exclusion criterion.

For the purpose of this study, C282Y homozygotes were classified as *cases* if they had histological evidence of cirrhosis evidenced by a METAVIR score of F4,[29] or unequivocal evidence of significant liver damage based on a combination of *two* or more of the following sets of conditions: (a) compatible laboratory abnormalities, *viz*. hyperbilirubinaemia, transaminitis, hypo-albuminaemia, impaired coagulation and thrombocytopaenia; (b) a history or evidence of hepatic decompensation manifest as jaundice, ascites, variceal haemorrhage or hepatic encephalopathy; (c) evidence on imaging of chronic liver disease including changes in liver outline and texture, splenomegaly and the presence of a collateral

circulation; (d) a liver stiffness measurement (Fibroscan, Echosens) of >12 kPa (IQR <20%) and (e) the presence of gastro-oesophageal varices on upper gastrointestinal endoscopy.

Controls were defined as C282Y homozygotes who had no historical, clinical or laboratory evidence of advanced fibrosis confirmed either by a liver stiffness measurement (Fibroscan, Echosens) of <8 kPa (IQR <20%) or by a histological METAVIR score of F0 to F2.[29]

Patients with advanced fibrosis evidenced by a METAVIR score of F3 (n = 15) were not assigned as either cases or controls and were excluded from the main association analyses.

2.2 | Genotyping

Genomic DNA was extracted from the British and Irish peripheral blood samples using the Illustra Nucleon® Genomic Kit (GE Healthcare Life Sciences) and from the German, Austrian, Swiss and Italian samples using the Invisorb Blood Giga Kit (Invitec). All DNA samples were quality checked on agarose gels. Genomic DNA (10 ng) was used for direct genotyping in the UK, whereas DNA (1 μl) from the German, Austrian, Swiss and Italian samples was amplified, before genotyping, using the GenomiPhi Amplification Kit (GE Healthcare, Life Sciences). Genotyping of the five single nucleotide polymorphisms (SNPs) of interest, *viz*. *PCSK7*:rs236918, *PNPLA3*:rs738409, *TM6SF2*:rs58542926; *MBOAT7*:rs641738 and *HSD17B13*:rs76213567, was performed using either the TaqMan® SNP Genotyping Assays and chemistries (Applied Biosystems) on an automated platform with TECAN Freedom EVO and 384 well TEMO liquid-handling robots (TECAN), or TaqMan 5′-Nuclease Assays (Life Technologies) or the K-Biosciences Competitive Allele Specific PCR (LGC Genomics) platform with amplification and detection undertaken using a LightCycler® 480 real-time PCR system (Roche Molecular Diagnostics). The sequences of the primers used for each SNP are detailed in Table S2.

Approximately 12% of the samples for each SNP were randomly selected and re-genotyped to verify the original genotyping calls.

2.3 | Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 25 (IBM Corp), the PLINK 2.0 genome analysis toolset[30,31] and R Version 1.18.1. (CRAN.R-project.org/doc/FAQ/R-FAQ.html). The genotypic data in each cohort were analysed for departures from Hardy-Weinberg equilibrium (HWE) in the entire sample and separately in the *cases* and *controls* using exact test statistics. The association between each genetic variant and the risk for developing cirrhosis was estimated from multivariate logistic regression analysis, adjusted for age and sex, assuming a multiplicative (log-additive) genetic model. Results, expressed as odds ratios (OR) with their 95% confidence intervals (CI), were derived from beta coefficients and their standard deviations. Nominal two-sided asymptotic *P*-values are reported for all tests.

A power analysis, based on the 171 cases and 1148 controls available for study, was performed using PS-Power (https://vbiostatps.app.vumc.org/ps/), adopting various allelic odds ratio between 1 and 2 and a nominal significance level of 0.05.

Both fixed effects and random effects models were used to meta-analyse the adjusted summary data, for each SNP, in each of the six country cohorts. Inter-cohort heterogeneity was quantified using the I^2 statistic, which was calculated using the formula:

$$I^2 = 100\% \times (Q - df)/Q$$

where Q is Cochran's homogeneity test statistic and df the degrees of freedom.

The DerSimonian-Laird random effects model was used if the degree of heterogeneity was significant ($P < 0.05$); otherwise, an inverse variance weighted fixed effects model was used to achieve pooled effect sizes of the adjusted summary data using the Metafor package in R.[32] Sensitivity analyses were undertaken to assess the robustness of the association findings when the observed heterogeneity between studies was high (Q-statistics $P < 0.05$) using MetaXL software (http://www.epigear.com/index_files/metaxl.html). The effect of excluding studies in which genotype distribution in the controls deviated significantly ($P < 0.05$) from HWE was also assessed.

Post-hoc meta-analyses of the data were undertaken in three sub-cohorts, *viz*. (a) men only, adjusted for age; (b) participants with histological confirmation of their case/control status, adjusted for age and sex, and (c) participants with histological confirmation of their case/control status, adjusted for age, sex and obesity.

Co-carriage of significantly associated risk variants and genotype proportions in groups defined by increasing fibrosis stage was investigated in the cases and controls with available histology using a linear-by-linear test for ordered contingency tables in R.[33] This test is similar to a Chi-square test, and is used to test the association among variables in a contingency table with ordered categories. All available subjects were included and were evaluated according to single or grouped METAVIR fibrosis staging scores as follows: absent (stage: F0), mild-moderate fibrosis (stages: F1 and F2) and severe fibrosis/cirrhosis (stages: F3 and F4).

Possible interactions between any significantly associated SNPs were examined pair-wise by logistic regression adjusted for age, sex and country as categorical variables, including both main allelic SNP effects and the allelic SNP*SNP interaction term.

The population-attributable fraction (PAF) provides an epidemiological estimate of the proportion of a disorder that is attributable to a given risk factor. Thus, in this instance, it is an estimate of how much lower the frequency of cirrhosis would be in C282Y homozygotes if any risk genotypes were eliminated from the population. Calculation of the PAF for each of the significantly associated risk allele was based on the pooled adjusted allelic OR from the fixed effects meta-analysis and risk genotype frequency of the SNP using the formula[34]:

$$\%\ \mathrm{PAF} = P_E \times (\mathrm{RR} - 1)/[1 + P_E(\mathrm{RR} - 1)] \times 100$$

where P_E is the proportion of exposed controls (risk allele frequency in the C282Y homozygotes without cirrhosis) and RR is the relative risk.

The RR is approximated by the ORs obtained from the meta-analysis of the six country cohorts, adjusted by age and sex.

Combined PAF estimates were calculated based on the individual PAFs for each associated SNP, assuming no multiplicative interaction between them.[35]

2.4 | Ethics

The study protocol was approved by the ethics committees of the participating institutions; all included subjects provided written informed consent prior to inclusion into the study.

3 | RESULTS

3.1 | Study population

The study population comprised of 1319 unselected C282Y homozygotes recruited from six countries across Europe (Table S1). Participants were predominantly middle aged and approximately two thirds were men; these findings were consistent across cohorts except that there were proportionately more men in the Swiss cohort (Table 1). A total of 171 (13%) participants were classified as cases; the proportions varied from 6% in the Irish cohort to 30% in the Swiss cohort. The cases were, on average, a decade older than the controls; proportionately more were men (88% *cf.* 62%; $P < 0.0001$) and proportionately more were diabetic (25% *cf.* 7%; $P < 0.0001$) (Table 1).

Case-control status was confirmed by histological examination of liver biopsy tissue in 131 (76.6%) of the 171 cases and in 299 (26.0%) of the 1148 controls (Figure 1). The characteristics of this subpopulation closely mirrored those of the total population (Table S3).

3.2 | Genotyping

Genotyping for all five SNPs was completed with call rates greater than 99% except for *HSD17B13*:rs72613567, which had a call rate of 93.6%. All markers followed HWE (cut-off $P > 0.05$) with the exception of *PCSK7*:rs236918 in the Irish control population (HWE $P = 0.026$) (Table S4). The minor allele frequencies (MAFs) of three of the five SNPs, *viz*. *PCSK7*:rs236918, *PNPLA3*:rs738409 and *HSD17B13*:rs72613567, showed moderate heterogeneity in control populations across the cohorts but the observed heterogeneity was comparable with that of European populations in public databases[36] (Table S5), and in previous publications.[18-21,23,27,28] (Table 2).

3.3 | Association analyses

The systematic power calculation based on the 171 cases and 1148 controls included in the study indicated that risk associations with an

TABLE 1 Demographic, clinical and laboratory information on the entire study population of C282Y homozygotes, at the time of diagnosis

	Cases (n = 171)							Controls (n = 1148)						
Cohort (n)	Total (n: %)	Men (n: %)	Age (yr)	Serum ferritin (μg/L)	BMI	Obese[a] (n: %)	Diabetes (n: %)	Total (n)	Men (n: %)	Age (yr)	Serum ferritin (μg/L)	BMI	Obese[a] (n: %)	Diabetes (n: %)
Austria (392)	56 (14%)	52 (93%)	62 (52-68)	2720 (1525-4105)	26.1 (23.9-27.5)	3/26	1/10	336	211 (63%)	49 (38-60)	917 (534-1457)	25.9 (24.1-28.3)	11/87	8/68
England (148)	22 (15%)	19 (86%)	55 (47-65)	2628 (1280-4255)	<30	0/22	10/22	126	74 (59%)	54 (42-63)	800 (444-1368)	<30	0/126	9/106
Germany (163)	25 (15%)	22 (88%)	59 (53-63)	2776 (2000-5250)	27.3 (25.3-31.9)	3/10	6/11	138	90 (65%)	51 (41-61)	1096 (600-2200)	25.9 (23.5-28.4)	15/106	8/117
Ireland (372)	22 (6%)	18 (82%)	49 (45-55)	1518 (1069-2148)	n/a	n/a	5/16	350	215 (61%)	47 (39-55)	538 (317-800)	n/a	n/a	10/184
Italy (174)	25 (14%)	21 (84%)	48 (43-57)	2353 (1570-4000)	24.0 (22.5-25.0)	0/6	9/29	149	88 (59%)	45 (34-59)	800 (471-1071)	25.0 (22.0-27.0)	7/58	8/145
Switzerland (70)	21 (30%)	19 (90%)	57 (50-63)	3313 (2280-4940)	n/a	n/a	5/21	49	34 (69%)	50 (41-58)	1088 (671-2114)	n/a	n/a	4/49
TOTAL 1319	171 (13%)	151 (88%)	56 (48-64)	2560 (1570-4000)	26.0 (24.0-28.3)	9% (6/70)[a]	25% (36/145)	1148	712 (62%)*	48** (39-59)	748** (437-1216)	25.7 (23.8-28.3)	8% (33/410)	7%** (47/716)

Note: The study population comprised of 1319 unselected, unrelated C282Y homozygotes recruited from six countries across Europe. Of these 171 participants with histological or unequivocal surrogate evidence of cirrhosis were classified as CASES, while the remaining 1148 participants with no historical, or surrogate evidence cirrhosis were classified as CONTROLS.

Data expressed as median (interquartile [IQR: Q1-Q3] range).

Abbreviations: BMI, body mass index; n/a, not available.

Significance of the differences between cases and controls *$P < 0.01$; **$P < 0.0001$.

[a]Based on a BMI ≥30 but <40.

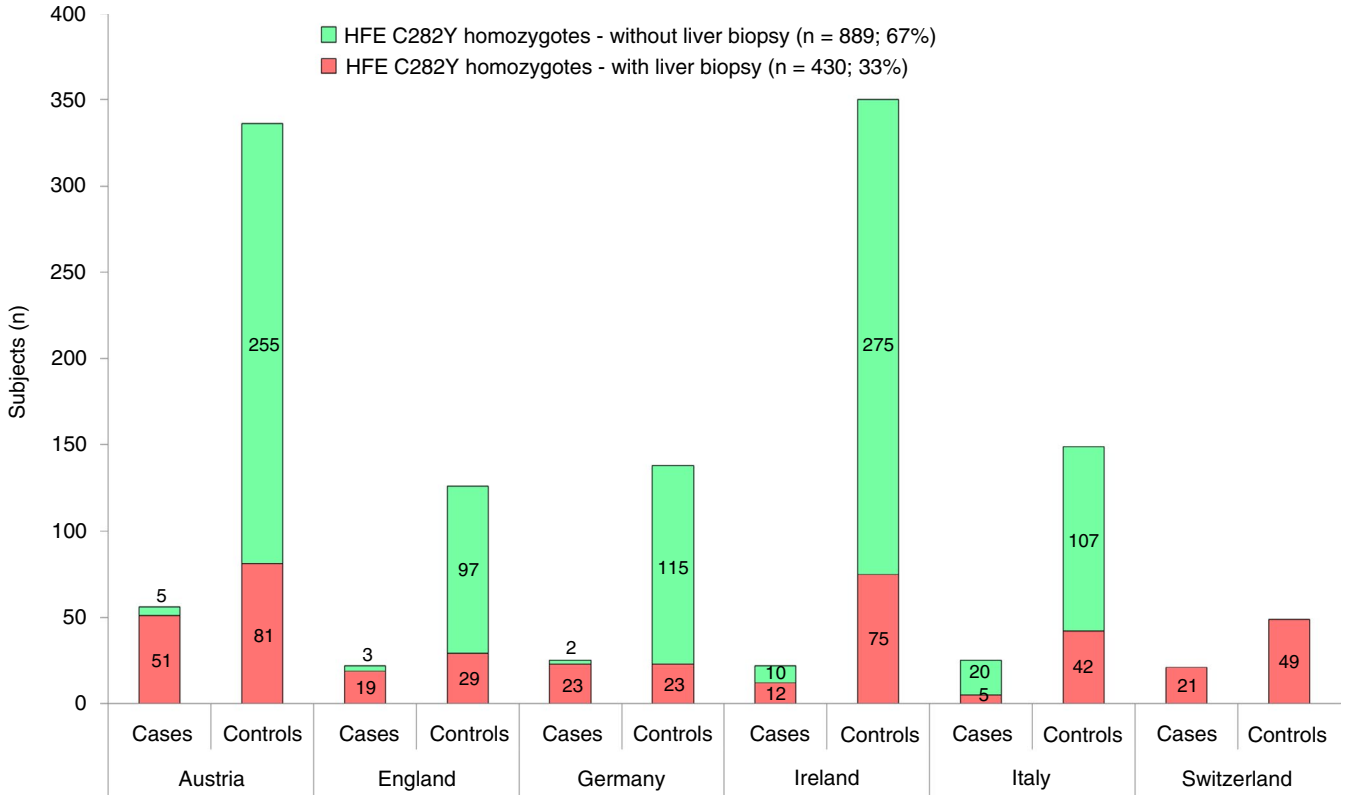

FIGURE 1 Proportion of C282Y homozygotes in whom case-control status was confirmed by examination of liver histology, by country cohort

odds ratios >1.6 would be detectable with a power >80% for allele frequencies ≥0.3 (Figure S1).

The associations between the five SNPs of interest and the risk of developing cirrhosis were not significant in all cohorts (Table 2; Figure 2). However, fixed effects meta-analysis, on the pooled age- and sex–adjusted data, confirmed significant associations between the risk for developing cirrhosis, in C282Y homozygotes, and carriage of *PCSK7*:rs236918 (OR = 1.52 [95% CI 1.06-2.19]; $P = 0.022$; $I^2 = 0\%$); *PNPLA3*:rs738409 (OR = 1.60 [95% CI 1.22-2.11]; $P = 7.37 \times 10^{-4}$; $I^2 = 45.5\%$) and *TM6SF2*:rs58542926 (OR = 1.94 [95% CI 1.28-2.95]; $P = 1.86 \times 10^{-3}$; $I^2 = 0\%$) (Figure 2, Table S5). The associations with *MBOAT7*:rs641738 (OR = 1.17 [95% CI 0.91-1.51]; $P = 0.219$; $I^2 = 31.8\%$) and *HSD17B13*:rs72613567 (OR = 0.94 [95% CI 0.69-1.27]; $P = 0.685$; $I^2 = 16.8\%$) were not significant (Figure 2, Table S6). There was no significant heterogeneity between the cohorts. The association results obtained utilising a random effects model were confirmatory (Figure 2, Table S6).

The Irish control population did not follow HWE for *PCSK7*:*rs236918* ($P = 0.026$). However, exclusion of the Irish cohort from the associational analysis for this SNP did not affect the significance of the association with cirrhosis risk (OR = 1.72 [95% CI 1.15-2.58]; $P = 0.008$; $I^2 = 0\%$) (Table S7).

A post-hoc fixed effects meta-analysis confined to male cases and controls confirmed the significance of the associations between the risk for developing cirrhosis and *PCSK7*:rs236918 (OR = 1.65 [95% CI 1.12-2.41]; $P = 0.011$; $I^2 = 0\%$); *PNPLA3*:rs738409 (OR = 1.51 [95% CI 1.12-2.03]; $P = 6.14 \times 10^{-3}$; $I^2 = 54.4\%$) and *TM6SF2*:rs58542926 (OR = 1.98 [95% CI 1.25-3.12]; $P = 3.35 \times 10^{3}$; $I^2 = 0\%$) (Table S8). The significance of the association with *PNPLA3*:rs738409 was lost if a random effects model was applied reflecting the moderate degree of inter-cohort heterogeneity for this SNP (Table S8).

A post-hoc fixed effects meta-analysis confined to homozygous C282Y cases and controls, of both sexes, whose status had been confirmed by histological examination of liver biopsy material, confirmed the presence of significant associations between *PCSK7*:rs236918 (OR = 2.13 [95% CI 1.25-3.66]; $P = 5.80 \times 10^{-3}$; $I^2 = 18.6\%$); *PNPLA3*:rs738409 (OR = 1.80 [95% CI 1.20-2.68]; $P = 4.09 \times 10^{-3}$; $I^2 = 75.6\%$) and *TM6SF2*:rs58542926 (OR = 2.06 [95% CI 1.18-3.61]; $P = 0.011$; $I^2 = 0\%$) and the risk for developing cirrhosis (Figure 3, Table S8). However, significant inter-cohort heterogeneity was observed for *PNPLA3*:rs738409; hence, the significance of this association was lost with the random effects model, *viz*. *PNPLA3*:rs738409 (OR = 1.59 [95% CI 0.70-3.65]; $P = 0.271$).

A sensitivity analysis applied to this subpopulation identified the German cohort (23 cases, 23 controls) as the major source of the substantial inter-cohort heterogeneity; its removal did not change the direction of the effect but reduced the heterogeneity ($I^2 = 57.2\%$; $P = 0.053$) and resulted in retention of the significant association between *PNPLA3*:rs738409 and cirrhosis risk with both the fixed effects (OR 2.32 [95% CI 1.52, 3.56]; $P = 1.10 \times 10^{-4}$, I^2 52.8%) and random effects models (OR = 2.21 [95% CI 1.13-4.34]; $P = 0.021$, I^2 57.2%) (Table S10).

TABLE 2 Raw genotype counts and association analyses for the five SNPs of interest and the risk for developing cirrhosis in C282Y homozygotes, adjusted for age and sex, by individual study cohort

		Cases		Controls			[a]Odds ratio
Gene: SNP	Cohort	Genotype count	MAF (%)	Genotype count	MAF (%)	Significance (*P* value)	(95% CI)
PCSK7 rs236918	Austria	GG/GC/CC 40/13/2	15.5	GG/GC/CC 275/58/2	9.3	0.143	1.60 (0.85-3.02)
	England	GG/GC/CC 15/7/0	15.9	GG/GC/CC 97/28/0	11.2	0.468	1.46 (0.52-4.07)
	Germany	GG/GC/CC 15/9/1	22.0	GG/GC/CC 117/17/3	8.4	0.061	2.22 (0.97-5.09)
	Ireland	GG/GC/CC 16/4/1	14.3	GG/GC/CC 252/82/15	16.0	0.811	0.90 (0.39-2.08)
	Italy	GG/GC/CC 17/8/0	16.0	GG/GC/CC 125/24/0	8.1	0.324	1.93 (0.52-7.11)
	Switzerland	GG/GC/CC 15/6/0	14.3	GG/GC/CC 41/7/1	9.2	0.503	1.50 (0.46-4.96)
PNPLA3 rs738409	Austria	CC/CG/GG 20/25/10	40.9	CC/CG/GG 172/130/29	28.4	0.004*	1.98 (1.24-3.14)
	England	CC/CG/GG 8/12/2	36.4	CC/CG/GG 69/48/8	25.8	0.110	1.86 (0.87-3.98)
	Germany	CC/CG/GG 13/11/1	26.0	CC/CG/GG 63/66/9	30.4	0.447	0.75 (0.35-1.59)
	Ireland	CC/CG/GG 7/11/3	40.5	CC/CG/GG 224/111/14	19.9	0.006*	2.67 (1.38-5.16)
	Italy	CC/CG/GG 10/9/6	42.0	CC/CG/GG 60/64/24	37.8	0.842	0.92 (0.43-2.01)
	Switzerland	CC/CG/GG 8/10/2	35.0	CC/CG/GG 26/21/2	25.5	0.444	1.42 (0.58-3.48)
TM6SF2 rs58542926	Austria	CC/CT/TT 41/14/0	12.7	CC/CT/TT 289/45/1	7.0	0.040*	2.17 (1.04-4.55)
	England	CC/CT/TT 18/4/0	9.1	CC/CT/TT 101/24/0	9.6	0.871	0.90 (0.27-3.07)
	Germany	CC/CT/TT 19/2/1	9.1	CC/CT/TT 112/24/0	8.8	0.691	1.27 (0.39-4.09)
	Ireland	CC/CT/TT 13/5/2	22.5	CC/CT/TT 298/48/2	7.5	0.004*	3.31 (1.47-7.44)
	Italy	CC/CT/TT 22/3/0	6.0	CC/CT/TT 134/15/0	5.0	0.228	2.47 (0.57-10.76)
	Switzerland	CC/CT/TT 17/4/0	9.5	CC/CT/TT 40/9/0	9.2	0.906	1.09 (0.27-4.38)
MBOAT7 rs641738	Austria	CC/CT/TT 19/25/11	42.7	CC/CT/TT 107/172/53	41.9	0.995	1.00 (0.63-1.58)
	England	CC/CT/TT 9/10/3	36.4	CC/CT/TT 42/67/16	39.6	0.690	0.86 (0.40-1.83)
	Germany	CC/CT/TT 10/9/5	39.6	CC/CT/TT 47/60/31	44.2	0.910	0.97 (0.52-1.78)
	Ireland	CC/CT/TT 6/8/7	52.4	CC/CT/TT 136/150/63	39.5	0.097	1.66 (0.91-3.00)
	Italy	CC/CT/TT 2/15/7	60.4	CC/CT/TT 52/66/30	42.6	0.015*	2.99 (1.23-7.25)
	Switzerland	CC/CT/TT 7/9/5	45.2	CC/CT/TT 15/22/12	46.9	0.948	0.98 (0.48-1.99)
HSD17B13 rs72613567	Austria	--/-A/AA 32/16/4	23.1	--/-A/AA 203/104/19	21.8	0.736	0.92 (0.55-1.53)
	England	--/-A/AA 8/9/2	34.2	--/-A/AA 59/40/7	25.5	0.375	1.42 (0.65-3.11)
	Germany	--/-A/AA 10/8/2	30.0	--/-A/AA 71/53/14	29.3	0.931	0.97 (0.46-2.05)
	Ireland	--/-A/AA 11/6/2	26.3	--/-A/AA 166/129/21	27.1	0.827	0.92 (0.43-1.97)
	Italy	--/-A/AA 14/10/1	24.0	--/-A/AA 97/37/9	19.2	0.747	1.15 (0.49-2.70)
	Switzerland	--/-A/AA 17/4/0	9.5	--/-A/AA 25/21/3	27.6	0.024*	0.23 (0.07-0.82)

Note: Genotype counts for the five investigated SNPs by case/control designation in the 1319 C282Y homozygotes from six European countries—listed alphabetically.

Abbreviations: CI, confidence interval; MAF, mean allele frequency.

[a]Odds ratios show the increase in risk of developing cirrhosis in C282Y homozygotes associated with carriage of each copy of the minor allele.

*Significance values $P < 0.05$.

A post-hoc fixed effects analysis in the population whose case-control status had been confirmed by histological examination, with additional adjustment for obesity, confirmed the significant associations with cirrhosis risk for *PCSK7*:rs236918 (OR = 3.65 [95% CI 1.18-11.23]; $P = 0.024$; $I^2 = 0\%$) and *PNPLA3*:rs738409 (OR = 2.78 [95% CI 1.25-6.20]; $P = 0.012$; $I^2 = 38.9\%$) (Table S10). The significance of the association with *PNPLA3*:rs738409 was not retained when the random effects model was applied but the same caveats in relation to the German cohort also pertained; exclusion of this cohort, as above, maintained the significance of the association with *PNPLA3*:rs738409 irrespective of which effects model was applied (data not shown).

In the combined case-control sample of homozygous C282Y homozygotes with available liver histology, the proportion of subjects carrying the risk-associated alleles significantly increased with increasing fibrosis stage depending on the groupings used. Thus, significant trends were observed between carriage of both *PNPLA3*:rs738409:G ($P = 0.009$) and *TM6SF2*:rs58542926:T

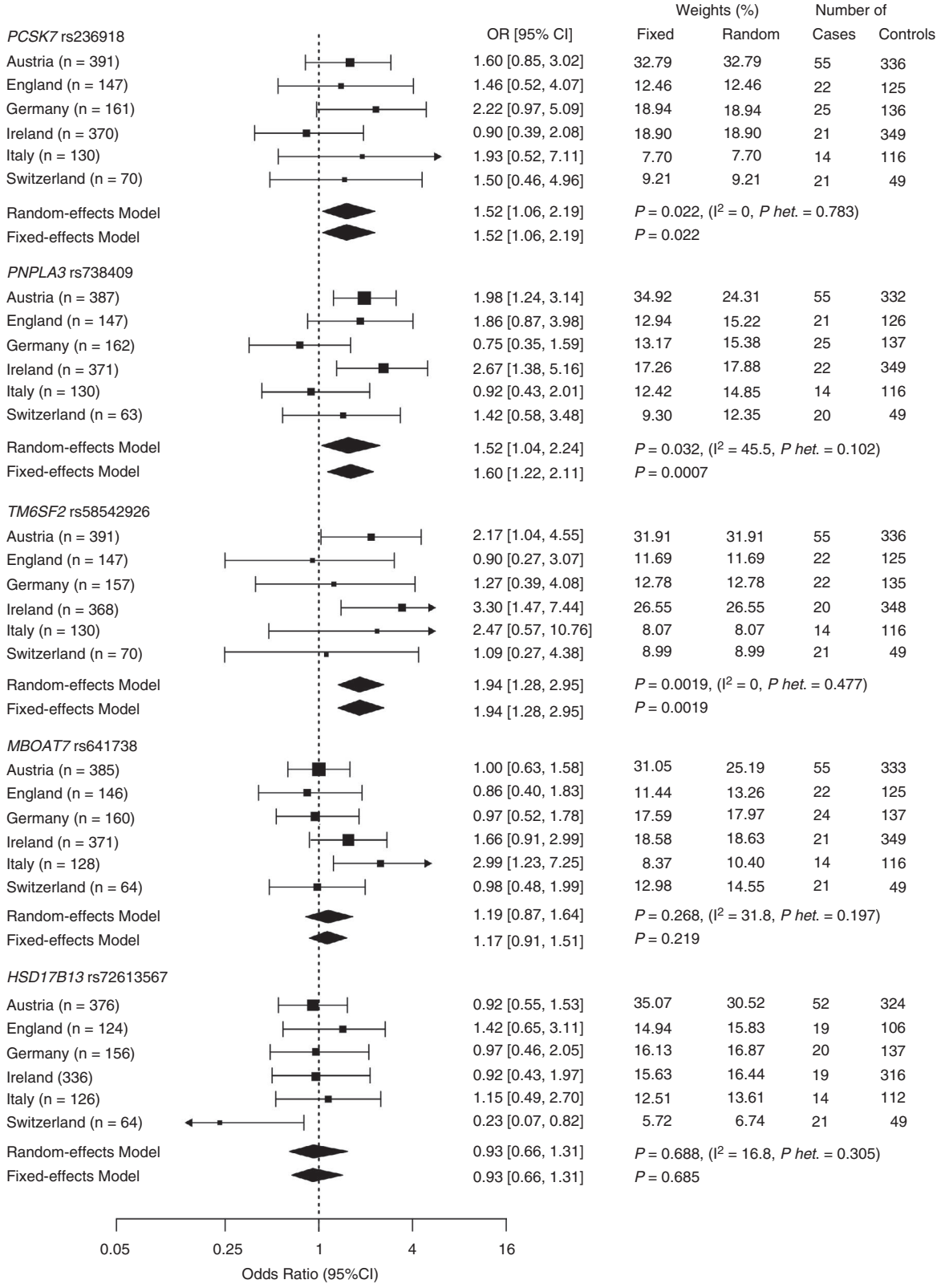

FIGURE 2 Forest plots of the fixed effects meta-analyses of the associations between the five SNPs of interest and the risk of developing cirrhosis in C282Y homozygotes, adjusted for age and sex, by country listed alphabetically. CI, confidence interval; I^2, between cohort heterogeneity index; OR, odds ratio; P_{het}., *P* value of the Cochran's Q test for heterogeneity between cohorts; weights %, relative cohort weight/importance; weights % is based on the variance in scores within a cohort; it closely relates to the sample size and is reflected in the size of the squares on the plot; weights are assigned under fixed effects and random effects models; I^2 describes the percentage of variation across studies due to heterogeneity rather than chance. In the absence of inter-cohort heterogeneity ($I^2 = 0$), the weights will be identical for both models

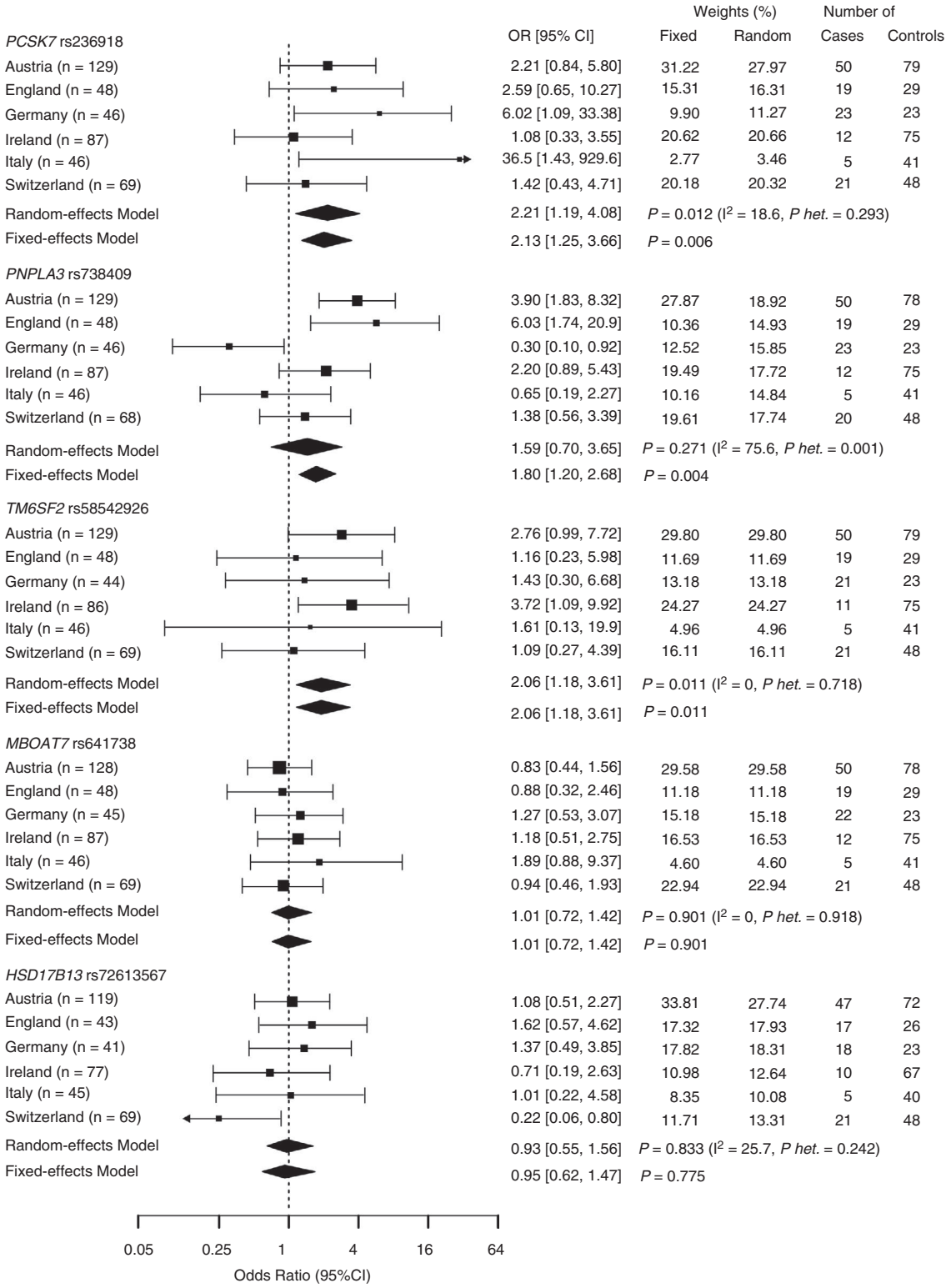

FIGURE 3 Forest plots of the fixed effects meta-analyses of the associations between the five SNPs of interest and the risk of developing cirrhosis in C282Y homozygotes with histological confirmation of their case-control status, adjusted for age and sex, by country listed alphabetically. CI, confidence interval; I^2, between cohort heterogeneity index; OR, odds ratio; P_{het}., *P* value of the Cochran's Q test for heterogeneity between cohorts; weights %, relative cohort weight/importance; weights % is based on the variance in scores within a cohort; it closely relates to the sample size and is reflected in the size of the squares on the plot; weights are assigned under fixed effects and random effects models; I^2 describes the percentage of variation across studies due to heterogeneity rather than chance. In the absence of inter-cohort heterogeneity ($I^2 = 0$), the weights will be identical for both models

(P = 0.01) and increasing fibrosis when comparisons were made between F0 vs F1-2 vs F3-4 and also between *PNPLA3*:rs738409:G (P = 0.045) and *PCSK7*:rs236918:C (P = 0.01) when comparisons were made between F0-F2 vs F3-4 (Figure S2).

3.4 | Population-attributable fractions

In the population overall the PAF% for *PCSK7*:rs236918 was 5.6%, for *PNPLA3*:rs738409, 13.8% and for *TM6SF2*:rs585429265, 6.5%; the combined PAF% for the three loci was 24.0% (Table 3). The PAFs in the sub-population in whom case/control status was verified by examination of liver histology were higher (Table 3); the combined PAF% in this sub-population was 36.8%.

The proportion of cases and controls carrying either none of the variants of interest in *PCSK7*, *PNPLA3* and *TM6SF2*, or one of three, two of three or all three was used to determine the aggregated risk of co-carriage of the risk loci (Figure 4). Thus, while only 14.9% and 0.9% of the controls carried either two or three of the variants these proportions increased significantly to 31.1% and 2.4% in the cases ($P = 9.98 \times 10^{-8}$, linear-by-linear test for trend).

3.5 | SNP: SNP interactions

Significant association was observed between the *PNPLA3*:rs738409 by *TM6SF2*:rs58542926 interaction term and the risk for developing cirrhosis in C282Y homozygotes (P = 0.042; OR 1.94 [1.02-3.67]) (Table S12). No other pair-wise SNP*SNP interaction was significant in the applied model.

4 | DISCUSSION

HH is a complex disorder of iron dysregulation predominantly attributable to a C282Y substitution in *HFE*. It has a very wide phenotypic spectrum. Thus, only 30% to 50% of C282Y homozygotes develop significant iron overload,[6] and only a minority of these develop significant liver injury.[37] A variety of constitutional and environmental factors are known to increase the risk for developing cirrhosis and HCC in these individuals but the nature and extent of any additional genetic modulation is largely unknown. Identification of these additional genetic elements is important as it will enable an individual's risk for developing progressive liver damage to be assessed more precisely, and will allow greater insight into currently unknown molecular aspects of the underlying liver injury.

4.1 | Study findings

The present study provides further confirmation that carriage of *PCSK7*:rs236918[21,22] and *PNPLA3*:rs7384093[18] are associated with the risk for developing cirrhosis in C282Y homozygotes, attesting to the robustness of these associations. In addition, *TM6SF2*:rs58542926, which is a known risk factor for the development of alcohol- and NAFLD-related cirrhosis,[19,23-25] was also shown to be a risk factor for cirrhosis development in this patient population, with ORs and attributable fractions comparable to those of *PCSK7*:rs236918. These associations remained significant in sub-populations of men only, and in men and women with available liver histology, indicating that the findings are generally applicable.

Carriage of these three genetic risk modulators between them account for 24.0% of the excess risk for developing cirrhosis in C282Y homozygotes. However, this is probably an underestimate as the PAF in those homozygotes in whom the presence of cirrhosis was confirmed or excluded by examination of liver histology was almost 37.0%.

The association with *MBOAT7*:rs641738, a risk factor for the development of alcohol- and NAFLD-related cirrhosis,[19,24,26] was not significant. There are no studies with which to compare these results but a recent candidate gene study in people with chronic hepatitis C showed that the influence of this variant is restricted to the transition from absence of fibrosis to mild fibrosis.[38] However, the allelic effect size of *MBOAT7*:rs641738, in the present study, was below the significance threshold identified in the power analysis; this suggests that the study may be underpowered to detect a modulating effect for this SNP on cirrhosis risk in HH.

Likewise, there was no significant association with *HSD17B13*:rs72613567, which confers protection against the development of NAFLD-related and alcohol-related cirrhosis and the subsequent development of HCC.[27,28] However, the study may also be underpowered to detect a significant modulating effect in HH. Less is known about the genetic associations of this locus with chronic liver disease of other aetiologies, although carriage of rs72613567: TA appears to protect against the development of severe hepatic fibrosis in people with chronic hepatitis C infection.[39]

4.2 | Mechanisms of liver injury

Several genetic mutations, primarily of *HFE*, are associated with the risk for developing HH, although the mechanism(s) by which these genetic variants disrupt iron metabolism/regulation/homeostasis is not entirely understood.[40] However, hepcidin deficiency is believed to play a central role as it results in enhanced intestinal iron absorption and release of iron from reticuloendothelial cells. The C282Y variant in *HFE* alters a key amino acid, which results in impairment of HFE protein signalling; this leads to reduced expression of hepcidin mRNA and decreased plasma hepcidin levels resulting in excessive systemic iron accumulation.

A number of hypotheses link the protein product of *PCSK7* to iron metabolism and liver fibrosis. PCSK7 is a convertase, which is thought to function in iron homeostasis by generating soluble hemojuvelin,[41] a protein which plays a crucial role in the regulation of hepcidin, and also processes prohepcidin.[42] In zebrafish, PCSK7 has been shown to contribute to mRNA expression and

TABLE 3 Population-attributable fraction[a] of each variant relating to the genetic risk of developing cirrhosis in C282Y homozygotes assessed both individually, and in combination in the entire population and in those in whom the case-control status was verified histologically

Genetic variant	[b]Population-attributable fraction % [95% CI]
Entire population	
PCSK7:rs236918	5.6 [0.7-11.9]
PNPLA3:rs738409	13.8 [5.6-22.9]
TM6SF2:rs58542926	6.5 [2.0-12.7]
PCSK7 + *PNPLA3* + *TM6SF2* (combined)	24.0 [8.1-40.7]
Cohort with available liver histology[c]	
PCSK7:rs236918	7.5 [0.3-18.3]
PNPLA3:rs738409	24.1 [3.3-46.6]
TM6SF2:rs58542926	10.1 [1.8-22.1]
PCSK7 + *PNPLA3* + *TM6SF2* (combined)	36.8 [4.5-62.6]

[a]Population-attributable fraction is an estimate of the proportion of cirrhosis cases in the population of C282Y homozygotes that can be attributed to exposure to the risk variants.

[b]Estimated from the pooled adjusted allelic odds ratio from the random effects meta-analyses and the risk genotype frequency of the SNP.

[c]German cohort excluded from the risk calculation.

proteolytic cleavage of transforming growth factor beta 1a (TGFβ), a cytokine key for the activation of hepatic stellate cells/myofibroblasts.[43] Carriage of the risk allele has been shown to increase PCSK7 expression in humans, while in human hepatocytes PCSK7 downregulation has been shown to facilitate intracellular fat accumulation and reduce (TGFβ) activation.[44] Thus, genetic modulation of PCSK7 expression or protein function may impact on hepatic fibrosis development.

PNPLA3:rs738409 and *TM6SF2*:rs58542926 are established genetic risk factors for the development of cirrhosis in response to a variety of insults including alcohol misuse and NAFLD. The gene products of *PNPLA3* and *TM6SF2* are enzymes which play a role in lipid/fatty acid turnover and are expressed in liver tissue. PNPLA3 has both triacylglycerol lipase and acylglycerol O-acyltransferase activities. The loss-of-function variant rs738409 is associated with a reduction in triglyceride hydrolysis,[45] and hence impaired triglyceride mobilisation.[46] *TM6SF2* is incompletely characterised, but the risk variant rs58542926:T encodes a loss-of-function protein, which impairs very low-density lipoprotein export, resulting in increased hepatocellular lipid content.[47]

It is possible that HFE-mediated hepatic iron overload may interact adversely with the lipid accumulation in hepatocytes, engendered by carriage of *PNPLA3*:rs738409 and *TM6SF2*:rs58542926, resulting in increased production of lipid peroxides via iron-mediated oxidation of fatty acids such as 4-hydroxynonenal and/or

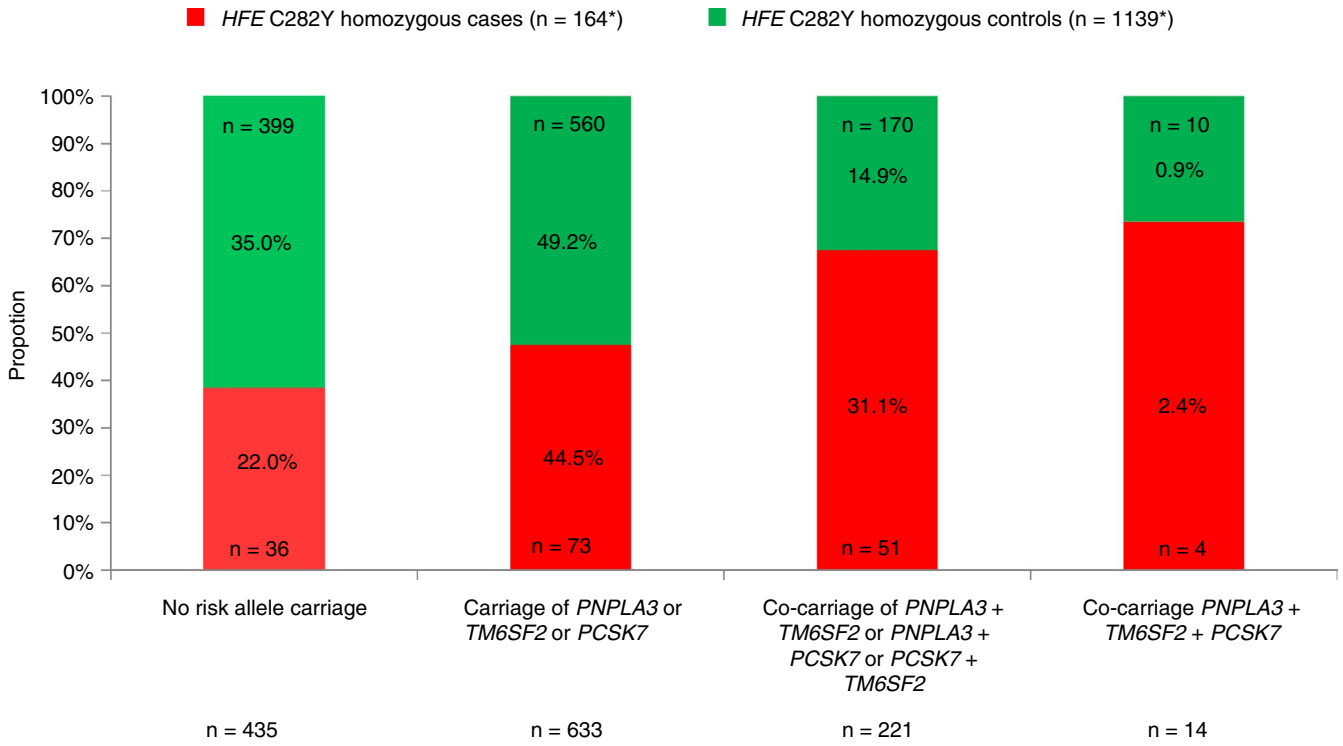

FIGURE 4 Carriage and co-carriage of the *PCSK7*/*PNPLA3*/*TM6SF2* risk variants in the 164 C282Y homozygotes cases with cirrhosis and the 1139 C282Y homozygotes controls without significant liver disease in whom complete genotypic information was available. The proportion of cases and controls carrying either none of the variants of interest in *PCSK7*, *PNPLA3* and *TM6SF2*, or one of three, two of three or all three. Only 14.9% and 0.9% of the controls carried either two or three of the variants, while these proportions increased significantly to 31.1% and 2.4% in the cases ($P = 9.08 \times 10^{-8}$, linear-by-linear trend test)

malondialdehyde, which are well-known drivers of liver fibrogenesis.[48,49] This proposition will need further exploration.

4.3 | Strengths and limitations

This study has a number of strengths. First, it is the largest single study, to date, designed to identify genetic modulators of the risk for developing cirrhosis among C282Y homozygotes. Second, the participants were unselected and unrelated and were recruited from centres across Europe. As such they are likely to be representative of the broader HH population. Third, the cohorts were all assembled using a consistent study/data collection protocol with predetermined inclusion/exclusion criteria. Fourth, the selection of cases was based on rigorous criteria, which not only robustly identified the presence of significant liver disease but also ensured that no cause for the development of cirrhosis, other than HH, was likely. Finally, the data analyses were stringent and comprehensive; the heterogeneity observed within the much smaller cohorts with available histological confirmation of their case/control status was explored, and most likely results from sampling error rather than genuine differences in effect sizes between cohorts.

The study also has its limitations. First, the data were collected retrospectively although missing data were obtained, where possible, during subsequent routine clinic visits. The study therefore lacked the granularity that would be available if the data have been collected prospectively. Thus, for example, a high proportion of the cases had 'diabetes' but it is uncertain whether this reflected pancreatic involvement in the disease process, disturbances of glucose homeostasis secondary to cirrhosis or co-morbid type 2 diabetes, although, given the low prevalence of obesity, the latter is less likely. Second, the criteria for case selection were so stringent that it is likely that some people with incipient or asymptomatic cirrhosis may have been misclassified as controls. It is also likely that a proportion of the controls might develop cirrhosis over time if left untreated. The net result is that the control group may have contained individuals predisposed to develop cirrhosis. This, however, strengthens the findings of the identified associations ensuring that they are real and robust. Third, the study participants were European so the findings may not be applicable to populations of non-European ancestry. However, C282Y allele frequencies in people of non-European ancestry are likely to be very low.[50] Fourth, considerable inter-cohort heterogeneity was observed in the association analyses for *PNPLA3*:rs738409, which hampered the assessment of its association with cirrhosis risk in the subgroup analyses. Sensitivity analysis determined that the major source of this heterogeneity was the German cohort and its exclusion allowed for a robust assessment of disease modulation.

4.4 | Clinical and public consequences

The diagnosis of HH is often made at a stage when advanced or decompensated liver disease and HCC are already established. A better understanding of the genetic risk factors associated with the development of cirrhosis in C282Y homozygotes could lead to a more comprehensive approach to risk stratification. This study has provided evidence for risk associations with carriage of specific variants in three genes, *viz. PCSK7*, *PNPLA3* and *TM6SF2*. However, it is likely that further genetic risk factors, at present unrecognised, also exist.

A GWAS of unrelated patients with HH, undertaken in 2015, involving 474 C282Y homozygotes identified an association between rs3811647 in the transferrin gene and serum transferrin and iron levels, but only indirectly with fibrosis.[51] However, only 72 (15%) of the 474 participants had evidence of hepatic fibrosis and so the study was under-powered to study liver phenotypes. A large well-conducted GWAS would be the best way to identify other possible genetic modulators not only associated with the susceptibility to develop cirrhosis but also with other HH disease phenotypes.

More comprehensive genetic testing at the time of diagnosis would allow necessary adjustments to be made to the standard approach to treatment to accommodate any additional risks, for example, early modification of any risky lifestyle behaviours such as excess calorie and alcohol ingestion, adopting a more rigorous approach to depletion of iron stores and placing more emphasis on screening for the development of liver injury.

Further understanding of the mechanisms by which genetic variants confer the risk for developing cirrhosis could pave the way for the development of new treatments if the risk loci and their corresponding gene products were potential drug targets.

In conclusion: approximately 30% of the variance in susceptibility of C282Y homozygotes to develop cirrhosis can be explained by carriage of *PCSK7*:rs236918, *PNPLA3*:rs738409 and *TM6SF2*:rs58542926. More comprehensive genetic testing will identify individuals at particular risk and a better understanding of the modulating effects of these gene variants may lead to the development of alternative therapeutic approaches.

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AUTHORSHIP

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Author contributions: Stephan Buch: Undertook the majority of the genotyping of the DNA samples contributed by the Austrian, German, Swiss and Italian collaborators; performed the initial data analyses on these cohorts and the meta-analyses and more detailed analyses of the entire cohort; critically revised the manuscript and approved the submitted version. Aneesh Sharma: Extracted and

quantified genomic DNA from the second set of Irish and English case-control samples; undertook genotyping of the initial four SNPs of interest; consolidated the sample phenotypic data; ran the initial data analyses on the whole study cohort; critically revised the manuscript and approved the submitted version. Eleanor Ryan: Recruited the entire Irish cohort; prospectively collected blood for DNA extraction; retrieved stored blood and archived phenotypic data; classified cases and controls; critically revised the manuscript and approved the submitted version. Christian Datz: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. William J. H. Griffiths: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Michael J. Way: Extracted and quantified genomic DNA from the first set of Irish and English case-control samples; undertook genotyping of the initial four SNPs of interest and processed the results; critically revised the manuscript and approved the submitted version. Thomas Buckley: Undertook genotyping of rs72613567 in *HSD17B13* in the Irish and English samples; critically revised the manuscript and approved the submitted version. John D. Ryan: Contributed to the collection of the Irish biological and phenotypic information; critically revised the manuscript and approved the submitted version. Stephen Stewart: Oversaw and approved the collection of the Irish samples; critically revised the manuscript and approved the submitted version. Callum Wright: Assisted in the retrieval of the phenotypic information for the Cambridge cases and controls; critically revised the manuscript and approved the submitted version. Paola Dongiovanni: Contributed biological material and phenotypic information for study cases and controls; undertook additional genotyping; critically revised the manuscript and approved the submitted version. Anna Fracanzani: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Jochen Zwerina: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Uta Merle: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Karl Heinz Weiss: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Elmar Aigner: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Elisabeth Krones: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Christian Dejaco: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Janet Fischer: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Thomas Berg: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Luca Valenti: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Heinz Zoller: Contributed biological material and phenotypic information for study cases and controls; critically revised the manuscript and approved the submitted version. Andrew McQuillin: Oversaw the DNA extraction and genotyping of the Irish and English samples, undertook the final analysis of the data for the full study cohort; critically revised the manuscript and approved the submitted version. Jochen Hampe: Conceived the study; provided the genotyping infrastructure; critically revised the manuscript and approved the submitted version. Felix Stickel: Conceived the study; recruited collaborating centres in Austria, Germany, Switzerland and Italy; coordinated the sample collection and data collection from these sites; critically revised the manuscript and approved the submitted version. Marsha Y. Morgan: Conceived the study; recruited the collaborating centres in the UK and Ireland and created the study database; retrieved phenotypic data for the UK cohort; determined the case-control status of the Irish and UK samples interpreted the data analyses; wrote and revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supplementary Material of this article.

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REFERENCES

1. Powell LW, Seckington RC, Deugnier Y. Haemochromatosis. *Lancet*. 2016;388:706-716.
2. Adams PC. Epidemiology and diagnostic testing for hemochromatosis and iron overload. *Int J Lab Hematol*. 2015;37(Suppl.1):25-30.
3. European Association for the Study of the Liver. EASL clinical practice guidelines for HFE hemochromatosis. *J Hepatol*. 2010;53:3-22.
4. Allen KJ, Gurrin LC, Constantine CC, et al. Iron-overload-related disease in HFE hereditary hemochromatosis. *N Engl J Med*. 2008;358:221-230.
5. Byrnes V, Ryan E, Barrett S, Kenny P, Mayne P, Crowe J. Genetic hemochromatosis, a Celtic disease: is it now time for population screening? *Genet Test*. 2001;5:127-130.
6. Altes A, Ruiz A, Barceló MJ, et al. Prevalence of the C282Y, H63D, and S65C mutations of the HFE gene in 1,146 newborns from a region of Northern Spain. *Genet Test*. 2004;8:407-410.
7. Whitlock EP, Garlitz BA, Harris EL, Beil TL, Smith PR. Screening for hereditary hemochromatosis: a systematic review for the U.S. Preventive Services Task Force. *Ann Intern Med*. 2006;145:209-223.
8. Moirand R, Jouanolle A, Brissot P, Le Gall J, David V, Deugnier Y. Phenotypic expression of *HFE* mutations: a French study of 1110

unrelated iron-overloaded patients and relatives. *Gastroenterology*. 1999;2:372-377.
9. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet*. 2002;359:211-218.
10. Pilling LC, Tamosauskaite J, Jones G, et al. Common conditions associated with hereditary haemochromatosis genetic variants: cohort study in UK Biobank. *BMJ*. 2019;64:k5222.
11. Fargion S, Mandelli C, Piperno A, et al. Survival and prognostic factors in 212 Italian patients with genetic hemochromatosis. *Hepatology*. 1992;15:655-659.
12. Fletcher LM, Dixon JL, Purdie DM, Powell LW, Crawford DHG. Excess alcohol greatly increases the prevalence of cirrhosis in hereditary hemochromatosis. *Gastroenterology*. 2002;122:281-289.
13. Scotet V, Mérour MC, Mercier AY, et al. Hereditary hemochromatosis: effect of excessive alcohol consumption on disease expression in patients homozygous for the C282Y mutation. *Am J Epidemiol*. 2003;158:129-134.
14. Wood MJ, Powell LW, Dixon JL, Ramm GA. Clinical cofactors and hepatic fibrosis in hereditary hemochromatosis: the role of diabetes mellitus. *Hepatology*. 2012;56:904-911.
15. Powell EE, Ali A, Clouston AD, et al. Steatosis is a cofactor in liver injury in hemochromatosis. *Gastroenterology*. 2005;129:1937-1943.
16. Adams LA, Angulo P, Abraham SC, Torgerson H, Brandhagen D. The effect of the metabolic syndrome, hepatic steatosis and steatohepatitis on liver fibrosis in hereditary hemochromatosis. *Liver Int*. 2006;26:298-304.
17. Tung BY, Emond MJ, Bronner MP, Raaka SD, Cotler SJ, Kowdley KV. Hepatitis C, iron status, and disease severity: relationship with *HFE* mutations. *Gastroenterology*. 2003;124:318-326.
18. Valenti L, Maggioni P, Piperno A, et al. *Patatin-like phospholipase domain containing-3* gene I148M polymorphism, steatosis, and liver damage in hereditary hemochromatosis. *World J Gastroenterol*. 2012;18:2813-2820.
19. Buch S, Stickel F, Trépo E, et al. A genome-wide association study confirms *PNPLA3* and identifies *TM6SF2* and *MBOAT7* as risk loci for alcohol-related cirrhosis. *Nat Genet*. 2015;47:1443-1448.
20. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in *PNPLA3* confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet*. 2008;40:1461-1465.
21. Stickel F, Buch S, Zoller H, et al. Evaluation of genome-wide loci of iron metabolism in hereditary hemochromatosis identifies *PCSK7* as a host risk factor of liver cirrhosis. *Hum Mol Genet*. 2014;23:3883-3890.
22. Pelucchi S, Galimberti S, Greni F, et al. *Proprotein convertase 7* rs236918 associated with liver fibrosis in Italian patients with *HFE*-related hemochromatosis. *J Gastroenterol Hepatol*. 2016;31:1342-1348.
23. Kozlitina J, Smagris E, Stender S, et al. Exome-wide association study identifies a *TM6SF2* variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet*. 2014;46:352-356.
24. Krawczyk M, Rau M, Schattenburg JM, et al. Combined effects of the *PNPLA3*rs738409, *TM6SF2*rs58542926, and *MBOAT7*rs641738 variants on NAFLD severity: a multicenter biopsy-based study. *J Lipid Res*. 2017;58:247-255.
25. Dongiovanni P, Petta S, Maglio C, et al. Transmembrane 6 superfamily member 2 gene variant disentangles nonalcoholic steatohepatitis from cardiovascular disease. *Hepatology*. 2015;61:506-514.
26. Mancina RM, Dongiovanni P, Petta S, et al. The *MBOAT7-TMC4* variant rs641738 increases risk of nonalcoholic fatty liver disease in individuals of European descent. *Gastroenterology*. 2016;150:1219-1230.
27. Abul-Husn NS, Cheng X, Li AH, et al. A protein-truncating *HSD17B13* variant and protection from chronic liver disease. *N Engl J Med*. 2018;378:1096-1106.
28. Stickel F, Lutz P, Buch S, et al. Genetic variation in *HSD17B13* reduces the risk of developing cirrhosis and hepatocellular carcinoma in alcohol misusers. *Hepatology*. 2020;72:88-102.
29. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology*. 1994;20:15-20.
30. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81:559-575.
31. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience*. 2015;4:7.
32. Viechtbauer W. Conducting met-analyses in R with the Meafor package. *J Stat Softw*. 2010;36:1-48.(https://www.jstatsoft.org/v36/i03/)
33. Mangiafico SS. *Summary and Analysis of Extension Program Evaluation in R, version 1.18.1*; 2016. rcompanion.org/documents/RHandbookProgramEvaluation.pdf
34. Witte JS, Visscher PM, Wray NR. The contribution of genetic variants to disease depends on the ruler. *Nat Rev Genet*. 2014;15:765-776.
35. Anderson CA, Pettersson FH, Clarke GM, Cardon LR, Morris AP, Zondervan KT. Data quality control in genetic case-control association studies. *Nat Protoc*. 2010;5:1564-1573.
36. 1000 Genomes Project Consortium, Auton A, Brooks LD, et al. A global reference for human genetic variation. *Nature*. 2015;526:68-74.
37. Grosse SD, Gurrin LC, Bertalli NA, Allen KJ. Clinical penetrance in hereditary hemochromatosis: estimates of the cumulative incidence of severe liver disease among *HFE* C282Y homozygotes. *Genet Med*. 2018;20:383-389.
38. Thabet K, Asimakopoulos A, Shojaei M, et al. *MBOAT7* rs641738 increases risk of liver inflammation and transition to fibrosis in chronic hepatitis C. *Nat Commun*. 2016;7:12757.
39. About L, Abel F, Cobat A. HCV-associated liver fibrosis and *HSD17B13*. *N Engl J Med*. 2018;379:1875-1876.
40. Hollerer I, Bachmann A, Muckenthaler MU. Pathophysiological consequences and benefits of *HFE* mutations: 20 years of research. *Haematologica*. 2017;102:809-817.
41. Lin L, Nemeth E, Goodnough JB, Thapa DR, Gabayan V, Ganz T. Soluble hemojuvelin is released by proprotein convertase-mediated cleavage at a conserved polybasic RNRR site. *Blood Cells Mol Dis*. 2008;40:122-131.
42. Schranz M, Bakry R, Creus M, Bonn G, Vogel W, Zoller H. Activation and inactivation of the iron hormone hepcidin: biochemical characterization of prohepcidin cleavage and sequential degradation to N-terminally truncated hepcidin isoforms. *Blood Cells Mol Dis*. 2009;43:169-179.
43. Turpeinen H, Oksanen A, Kivinen V, et al. *Proprotein convertase subtilisin/kexin type 7 (PCSK7)* is essential for the zebrafish development and bioavailability of transforming growth factor β1a (TGFβ1a). *J Biol Chem*. 2013;288:36610-36623.
44. Dongiovanni P, Meroni M, Baselli G, et al. *PCSK7* gene variation bridges atherogenic dyslipidemia with hepatic inflammation in NAFLD patients. *J Lipid Res*. 2019;60:1144-1153.
45. He S, McPhaul C, Li JZ, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. *J Biol Chem*. 2010;285:6706-6715.
46. BasuRay S, Smagris E, Cohen JC, Hobbs H. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. *Hepatology*. 2017;66:1111-1124.
47. Sookoian S, Castaño GO, Scian R, et al. Genetic variation in transmembrane 6 superfamily member 2 and the risk of nonalcoholic fatty liver disease and histological disease severity. *Hepatology*. 2015;61:515-525.

48. Zamara E, Novo E, Marra F, et al. 4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells. *J Hepatol*. 2004;40:60-68.
49. Poli G, Biasi F, Leonarduzzi G. 4-Hydroxynonenal-protein adducts: a reliable biomarker of lipid oxidation in liver diseases. *Mol Aspects Med*. 2008;29:67-71.
50. Steinberg KK, Cogswell ME, Chang JC, et al. Prevalence of C282Y andH63D mutations in the hemochromatosis (HFE) gene in the United States. *JAMA*. 2001;285:2216-2222.
51. de Tayrac M, Roth M-P, Jouanolle A-M, et al. Genome-wide association study identifies *TF* as a significant modifier gene of iron metabolism in *HFE* hemochromatosis. *J Hepatol*. 2015;622:664-672.

SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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APPENDIX 1

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