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

Konrad Oexle\textsuperscript{1,*}, Janina S. Ried\textsuperscript{3,†}, Andrew A. Hicks\textsuperscript{4,†}, Toshiko Tanaka\textsuperscript{5}, Caroline Hayward\textsuperscript{6}, Mathias Bruegel\textsuperscript{7}, Martin Gögele\textsuperscript{4}, Peter Lichtner\textsuperscript{8}, Bertram Müller-Myhsok\textsuperscript{9}, Angela Döiring\textsuperscript{3}, Thomas Illig\textsuperscript{3}, Christine Schwienbacher\textsuperscript{4,10}, Cosetta Minelli\textsuperscript{4}, Irene Pichler\textsuperscript{4}, G. Martin Fiedler\textsuperscript{7}, Joachim Thiery\textsuperscript{7}, Igor Rudan\textsuperscript{11,12}, Alan F. Wright\textsuperscript{6}, Harry Campbell\textsuperscript{12}, Luigi Ferrucci\textsuperscript{5}, Stefania Bandinelli\textsuperscript{13}, Peter P. Pramstaller\textsuperscript{4,14,15}, H.-Erich Wichmann\textsuperscript{3,16}, Christian Gieger\textsuperscript{3,†}, Juliane Winkelmann\textsuperscript{1,2,†} and Thomas Meitinger\textsuperscript{1,8,†}

\textsuperscript{1}Institute of Human Genetics, MRI and \textsuperscript{2}Department of Neurology, MRI, Technische Universität München, Munich, Germany, \textsuperscript{3}Institute of Epidemiology, Helmholtz Zentrum München, Neuherberg, Germany, \textsuperscript{4}Institute of Genetic Medicine, European Academy Bozen/Bolzano (EURAC), Bolzano, Italy, affiliated institute of the University of Lübeck, Lübeck, Germany, \textsuperscript{5}NIA at Harbor Hospital, Baltimore, MD, USA, \textsuperscript{6}MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Edinburgh, UK, \textsuperscript{7}Institute of Laboratory Medicine, Universitätsklinikum Leipzig, Leipzig, Germany, \textsuperscript{8}Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany, \textsuperscript{9}Max Planck Institute of Psychiatry, Munich, Germany, \textsuperscript{10}Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy, \textsuperscript{11}Croatian Centre for Global Health, University of Split Medical School, Split, Croatia, \textsuperscript{12}Centre for Population Health Sciences, The University of Edinburgh Medical School, Edinburgh, UK, \textsuperscript{13}Geriatric Unit, Azienda Sanitaria Firenze (ASF), Florence, Italy, \textsuperscript{14}Department of Neurology, General Central Hospital, Bolzano, Italy, \textsuperscript{15}Department of Neurology, University of Lübeck, Lübeck, Germany and \textsuperscript{16}Institute of Medical Informatics, Biometry and Epidemiology, Ludwig-Maximilians-Universität and Klinikum Großhadern, Munich, Germany

Received October 25, 2010; Revised November 29, 2010; Accepted December 6, 2010

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.

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, \( P = 10^{-23} \)) 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 ≤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 \times 10^{-15}, respectively, in the unconditioned analysis without InChianti to 0.01 and 6 \times 10^{-16}, 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^{-22} 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 Catalon 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
(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 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 mutations of TMPRSS6 with 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 ($P_{\text{min}} = 0.0067$, 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...
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 (Abbott) for ferritin, particle-enhanced immunonephelometry (Siemens) for sTfR, photometry (ADVA, 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 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.
ACKNOWLEDGEMENTS

We thank the primary care practitioners Raffaela Stocker, Stefan Waldner, Toni Pizzecco, Josef Plangger, Ugo Marka- dent and the personnel of the Hospital of Silandro (Department of Laboratory Medicine) for their participation and collaboration in the research project. We thank Professor Pavao Rudan and staff of the Institute for Anthropological Research in Zagreb, Croatia; Professor Ariana Vorko-Jovic and staff and medical students of the Andrija Stamper School of Public Health of the Faculty of Medicine, University of Zagreb and our colleagues in MRC Human Genetics Unit and the Centre for Population Health Sciences; The University of Edinburgh Medical School. SNP Genotyping was carried out by the Genetics Core Laboratory at the Wellcome Trust Clinical Research Facility, WGH, Edinburgh. We are grateful for the insightful comments of an unknown reviewer.

Conflict of Interest statement. None declared.

FUNDING

J.W., K.O. and T.M. were supported by a grant (#81620/ 4-1) from the German Research Council (DFG). The KORA research platform was initiated and financed by the Helmholtz Center Munich, German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. Part of the financing was provided by the German National Genome Research Network (NGFN-2 and NGFNPlus: 01GS0823). KORA research was also supported within the Munich Center of Health Sciences (MC Health) as part of LMUInnovativ. The MICROS study was supported by the Ministry of Health and Department of Educational Assistance, University and Research of the Autonomous Province of Bolzano and the South Tyrolean Sparkasse Foundation. The InCHIANTI study baseline (1998–2000) was supported as a ‘targeted project’ (ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the US National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336). The CROATIA-VIS study was supported by the Medical Research Council and a grant, #216-1080315-0302 (to I.R.) from the Croatian Ministry of Science, Education and Sport.

REFERENCES


