Novel association to the proprotein convertase \textit{PCSK7} gene locus revealed by analysing soluble transferrin receptor (sTfR) levels

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The level of body iron storage and the erythropoietic need for iron are indicated by the serum levels of ferritin and soluble transferrin receptor (sTfR), respectively. A meta-analysis of five genome-wide association studies on sTfR and ferritin revealed novel association to the \textit{PCSK7} and \textit{TMPRSS6} loci for sTfR and the \textit{HFE} locus for both parameters. The \textit{PCSK7} association was the most significant (rs236918, $P = 1.1 \times 10^{-27}$) suggesting that proprotein convertase 7, the gene product of \textit{PCSK7}, may be involved in sTfR generation and/or iron homeostasis. Conditioning the sTfR analyses on transferrin saturation abolished the \textit{HFE} signal and substantially diminished the \textit{TMPRSS6} signal while the \textit{PCSK7}-associated effect on sTfR generation appears to be more direct.

\textbf{INTRODUCTION}

Iron is indispensable for oxygen transport and oxidative metabolism. The homeostatic distribution of iron to functional sites (predominantly to haemoglobin in erythrocytes) and storage (predominantly to ferritin in the liver) is controlled by a network of local and systemic regulators. The systemic regulation relies on the hormone hepcidin and the iron...
exporter ferroportin, the local regulation implies iron-regulatory proteins that bind iron-responsive elements in messenger RNAs (1). For transport in serum, most iron is loaded to transferrin that attaches to transferrin receptors (TfR) on target cells. In case of iron deficiency, the fraction of iron-loaded transferrin, i.e. the transferrin saturation, is low. The level of iron storage is also indicated by the serum concentration of ferritin leaking into the blood. The erythropoietic need for iron is revealed by the soluble transferrin receptor (sTfR) in serum. sTfR reflects the TfR expression on erythroid precursors (2). Moreover, sTfR generation by TfR cleavage appears to be inhibited by iron-loaded transferrin (3). As serum ferritin may rise disproportionately to iron storage status during inflammation or neoplasia (ferritin being an acute-phase reactant), sTfR is considered to be a more reliable index of iron deficiency in anaemia of inflammation (or of chronic disease) (2).

We measured ferritin and sTfR in five independent population cohorts and subjected the log-transformations of these parameters to a meta-analysis of the individual cohort genome-wide association study (GWAS) data. While GWAS on ferritin, on other common iron parameters, and on erythrocytes measures have been presented before (4–8), GWAS results on sTfR have not yet been published. We identified a strong signal in a gene locus (PCSK7) that is entirely new to the field of iron homeostasis alongside genes (HFE and TMPRSS6) that are already known to influence iron parameters.

RESULTS

Meta-analysis of GWAS on log$_{10}$(ferritin) and log$_{10}$(sTfR) revealed three gene loci with genome-wide significance (Fig. 1; see Supplementary Material, Tables S4 and S5 for $P$-values and effect sizes). The strongest and most novel association was found on chromosome 11q in the analysis of log$_{10}$(sTfR). This association signal comprised a 0.4 Mb region delimited at the distal end by a recombination hot spot (Fig. 1). The most significantly associated SNP (rs236918 at 116.6 Mb, $P = 2.4 \times 10^{-9}$) was identified within intron 9 of PCSK7. At the other end of the region, close to the cluster of apolipoprotein A (APOA) genes, SNPs such as rs10047462 at 116.2 Mb demonstrated almost equally significant association. We tested for possible correlation between iron (ferritin, sTfR) and lipid (triglycerides, total cholesterol, HDL, LDL) parameters to see whether such a correlation drives the association signal since the APOA gene locus is known to be associated with lipid parameters (9,10). However, in keeping with previous findings (11), Pearson coefficients revealed no correlation in our cohorts (absolute value of correlation $\leq 0.13$; Supplementary Material, Table S6). Association analysis conditioned on top hit rs236918 did not show any independent signals at the APOA cluster with $P$-values below $10^{-4}$ but conserved independent signals within PCSK7 at a level of almost genome-wide significance ($P = 1.4 \times 10^{-6}$, Fig. 2).

Two further loci were found to have genome-wide significance in association with log$_{10}$(sTfR). One was a pointed signal on chromosome 22q (Fig. 1) with the most significant SNP, rs855791, being a common missense mutation (A736V) in the protease domain of the matriptase-2 gene TMPRSS6. The other was identical to the most common missense mutation in the HFE (C282Y) gene (rs1800562) causing autosomal recessive haemochromatosis type 1 in the homozygous state. The rs1800562 (C282Y) variant of HFE was associated with both log$_{10}$(sTfR) and log$_{10}$(ferritin).

As the transferrin saturation appears to influence sTfR (3), a meta-analysis of association tests conditioned on transferrin saturation, i.e. log$_{10}$(transferrin saturation), was performed for the top hit in each of the three sTfR associated loci in order to evaluate whether these hits were secondary to an association with transferrin saturation (see Supplementary Material, Table S7; transferrin saturation was available from all cohorts except for InChianti.) The HFE hit (rs1800562) was abolished in the conditional analysis. Similarly, effect size and significance of the TMPRSS6 hit (rs855791) substantially declined (from 0.02 and $3 \times 10^{-15}$, respectively, in the unconditioned analysis without InChianti to 0.01 and $6 \times 10^{-6}$, respectively, in the analysis conditioned on transferrin saturation). The effect of the top hit (rs236918) at the PCSK7 locus, however, was not affected by the conditioning and its $P$-value was even diminished (compared with the unconditioned analysis without InChianti). In keeping with these findings, rs236918 was not associated with transferrin saturation ($P = 0.3$), whereas the top SNPs at the HFE and TMPRSS6 loci showed associations of genome-wide significance ($2.4 \times 10^{-25}$ and $1.6 \times 10^{-16}$, respectively).

DISCUSSION

Our GWAS meta-analysis identified both novel and previously known genetic associations to iron-related parameters. The association signals detected on rs1800562, the most common missense mutation in the HFE (C282Y) gene, with both log$_{10}$(ferritin) and log$_{10}$(sTfR) levels indicated our ability to detect true associations, thus confirming the sensitivity of our study. C282Y causes autosomal recessive haemochromatosis type 1 (MIM +235200) and is known to influence common parameters of iron metabolism (including ferritin) in both homozygous and heterozygous states (12). The association of pathogenic HFE mutations with sTfR has been examined before in a relatively small Catalan population sample (13) revealing association with another HFE mutation, rs1799945 (H63D), but not with rs1800562. The minor allele frequency (MAF) of rs1800562 is low (5%), possibly explaining the variability of the association results that was also evident among the different cohorts in the present study (Supplementary Material, Tables S4 and S5). While the mode of action of HFE mutations on sTfR levels remains to be determined, it may reflect an indirect consequence of the change in iron homeostasis, i.e. a change in transferrin saturation (3). Indeed, the association of sTfR with rs1800562 vanished when the analysis was conditioned on transferrin saturation (Supplementary Material, Table S7).

Association analysis of log$_{10}$(sTfR) revealed two other loci with $P$-values of genome-wide significance. One was located at the TMPRSS6 gene locus on chromosome 22q (Fig. 1) and had the lowest $P$-value on a common missense mutation.
decreased hepatic iron stores (18). However, neither our data
mutations are more susceptible to iron deficiency and have
ation (3). Indeed, the associations of the
transferrin (i.e. transferrin saturation) that inhibits sTfR gener-
association of TMPRSS6
Supplementary Material, Table S7).
transferrin saturation greatly reduced the sTfR-rs855791 association
sTfR had opposite directions, and the conditioning on transfer-
mutation A736V (rs855791) with transferrin saturation and
plasma to hepcidin expression in the liver (15–18). The
signal cascade that leads from iron-loaded transferrin in
morphogenetic protein (BMP)–Smad negative feedback
juvelin at the cell membrane, thus downregulating the bone
morphogenetic protein (BMP)–Smad negative feedback
is assumed to function in iron homeostasis by cleaving haemo-
#206200) (14). Matriptase-2, the gene product of
cause iron-refractory iron deficiency anaemia (MIM
8). Homozygous loss-of-function mutations of
transferrin stores) and
hemochromatosis type 7, PC7/8) is a convertase similar to furin (PCSK3) that
physiologically plausible candidate for affecting sTfR levels.
The gene product (proprotein convertase subtilisin/kexin
type 7, PC7/8) is a convertase similar to furin (PCSK3) that
belongs to a family of proteases acting on various physiological
paths and having functional overlap in many but not all
circumstances (19). Two possible mechanisms of PCSK7
acting on sTfR can be considered.
First, furin and/or furin-like convertases function in iron
homeostasis by generating soluble haemojuvelin (sHJV)
from cellular haemojuvelin (20,21). Haemojuvelin has a
crucial role in regulation of hepcidin. Membrane-bound hae-
mojuvelin is a coreceptor for BMPs, while sHJV acts as a
decoy receptor (22). Molecular mechanisms of hepcidin down-
regulation by sHJV and by TMPRSS6 (see above) thus seem
to converge on the BMP pathway that transmits signals from
iron-loaded transferrin to hepcidin expression (18). Therefore,
one may speculate that PCSK7, similar to furin, modulates
hepcidin expression via the BMP pathway by influencing
sHJV levels. An effect by BMP activation is also conceivable
(23), but the RTTR amino acid sequence in BMP6, the key
player (16,17) among the BMPs, is only sufficient for furin
but not for PCSK7 cleavage (24). Prohepcidin processing is
another possible mechanism by which proprotein convertases
such as PCSK7 could influence sTfR levels indirectly via
the hepcidin regulation of iron homeostasis (25). Since the
other eight proprotein convertases also might have such
effects on sTfR generation, we tested them for association
with \log_{10}\(\text{sTfR}\) (20). However, we did not find a
P-value < 0.001 for any of their intragenic SNPs (data not shown).
PCSK7 is expressed in various tissues including liver (25). It
has a non-redundant function in MHC I-mediated antigen
presentation (19) and, again, an indirect influence via hepcidin
expression [responding to immune reactions (1)] cannot be
excluded.
Alternatively, however, PCSK7 may be involved more
directly in sTfR release from membrane-bound TfR. Indeed,
sTfR is shed from the cell surface by proteolytic cleavage,
primarily mediated by a metalloprotease, which is likely acti-
vated by a furin-like convertase, as the activation is sensitive to
inhibitors of these convertases (26). In keeping with this
hypothesis, the top hit (rs236918) at the PCSK7 locus was
not associated with transferrin saturation, and its strong associa-
tion with sTfR was not affected when the analysis was
conditioned on transferrin saturation (Supplementary Material, Table S7).

PCSK7 knockout mice have been generated and reportedly do not have an overt pathology (27). However, as emphasized by Lin et al. (20), iron-related phenotypes may have been overlooked in these knockout models. They may thus need further study in light of our association data.

In summary, we identified three gene loci that associate with sTfR levels in serum. Two of these genes, HFE and TMPRSS6, associate with other iron parameters, indicating that physiologically related traits may converge in genomic association studies. The third, novel association signal reveals variants at the PCSK7 locus to play a major role in sTfR generation.

MATERIALS AND METHODS

Ferritin and sTfR were measured in five independent population cohorts (KORA F3 and KORA F4, two surveys of the Cooperative Health Research in the Southern German region of Augsburg; MICROS, a study on microisolates in South Tyrol, Italy; InCHIANTI, a study on the population in the Chianti area of Tuscany, Italy; and CROATIA-VIS, a study on an island population in Croatia) and subjected to a meta-analysis of the individual GWAS. A total of 6616 and 6592 individuals were included in the meta-analysis on sTfR and ferritin, respectively. Furthermore, transferrin saturation was available in all cohorts except InCHIANTI. Serum parameters were measured by standard laboratory methods, i.e. by electrochemiluminescence immunoassay (Roche) for ferritin, Tina-quant® immunoturbidimetry (Roche) for sTfR, colorimetry (Roche) for iron and immunonephelometry (Siemens) for transferrin in case of KORA F3, KORA F4 and CROATIA-VIS, by microparticle enzyme immunoassay (Abott) for ferritin, particle-enchanced immunonephelometry (Siemens) for sTfR, photometry (ADVIA, Siemens) for iron and PEG-enhanced immunoturbidimetry (Siemens) for transferrin in case of MICROS, and by chemiluminescent immunoassays (Abbott) for ferritin as well as sTfR, and colorimetry (Roche) for iron in case of InCHIANTI (see Supplementary Material, Table S2). Genotyping was performed on different platforms, i.e. on Affymetrix platforms in case of KORA F3 and KORA F4, and on Illumina platforms in case of MICROS, InCHIANTI and CROATIA-VIS. All studies used MACH for imputation (28).

The genetic effects were assumed to be additive. The GWAS on the log_{10}-transformation of ferritin (ng/ml) and sTfR (mg/l) with age and sex as covariates were calculated in each cohort separately. Different software was applied according to the special properties of each cohort data (see Supplementary Material, Table S3 for details).

In the subsequent meta-analysis, only SNPs with a call rate ≥0.95, a MAF ≥0.01 and an imputation quality r^2 hat ≥0.3 were included. Overall significance values and effect sizes were derived by inverse variance-weighted meta-analysis using a fixed-effect model in METAL (www.sph.umich.edu/csg/abecasis/metal). The result of each cohort was corrected for residual inflation with the genomic control method with correction for familiarity where necessary. Overall inflation factor after meta-analysis was small (≤1.01). The threshold for genome-wide significance was set to the level of 5 × 10^{-8} (29).

In the analysis conditioned on the top hit at the PCSK7 locus, this SNPs’ genotype was treated as covariate in each study.

Three selected top hits were analysed in each cohort for association with transferrin saturation using log_{10}-transformation, age and sex as covariates, and assuming additive SNP effects. Furthermore, the association analysis of these top hits and sTfR (log_{10}-transformation) with conditioning on transferrin saturation was performed in each cohort (age and sex as covariates, additive SNP effect). Results were meta-analysed using METAL. Details on study populations, phenotypes, genotyping, quality controls and statistical analysis are given in online Supplementary Methods, S-Methods and Supplementary Material, Tables S1, S2 and S3.

This meta-analysis was conducted according to the principles expressed in the Declaration of Helsinki. All contributing studies were approved by the local ethics committees. All subjects provided written informed consent for the collection of samples and subsequent analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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