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Evaluation of genome-wide loci of iron metabolism in hereditary hemochromatosis identifies *PCSK7* as a host risk factor of liver cirrhosis

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Genome-wide association studies (GWAS) have revealed genetic determinants of iron metabolism, but correlation of these with clinical phenotypes is pending. Homozygosity for *HFE* C282Y is the predominant genetic risk factor for hereditary hemochromatosis (HH) and may cause liver cirrhosis. However, this genotype has a low penetrance. Thus, detection of yet unknown genetic markers that identify patients at risk of developing severe liver disease is necessary for better prevention. Genetic loci associated with iron metabolism (*TF*, *TMPRSS6*, *PCSK7*, *TFR2* and Chr2p14) in recent GWAS and liver fibrosis (*PNPLA3*) in recent meta-analysis were analyzed for association with either liver cirrhosis or advanced fibrosis in 148 German *HFE* C282Y

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homozygotes. Replication of associations was sought in additional 499 Austrian/Swiss and 112 *HFE* C282Y homozygotes from Sweden. Only variant rs236918 in the *PCSK7* gene (proprotein convertase subtilisin/kexin type 7) was associated with cirrhosis or advanced fibrosis ($P = 1.02 \times 10^{-5}$) in the German cohort with genotypic odds ratios of 3.56 (95% CI 1.29–9.77) for CG heterozygotes and 5.38 (95% CI 2.39–12.10) for C allele carriers. Association between rs236918 and cirrhosis was confirmed in Austrian/Swiss *HFE* C282Y homozygotes ($P = 0.014$; $OR_{allelic} = 1.82$ (95% CI 1.12–2.95) but not in Swedish patients. *Post hoc* combined analyses of German/Swiss/Austrian patients with available liver histology ($N = 244$, $P = 0.00014$, $OR_{allelic} = 2.84$) and of males only ($N = 431$, $P = 2.17 \times 10^{-5}$, $OR_{allelic} = 2.54$) were consistent with the premier finding. Association between rs236918 and cirrhosis was not confirmed in alcoholic cirrhotics, suggesting specificity of this genetic risk factor for HH. *PCSK7* variant rs236918 is a risk factor for cirrhosis in HH patients homozygous for the *HFE* C282Y mutation.

INTRODUCTION

The rapid progress of genotyping technologies has enabled genome-wide association studies (GWAS) to be performed for quantitative laboratory traits, measurable in peripheral blood. Following the GWAS paradigm, many genetic variants underlying a wide range of hematological and metabolic parameters have been mapped (1–3). However, investigation of these variants in relation to specific human diseases is now required to translate the laboratory-based findings into clinical medicine.

Hereditary hemochromatosis (HH) is an autosomal recessive trait (MIM #235200) characterized by elevated serum ferritin and transferrin saturation, which entails excessive iron accumulation in parenchymal cells of the liver, heart and endocrine organs (4). Progressive HH is potentially fatal, with a mean survival of only 1.5 years before the introduction of phlebotomy therapy in the 1950s. Today, decompensated liver cirrhosis and hepatocellular carcinoma represent the main causes of HH-related mortality, mostly due to delayed diagnosis (5,6), whereas adequately treated patients have a normal life expectancy (7).

The majority of HH cases are due to a variation in *HFE* gene located on chromosome 6 (8), and >80% of the patients with clinical HH are homozygous for *HFE* mutation C282Y (rs1800562, G845A) (9,10). HH is one of the most prevalent genetic disorders in Caucasians, with an allele frequency of C282Y of 6.2% and a homozygosity frequency of 0.38% in a pool of 127,613 individuals, but shows considerable variation across Europe with allele frequencies of >10% in Ireland and 0–3% in Mediterranean regions (11). However, the penetrance of C282Y is low. In a family-based screening, elevation of liver enzymes was observed in <30% of male homozygotes (12), and cirrhosis was present in only 6% of males and 2% of females (13). A population-based study revealed mildly elevated liver enzymes or liver disease in <10% of C282Y homozygotes (14), and the estimated prevalence of clinical HH was as low as 1%. Other studies reported liver disease in up to 16% of C282Y homozygotes (15). Studies on the penetrance in C282Y homozygote relatives of index cases with HH showed that 38% had at least one disease-related condition, particularly, in males (16). In addition to environmental factors, such as diet, alcohol consumption and coexisting chronic viral hepatitis, the course of HH-associated cirrhosis may be further modulated by host factors such as gender, age, obesity and additional genetic variants (4,11).

Genes associated with iron metabolism represent obvious candidates for the modulation of the iron overload and/or liver disease phenotype in HH. Several genetic analyses of iron metabolism have been published that identified variants associated with markers of iron status, such as an association of transferrin (*TF*) variants with serum ferritin levels (17), and of type II transmembrane serine protease 6 (*TMPRSS6*) mutations with hemoglobin, serum iron and transferrin saturation (18,19). A recent meta-analysis of five GWAS of soluble transferrin receptor (sTfR) and ferritin revealed a novel association between sTfR and variation in the proprotein convertase 7 (*PCSK7*) gene (20). In addition, mutations in the *TFR2* gene were identified as a cause of hemochromatosis type 3 (HFE3, MIM:604250) (21). Another locus on chromosome 2p14, tagged by SNP rs2698530, was implicated in iron deficiency (22). In addition, variant I148M (rs738409 C/G) within the gene coding for patatin-like phospholipase domain containing 3 (PNPLA3) was shown to promote the risk of developing liver fibrosis and cirrhosis in nonalcoholic fatty liver disease patients in a recent meta-analysis (23).

Here, we demonstrate that *PCSK7* variation is a strong host risk factor of liver cirrhosis in HH patients homozygous for C282Y.

RESULTS

Analysis of association with liver cirrhosis in hereditary hemochromatosis

Selected genetic variants recently robustly associated with iron metabolism in previous studies are listed in Table 1. For the present study, these variants were subjected to an allelic association test in German HH patients, adopting a Bonferroni-corrected significance level of 0.008 (six tests). An overview of the cohorts utilized for discovery, replication and *post hoc* subgroup analyses efforts is shown in Table 2. Any additional tests were performed *post hoc* in the combined German/Austrian/Swiss sample set. All HH patients were confirmed as being HFE C282Y homozygous. The clinical diagnosis of liver cirrhosis or advanced (stage 5 or 6) fibrosis compatible with cirrhosis on biopsy was used for the definition of 'cases' in the German, Austrian and Swiss samples. Patients without cirrhosis on biopsy and patients without clinical signs of liver fibrosis who did not undergo liver biopsy because of a clinically mild presentation were classified as 'controls'. Among the six lead variants tested, only rs236918 (*PCSK7*) was found to be significantly associated with liver disease ($P = 1.02 \times 10^{-5}$; $P_{adjusted} = 0.0044$) in German HH

Table 1. Genetic analysis of liver disease in German discovery samples

Locus		*TF*	*TMPRSS6*	*PCSK7*	*TFR2*	*Chr2p14*	*PNPLA3*
Chromosome		3q22.1	22q12.3	11q23.3	7q22.1	2p14	22q13.31
SNPs		rs3811647	rs855791	rs236918	rs7385804	rs2698530	rs738409
	Phenotype (reference).	Serum transferrin (17)	(i) Serum iron (18) (ii) transferrin saturation (19)	sTfR (20)	Serum iron (21)	Unsaturated iron-binding capacity (22)	Liver fibrosis (23)
$MAF_{population}$		0.330	0.435	0.115	0.328	0.252	0.224
German patient ($N = 148$)	$MAF_{C282Y/C+}$	0.308	0.348	0.25	0.304	0.217	0.25
	$MAF_{C282Y/C-}$	0.318	0.354	0.058	0.38	0.283	0.35
	$P_{allelic}$	0.946	0.93	1.02×10^{-5}	0.33	0.357	0.16
	$P_{adjusted^*}$	0.69	0.92	4.39×10^{-3}	0.29	0.29	0.49
	$OR_{allelic}$	0.98 (0.51–1.87)	0.97 (0.50–1.89)	5.38 (2.39–12.10)	0.71 (0.36–1.41)	0.70 (0.33–1.49)	0.64 (0.32–1.27)
	$OR_{heterozygosity}$	1.00 (0.41–2.41)	1.59 (0.61–4.11)	3.56 (1.29–9.77)	0.61 (0.24–1.56)	0.59 (0.23–1.52)	0.49 (0.19–1.24)
	$OR_{homozygosity}$	0.92 (0.17–4.73)	0.42 (0.04–3.60)	NA	0.55 (0.11–2.83)	0.69 (0.07–6.21)	0.50 (0.21–1.21)

For each marker, the initial GWAS, the studied iron-related phenotype and the genome-wide significance are provided in the rows labeled 'GWAS'. The minor allele frequency is given for a general German ($MAF_{population}$) population sample with normal liver function tests and for different liver cirrhosis phenotypes of HH patients. The allelic *P*-value in the patient sample, defined on the basis of the cirrhosis phenotype, is given as the primary statistical result, followed by logistic regression analysis with adjustment for sex and age. The values in parentheses are 95% CI. C+ and C− indicate the presence and absence of cirrhosis, respectively. NA, not applicable.

patients (Table 1). Odds ratio estimates were 3.56 (95% CI 1.30–9.77) for CG heterozygosity and 4.89 (95% CI 1.9–12.5) for carriage of the C allele. The frequency of the rs236918 C allele of was 25% in German cirrhotic patients ($N = 28$) compared with 5.8% in German non-cirrhotics ($N = 120$). All other genetic variants showed no association with liver cirrhosis. None of the additional tested tagging variants yielded nominal significant association findings neither in the adjusted nor in the unadjusted analyses all $P > 0.1$ (Supplementary Material, Table S1).

Replication in Austrian and Swiss C282Y homozygote patients

Association of *PCSK7* rs236918 C with cirrhosis in German patients was replicated independently in 499 Austrian/Swiss C282Y homozygotes and again found significantly associated with cirrhosis ($P = 0.014$, $P_{adjusted\ (sex\ and\ age)} = 0.028$) (Table 3). Odds ratio estimations were 1.82 (95% CI 1.03–3.21) for CG heterozygosity, 1.90 (95% CI 1.09–3.29) for C allele carriage and 3.43 (95% CI 0.61–19.2) for CC homozygosity thereby confirming the initial finding with the C allele being over-represented in cirrhotics.

Association findings in Swedish C282Y homozygotes

Swedish cirrhotic ($N = 8$) and non-cirrhotic patients ($N = 104$) were classified based on liver biopsy results as previously described (24). An age- and sex-adjusted analysis yielded an over-representation of the C risk allele in Swedish cirrhotic patients with a similar odds ratio [$OR_{adjusted} = 1.75$ (95% CI 0.34–9.16), $P_{adjusted} = 0.5$] as obtained in the replication analysis performed in the Austrian/Swiss sample set. However, formal replication and statistical significance could not be achieved in the full Swedish sample owing to the low number of cirrhosis patients ($N = 8$, Table 3).

Post hoc analysis of disease risk in German, Austrian and Swiss patients

All *post hoc* analyses were performed in the combined sample set of German, Austrian and Swiss HH patients (Table 4) as an ethnically relatively homogenous sample. A consistent association between *PCSK7* rs236918 C and cirrhosis (Ishak fibrosis stages 5 and 6) was also detected in the subgroup of 244 patients who underwent a liver biopsy prior to therapy [$P = 1.41 \times 10^{-4}$, allelic OR = 2.84 (95% CI 1.63–4.94) and 3.10 (95% CI 1.58–6.08) for CG heterozygosity]. An overview of the risk allele frequency of *PCSK7* in the different patients groups is provided in Figure 1. In further *post hoc* analyses, the subgroup of biopsied patients without fibrosis (stage 0; $n = 60$) showed a lower frequency (5.8%) of allele C than all non-cirrhotic patients with fibrosis stages 1 to 4 (9.2%; $n = 92$). When cirrhotic ($n = 93$) and non-cirrhotic male patients ($n = 338$) were compared in a male-only analysis, the association with rs236918 remained highly significant [$P = 2.17 \times 10^{-5}$, $OR_{allelic} = 2.54$ (95% CI 1.63–3.96)] even after controlling for advanced age by logistic regression analyses [$P_{adjusted} = 1.96 \times 10^{-4}$, $OR_{adjusted} = 2.51$ (95% CI 1.54–4.07)].

Ferritin levels in C282Y homozygotes with cirrhosis (median: 2760 ng/ml) were higher than those in non-cirrhotic individuals

Table 2. Cohorts of HFE C282Y homozygous HH patients used in this study

Patient sample	Cases				Controls			
	N	% Male	Age	Ferritin (ng/ml)	*N*	% Male	Age	Ferritin (ng/ml)
Discovery cohort								
Germany	28	89	61	2757	120	63	51	1142
Replication cohorts								
Austria/Switzerland	73	93	60	2795	426	61	50	914
Sweden	8	75	70	2600	104	70	61	705
Post hoc analyses cohorts								
Germany/Austria/Switzerland: biopsy only	92	91	60	2765	152	64	52	1200
Germany/Austria/Switzerland: male only	93	100	60	2690	338	100	49	1113

Cases and controls were defined based on the presence of liver cirrhosis in the German, Austrian and Swiss samples. Medians are given for age and ferritin levels.

Table 3. Genetic analysis of liver disease in the Austrian/Swiss and Swedish replication samples

PCSK7 rs236918	Switzerland/Austria All patients	Sweden All patients (all biopsied)
N total (cases/controls)	$N = 499$ (73/426)	$N = 112$ (8/104)
$MAF(C)_{C282Y/C+}$	0.171	0.125
$MAF(C)_{C282Y/C-}$	0.102	0.082
$P_{allelic}$*	0.014	0.55
$P_{adjusted}$**	0.028	0.50
$OR_{adjusted}$	1.85 (1.07–3.21)	1.75 (0.34–9.16)
$OR_{allelic}$	1.82 (1.12–2.95)	1.61 (0.34–7.66)
$OR_{heterozygosity}$	1.82 (1.03–3.35)	1.95 (0.36–10.61)
$OR_{homozygosity}$	3.43 (0.61–19.21)	NA

The allelic *P*-value refers to cirrhosis, followed by logistic regression analysis with adjustment for sex and age. The values in parentheses are 95% CI. C+ and C− indicate the presence and absence of cirrhosis, respectively. NA, calculation not possible.
*P (allelic, χ^2 test) nominal; **P-value adjusted for sex and age; $OR_{adjusted}$ under additive model (adjusted for sex and age).

Table 4. Combined *post hoc* risk analysis in German, Austrian and Swiss Patients

PCSK7 rs236918 *post hoc* analysis	Germany/Switzerland/Austria Biopsy only	 Male only
N total (cases/controls)	$N = 244$ (92/152)	$N = 431$ (93/338)
$MAF(C)_{C282Y/C+}$	0.196	0.204
$MAF(C)_{C282Y/C-}$	0.079	0.092
$P_{allelic}$*	0.00014	$2.17 \times 10-5$
$P_{adjusted}$**	0.015	$1.96 \times 10-4$
$OR_{adjusted}$	2.07 (1.15–3.72)	2.51 (1.54–4.07)
$OR_{allelic}$	2.84 (1.63–4.94)	2.54 (1.63–4.94)
$OR_{heterozygosity}$	3.10 (1.58–6.08)	2.33 (1.37–3.96)
$OR_{homozygosity}$	3.58 (0.83–15.46)	7.75 (1.80–33.31)

The allelic *P*-value refers to cirrhosis. The values in parentheses are 95% CI. C+ and C− indicate the presence and absence of cirrhosis, respectively. NA, calculation not possible.
*P (allelic, χ^2 test) nominal; **P (adjusted for sex and age); $OR_{adjusted}$ under additive model (adjusted for sex and age).

(median: 960 ng/ml), as expected. However, no significant association between ferritin level and *PCSK7* genotype was observed, neither in the non-cirrhotic individuals (mean: 1539 ng/ml in C carriers; 1322 ng/ml in wild-type homozygotes,

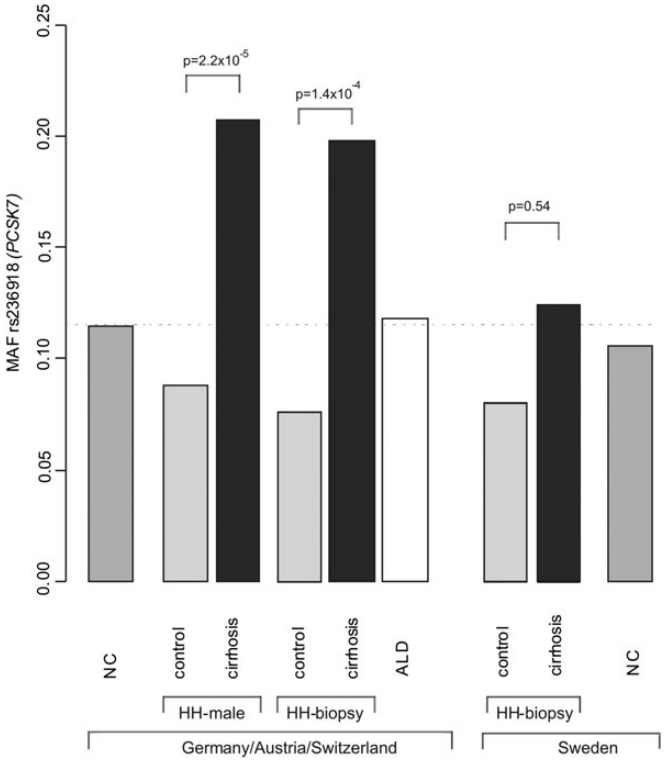

Figure 1. Allele frequency of *PCSK7* rs236918 C across study populations and subgroups. NC, normal controls; ALD, cirrhosis on the basis of alcoholic liver disease; HH, hereditary hemochromatosis as defined by C282Y homozygosity. *P*-values are given for an allelic test of association.

t-test: $P = 0.191$) nor in the HH patients with liver cirrhosis (mean: 3006 ng/ml in C carriers, 3711 ng/ml in wild-type homozygotes, $P = 0.22$). An analysis of ferritin levels in the total cohort of 647 patients produced no evidence of association (mean 1879 ng/ml in C carriers versus 1594 ng/ml in wild-type individuals, $P = 0.12$). A male-only analysis of ferritin levels and carriage of re236918 C allele in the total cohort ($N = 431$ male patients) also provided no evidence of association (mean 2231 ng/ml in C carriers versus 2006 ng/ml in wild-type individuals, $P = 0.56$). Similarly, no association between the *PCSK7* rs236918 C allele and transferring saturation was detected.

Allele frequencies in the general population and patients with alcoholic liver cirrhosis

To delineate the possible role of the tested genetic variants outside of HH, allele frequencies of all markers were also determined in unaffected individuals with normal liver enzyme levels and with no clinical evidence of liver disease. The frequency of the *PCSK7* rs236918 C allele (11.5%) among population-based control individuals from the EMIL (Ulm, Southern Germany, $n = 980$) and the SHIP study (Greifswald, Northern Germany, $n = 170$) (25,26) was higher than that among HH patients without apparent liver lesions (fibrosis stage 0) (5.8%, $P = 0.054$), higher than in HH controls (9.2%, $P = 0.046$) and significantly lower than in HH patients with confirmed liver cirrhosis or advanced fibrosis (19.3%, $P = 0.001$). A similar NC allele frequency as in German population controls (10.6%) was observed in the Swedish population, which was also higher than that in Swedish HH controls (8.2%, $P = 0.285$, Fig. 1).

Evaluation of the *PCSK7* allele distribution in patients with alcoholic cirrhosis [$n = 203$; for details see Stickel *et al.* (27)] revealed a similar frequency of the C allele as in the control population (11.8%, $P = 0.86$), indicating a disease-specific role of *PCSK7* in modifying the risk of progressive liver disease in C282Y homozygous HH patients.

In silico assessment of PCSK7 rs236918

In order to explore the putative functional effect of the rs236918 risk variant, we performed an *in silico* analysis at the *PCSK7* locus using data from ENCODE (28). The data contained therein point to a possible role of this variant in transcriptional regulation of *PCSK7* because the degree of histone H3 lysine 27 acetylation (H3K27ac) at CpG islands is associated with transcriptional activation. The analysis of chromatin state showed a cell-specific H3K27ac enrichment at *PCSK7* distal to the promoter. Rs236918 is located within this region that is predicted to serve as a strong enhancer in three of nine cell lines investigated by ENCODE (Supplementary Material, Fig. S1), thereby providing indirect evidence for a possible *cis*-effect of rs236918 on PCSK7 expression.

DISCUSSION

The present data identify the *PCSK7* rs236918 C allele as a genetic risk factor for cirrhosis in patients homozygous for *HFE* gene mutation C282Y. Apart from providing a partial explanation for the variability in clinical phenotype observed among homozygous carriers of C282Y, our result may potentially impact on screening of HH patients, particularly of males. Carriage of *PCSK7* rs236918 C allele may help to identify those at risk for progressive liver disease, and thereby open new research avenues for a better understanding of the molecular pathophysiology of HH. The lower frequency of rs236918 C risk allele among C282Y homozygotes without liver cirrhosis, compared with population controls, and the lack of association with alcoholic cirrhosis further illustrates how the phenotypic effect of this variant depends on the presence of the HFE mutation and points to a strong gene–gene interaction (Fig. 1).

Previous population studies have revealed that progressive liver disease affects only a minority of C282Y homozygotes (11,14). One obvious approach to find potential genetic disease modifiers is the evaluation of genes associated with conditions that are closely related to the disease of interest. An attractive strategy is to select these genes from GWAS of quantitative traits such as serum laboratory parameters employed in the routine diagnosis of the disease under scrutiny. Along this line, genetic variants significantly associated with markers of iron metabolism in previous GWAS were investigated in the present study. A multicenter cohort of HH patients recruited between 1997 and 2011 at several tertiary referral centers in Germany was used as a 'discovery sample'. Subsequent replication of association findings was then sought in an independent multicenter cohort of HH patients from Austria, Switzerland and Sweden. In retrospective multicenter studies, robust definition of the primary endpoint is pivotal. Considering that patients homozygous for the C282>Y mutation face a higher all-cause morbidity and mortality than heterozygotes or wild-type homozygotes, with end-stage liver cirrhosis and hepatocellular carcinoma being the predominant causes of death (6,7,29), we focused on the presence or absence of liver disease. For the latter, we chose an unambiguous phenotype, namely advanced fibrosis or cirrhosis as diagnosed on biopsy or liver cirrhosis unequivocally established by clinical and imaging studies that could be assessed consistently across centers. This might be one possible reason why the *PCSK7* risk variant identified here yielded very similar odds ratio for liver disease in different subgroups of HH patients.

Males with C282Y+/+ are more prone to develop iron overload and consecutive liver damage than females, mainly because women have regular blood losses owing to menstruation. However, iron accumulation commences similarly in postmenopausal women. Thus, premenopausal women with C282Y+/+ carriage may constitute a confounding factor that could skew genotype–phenotype associations. We therefore performed a *post hoc* analysis of male subjects only in the combined German/Austrian/Swiss cohort, which confirmed the association of the *PCSK7* rs236918 C allele and advanced liver fibrosis/cirrhosis. The presence of the *PCSK7* rs236918 C allele thus represents a significant risk marker in the subgroups of males who are most susceptible to develop liver disease underlining its potential impact on population screening strategies.

When restricting our analysis to those subjects in the total cohort of German/Austrian/Swiss patients in whom liver histology data staged for fibrosis (as a quantifiable trait) were available, *post hoc* calculations confirmed *PCSK7* rs236918 C allele as significantly associated with cirrhosis, showing a gradual increase in the allele frequency along with progression of fibrosis.

The loci selected for our study (i.e. *TF*, *TMPRSS6*, *PCSK7*, *TFR2* and Chr2p14) were identified in earlier studies, which focused on different laboratory parameters of iron metabolism (Table 1). The risk locus for liver disease in HH patients identified in the present study (*PCSK7*) yielded the strongest signal in a study investigating sTfR (20). Given the strong association of the *PCSK7* locus with sTfR levels in the iron metabolism GWAS, this variant could indeed be functionally relevant. Further *in silico* analyses show that *PCSK7* rs236918 is located within a region distal to the promoter that is predicted to serve as an enhancer in three of nine cell lines investigated by ENCODE. We thus speculate that rs236918 might affect the expression of *PCSK7* in a *cis*-regulatory fashion.

The gene product of *PCSK7* (proprotein convertase subtilisin/kexin type 7, PC7/8) is a convertase, similar to furin (*PCSK3*), that belongs to a larger family of proteases (30) and that is characterized by a distinct mode of intracellular assembly (31). Oexle and coworkers have recently demonstrated a significant genome-wide association of *PCSK7* SNP rs236918 with sTfR levels (20). It was speculated that PCSK7, being a furin-like convertase, functions in iron homeostasis by generating soluble hemojuvelin (sHJV) from cellular hemojuvelin (32,33), thereby modulating hepcidin activation. Further, direct shedding of the TfR was considered another possibility (20). Regarding the latter, Guillemot *et al.* have convincingly demonstrated in cell culture experiments using several hepatoma cell lines and primary murine hepatocytes coexpressing both human TfR1 and PCSK7 that PCSK7 is the only convertase that sheds TfR, whereas hepcidin is specifically processed by furin, but not by PCSK7 (34). A subsequent *in vitro* study in HeLa and LoVo cells found no evidence of PCSK7 cleavage activity on HJV supporting the role of PCSK7 as an exclusive sheddase of TfR (35).

Considering the association of *PCSK7* SNP rs236918 with cirrhosis in C282Y homozygotes, it would be important to decipher the molecular mechanism of how this variant may stimulate the production of excess hepatic scar tissue. There are no human or mammalian data on this issue, but a recent study in zebrafish demonstrated the ability of PCSK7 to increase both mRNA expression and proteolytic cleavage of transforming growth factor beta 1 (TGFβ1), the premier profibrogenic cytokine and activator of hepatic stellate cells/portal myofibroblasts (HSC/MFB) as the crucial source of collagenous matrix in liver fibrogenesis (36). Interestingly, an earlier study identified a specific TfR on isolated activated rat HSC/MFB, and treatment of cells with transferrin caused their activation and upregulation of procollagen-type gene expression indicating a significant role of TfR in iron-mediated liver fibrogenesis (37), Further research should therefore elucidate whether PCSK7-mediated TGFβ1 activation plays a significant role in human liver fibrosis and whether the postulated gain of function variant *PCSK7* rs236918 leads to increased TGFβ1 signaling and excess matrix formation.

Other markers associated primarily with serum transferrin levels (*TF*) (17), serum iron concentration (*TMPRSS6*) (18), total iron levels (*TFR2*) (18), iron deficiency (2p14) (22) and liver fibrosis (*PNPLA3*) (23) were negative with regard to this phenotype.

Regarding *TMPRSS6*, our data conflict with findings from a recent genetic case–control study (38) including 315 Italian HH patients with C282Y homozygosity ($n = 163$), compound heterozygotes ($n = 27$) and HFE genotypes not commonly associated with HH ($n = 125$). Authors found the p.A736V *TMPRSS6* polymorphism to confer protection against clinical HH, but independent replication was not demonstrated. In addition, subgroups were rather small, particularly cirrhotic patients ($n = 40$), and C282Y homozygotes afflicted by coexisting confounders (viral hepatitis and alcohol abuse). Moreover, the frequency of the protective p.A736V *TMPRSS6* CC genotype was similar in clinically healthy and diseased HH patients and only significantly different when compared with healthy control. Ethnic differences between Italian and Northern European subjects may account for the discrepancy between these and our findings.

In conclusion, our study shows how genetic markers of iron metabolism obtained through genome-wide scanning can be related to a clinically relevant trait, such as advanced liver fibrosis in HH. Admittedly, our study is limited by its retrospective nature preventing uniform sampling and more extensive phenotyping and explored only a small range of hypotheses (defined by previous iron metabolism GWAS). This notwithstanding, the large effect size of the *PCSK7* variant has allowed its discovery as the first disease modifier in HH. The potential for predictive testing in HH – a disorder that is easily treated and prevented by phlebotomy – has been thoroughly studied and discussed, and the current consensus is that population genetic screening is not indicated in HH (11,13,39). However, this view might change in the future with the possibility to genetically identify subgroups of HH who carry a high risk of progressive liver disease. To further this development, additional large-scale and population-based studies are however needed to confirm and better define the impact of *PCSK7* and other variants on HH phenotypes.

MATERIALS AND METHODS

Ethics approval

The study received Ethics committee approval from of all participating centers in which patients were recruited for the study, and only subjects were included who had given written consent to have their DNA tested in the context of a retrospective scientific study.

Patients

Recruitment of consecutive patients homozygous for the C282Y mutation from Germany ($N = 148$), Switzerland ($N = 60$), Austria ($N = 439$) and Sweden ($N = 112$) commenced in 1997 when routine genotyping for the *HFE* mutation became available in Erlangen, Rostock, Kiel, Frankfurt (Germany), Berne (Switzerland), Innsbruck, Salzburg, Oberndorf, Vienna, Graz (Austria), Örebro and Stockholm (Sweden). All patients included in the present study were confirmed homozygotes for the HFE C282Y mutation.

Phenotypes

Patients were clinically characterized as described earlier (40,41). Briefly, each patient underwent clinical examination, laboratory testing and abdominal ultrasound or computed tomography. In patients with confirmed or suspected cirrhosis, upper gastrointestinal endoscopy was performed. Chronic viral hepatitis was excluded in all patients by testing for hepatitis B virus surface antigen and anti-HBc, and by third-generation hepatitis C virus antibody ELISA. Daily alcohol intake of >30 g for men and >20 g for women led to exclusion. No clinical or serological signs of autoimmune liver disease were present. All patients and controls were European Caucasians and originated from Austria, Germany, Switzerland and Sweden.

Pretreatment liver biopsies ($N = 244$) were scored according to Ishak *et al.* (42). The presence of cirrhosis was established with fibrosis stages 5 and 6, or if there was unequivocal evidence

for liver damage (i.e. elevated liver enzyme activity, impaired coagulation, low serum albumin concentration and low platelet count) together with at least one of the following: (a) cirrhosis-related complications including encephalopathy, ascites or hepatocellular carcinoma, (b) cirrhosis on abdominal ultrasound and/or computed tomography and (c) esophageal varices in upper gastrointestinal endoscopy.

Swedish patients ($n = 112$) were diagnosed based upon C282Y homozygosity and laboratory parameters, including serum liver enzyme activities, bilirubin, prothrombin time, serum albumin and ferritin concentrations. From all patients, scored liver histologies were available and cirrhosis was established if biopsies revealed fibrosis stages 5 and 6 (42). Patients with alcoholic liver cirrhosis were identified as reported (27).

SNP selection

Loci known to be associated at genome-wide significance level with markers of iron status, namely ferritin, transferrin saturation, serum iron and soluble transferrin receptor and liver fibrosis were selected from previous GWAS and meta-analysis studies (17–23).

Additional tagging SNPs were selected for all iron loci from HAPMAP (www.hapmap.org) by the automated selection of haplotype tagging SNPs for Caucasians from the CEU dataset (settings – Mendel errors: 0, minor allele frequency: 0.1, HWE cutoff: 0.01) by using the tagger algorithm available through Haploview (http://www.broad.mit.edu/mpg/haploview/; http://www.broad.mit.edu/mpg/tagger/), using pairwise SNP selection with a minimum r^2 threshold of 0.8. We additionally selected SNPs based on functional impact according to the literature. Including the lead marker, this resulted in a selection of five SNPs for *PCSK7*, seven SNPs for *TMPRSS6*, four SNPs for *TFR2,* five SNPs for *TF* and four SNPs for the *Chr2p14* locus. This selection captures a high degree (>80%) of the known common variability in these loci. For *PNPLA3*, only the lead marker I148M (rs738409 C/G) was selected.

Genotyping and statistical analysis

Genotyping for *TF* (rs3811647)*, TMPRSS6* (rs855791)*, PCSK7* (rs236918)*, TFR2* (rs7385804) and for rs2698530 on chromosome 2p14 was performed using Sequenom and Taqman® allelic discrimination assays (43). All process data was logged and administered using a database-driven LIMS (44). Contingency tables were analyzed with a χ^2 test or Fisher's exact test, and differences between numerical values were evaluated using a *t*-test as implemented in R (www.r-project.org). Nominal *P*-values were reported for all tests. Primary association findings and *post hoc* analysis were also verified by controlling for advancing age and male gender as known risk factors for hemochromatosis using a multivariate logistic regression analysis with genotypes (additive model, defined as 0, 1 or 2 copies of the C allele), age and gender as independent variables. All logistic regression analyses were performed with SPSS software (version 21.0; SPSS, Inc., Chicago, IL, USA).

The investigated patient sample provides a power of >87% for the detection of a 5-fold higher risk of cirrhosis and >50% power for the detection of a 3-fold higher risk of cirrhosis at risk allele exposure level of 0.3 in controls. The Type I error probability was set to be 0.008, corresponding to the Bonferroni-corrected significance level of 0.05 for six tests. Power calculations were performed with PSpower (45).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

AUTHORS' CONTRIBUTION

All the authors have contributed significantly to patient recruitment, data generation and its interpretation and therefore qualify for authorship. All the authors have read and approved the present version.

Conflict of Interest statement. None declared.

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