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Novel association to the proprotein convertase *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 *PCSK7* and *TMPRSS6* loci for sTfR and the *HFE* locus for both parameters. The *PCSK7* association was the most significant (rs236918, $P = 1.1 \times 10E-27$) suggesting that proprotein convertase 7, the gene product of *PCSK7*, may be involved in sTfR generation and/or iron homeostasis. Conditioning the sTfR analyses on transferrin saturation abolished the *HFE* signal and substantially diminished the *TMPRSS6* signal while the *PCSK7* association was unaffected, suggesting that the former may be mediated by transferrin saturation whereas the *PCSK7*-associated effect on sTfR generation appears to be more direct.

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

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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, $P = 10^{-27}$) 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 theses 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 3×10^{-15}, respectively, in the unconditioned analysis without InCHIANTI to 0.01 and 6×10^{-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 (2.4×10^{-25} and 1.6×10^{-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

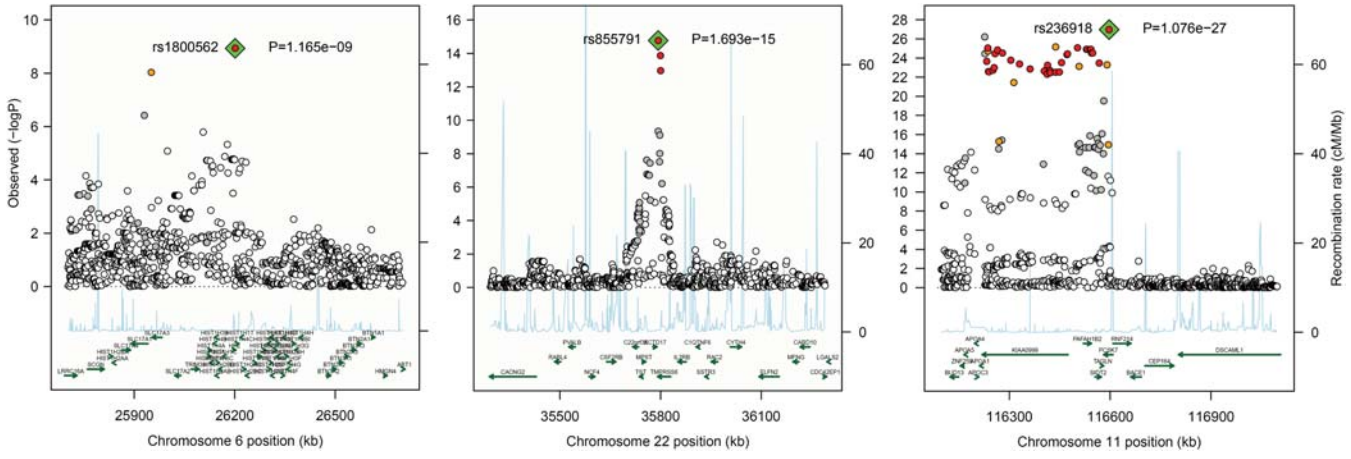

Figure 1. Loci with genome-wide significance for association with soluble transferrin receptor, $\log_{10}$(sTfR). The top hit of each locus is indicated by a green diamond. The level of linkage disequilibrium of the top hit with other SNPs in its vicinity is indicated by colouring of the circles that symbolize these SNP: red, $r^2 > 0.8$; orange, $0.8 > r^2 > 0.5$; grey, $0.5 > r^2 > 0.2$; white, $0.2 > r^2$. Genes are represented by green arrows, the recombination rate is displayed as a light blue graph.

(A736V, rs855791) within the protease domain of *TMPRSS6*. This and other *TMPRSS6* SNPs have been found recently to be associated with other parameters of iron status (i.e. iron and transferrin saturation) and with erythrocyte phenotypes (4–8). Homozygous loss-of-function mutations of *TMPRSS6* cause iron-refractory iron deficiency anaemia (MIM #206200) (14). Matriptase-2, the gene product of *TMPRSS6*, is assumed to function in iron homeostasis by cleaving haemojuvelin at the cell membrane, thus downregulating the bone morphogenetic protein (BMP)–Smad negative feedback signal cascade that leads from iron-loaded transferrin in plasma to hepcidin expression in the liver (15–18). The association of *TMPRSS6* with sTfR may be an indirect result of this matriptase-2 function, e.g. via a change in iron-loaded transferrin (i.e. transferrin saturation) that inhibits sTfR generation (3). Indeed, the associations of the *TMPRSS6* missense mutation A736V (rs855791) with transferrin saturation and sTfR had opposite directions, and the conditioning on transferrin saturation greatly reduced the sTfR-rs855791 association (Supplementary Material, Table S7).

Mice heterozygous for *TMPRSS6* loss-of-function mutations are more susceptible to iron deficiency and have decreased hepatic iron stores (18). However, neither our data ($P_{\min} = 0.0067$, rs5756505, not shown) nor previous reports (4,8) revealed an association of genome-wide significance between serum ferritin (which is a marker of body iron stores) and *TMPRSS6* SNPs. The effect of *TMPRSS6* variation on serum ferritin thus appears to be relatively small.

The strongest association to $\log_{10}$(sTfR) comprised a 0.4 Mb region on chromosome 11q (Fig. 1). The most significantly associated SNP (rs236918 at 116.6 Mb, $P = 10^{-27}$) was identified within intron 9 of the *PCSK7* gene. Although association does not necessarily mean causation, *PCSK7* is a 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 pathways 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 haemojuvelin is a coreceptor for BMPs, while sHJV acts as a decoy receptor (22). Molecular mechanisms of hepcidin downregulation 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 $\log_{10}$(sTfR). 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 activated 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 association with sTfR was not affected when the analysis was

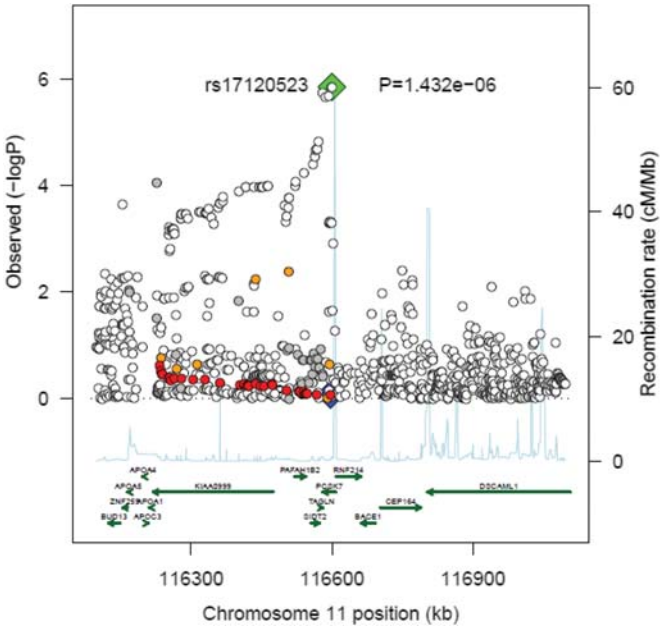

Figure 2. Meta-analysis of association studies of $\log_{10}$(sTfR) conditioned on rs236918 (blue diamond), the top hit at the *PCSK7* locus in unconditioned analysis. No signal with genome-wide significance is left in conditional analysis, indicating that there is no second hit in this region. However, SNPs within the *PCSK7* gene (e.g. rs17120532, green diamond) that are not in LD with rs236918 ($r^2 < 0.2$) keep a signal of almost genome-wide significance (10^{-6}), suggesting that there are several alleles of *PCSK7* influencing sTfR. The level of linkage disequilibrium of rs236918 with other SNPs in its vicinity is indicated as in Figure 1 by colouring of the circles that symbolize these SNP: red, $r^2 > 0.8$; orange, $0.8 > r^2 > 0.5$; grey, $0.5 > r^2 > 0.2$; white, $0.2 > r^2$. Genes are represented by green arrows, the recombination rate is displayed as a light blue graph.

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® immunoturbidometry (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 (Abbott) for ferritin, particle-enhanced immunonephelometry (Siemens) for sTfR, photometry (ADVIA, Siemens) for iron and PEG-enhanced immunoturbidometry (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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