Identification of a common variant in the **TFR2** gene implicated in the physiological regulation of serum iron levels

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The genetic determinants of variation in iron status are actively sought, but remain incompletely understood. Meta-analysis of two genome-wide association (GWA) studies and replication in three independent cohorts was performed to identify genetic loci associated in the general population with serum levels of iron and markers of iron status, including transferrin, ferritin, soluble transferrin receptor (sTfR) and sTfR–ferritin index. We identified and replicated a novel association of a common variant in the type-2 transferrin receptor (**TFR2**) gene with iron levels, with effect sizes highly consistent across samples. In addition, we identified and replicated an association between the **HFE** locus and ferritin and confirmed previously reported associations with the **TF**, **TMPRSS6** and **HFE** genes. The five replicated variants were tested for association with expression levels of the corresponding genes in a publicly available data set of human liver samples, and nominally statistically significant expression differences by genotype were observed for all genes, although only rs3811647

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in the TF gene survived the Bonferroni correction for multiple testing. In addition, we measured for the first time the effects of the common variant in TMPRSS6, rs4820268, on hepcidin mRNA in peripheral blood (n = 83 individuals) and on hepcidin levels in urine (n = 529) and observed an association in the same direction, though only borderline significant. These functional findings require confirmation in further studies with larger sample sizes, but they suggest that common variants in TMPRSS6 could modify the hepcidin-iron feedback loop in clinically unaffected individuals, thus making them more susceptible to imbalances of iron homeostasis.

INTRODUCTION

Iron is involved in several essential metabolic pathways, and thus balancing body iron levels is crucial for human health. Iron possesses features of both an essential nutrient and a potential toxin (1). It is primarily required for electron transfer and oxygen delivery, but an excess of free iron results in the production of free radicals and consequent tissue damage. Iron homeostasis is accomplished through the control of intestinal iron absorption and the recycling of heme iron after phagocytosis of senescent red blood cells by macrophages (2,3). Imbalance of iron acquisition at the cellular and systemic level can lead either to iron-overload disease due to excessive iron absorption, or to iron deficiency anaemia due to the inability to maintain normal plasma levels. Imbalanced iron status is also associated with disorders that include diabetes mellitus (4), inflammation (5) and neurological (6) and cardiovascular diseases (7).

Iron metabolism is meticulously regulated by the concerted action of several genes and proteins—a key role being played by hepcidin, a circulating peptide hormone produced mainly in the liver, which controls iron absorption and recycling via its interaction with the major cellular iron export protein ferroportin (8).

Because diseases of iron overload and deficiency are among the most frequent disorders worldwide (9), the underlying determinants of inter-individual variation of iron status are being actively sought. Heritability estimates of 20–30% suggest a substantial genetic contribution to iron regulation (10–13). Indeed, common variants in the transferrin (TF) gene have been confirmed to regulate serum transferrin levels, and the C282Y mutation in the HFE gene, found in patients with hereditary hemochromatosis (14), has been associated with iron transferrin and transferrin saturation (15,16). In addition, common variants in the TMPRSS6 gene, encoding the serine protease matriptase-2, which is required to sense iron deficiency (17), have been associated with iron and hematological traits, including hemoglobin levels (16,18–21).

To identify further genetic factors associated with variation in serum levels of iron markers, including iron, transferrin, ferritin, soluble transferrin receptor (sTfR) and sTfR–ferritin index in the general population, we performed a meta-analysis of two genome-wide association (GWA) studies and replicated top candidate variants in three independent cohorts. In addition, the replicated variants were investigated for association with the expression levels of the corresponding genes, using a publicly available database of human liver samples, and analyses of hepcidin levels were performed aimed at further elucidating the functional role of common variants in the TMPRSS6 gene.

RESULTS

Meta-analysis of GWA studies and replication

We performed a meta-analysis of GWA studies of serum iron and transferrin from two Italian cohorts [MICROS (Microisolates in South Tyrol Study), n ~ 1300; SardiNIA study, n ~ 4300] and used a two-stage design to perform replication in an additional three cohorts [SardiNIA stage 2 study, n ~ 1500; InCHIANTI study, n ~ 1200; BLSA (Baltimore Longitudinal Study on Aging), n ~ 500], using de novo (SardiNIA stage 2) or in silico (InCHIANTI and BLSA) genotyping. For the other traits, for which data were not available in the SardiNIA study, results from the MICROS GWA study were tested for replication in the InCHIANTI and BLSA studies (sTfR and sTfR–ferritin index) and in the BLSA study (ferritin). The study workflow is presented in Figure 1, and characteristics of each study, including sample size and trait distribution, are described in Supplementary Material, Table S1. Whereas the BLSA study was carried out in an outbred population, all other studies recruited individuals in semi-isolated regions. Manhattan plots of the discovery analyses, along with the quantile–quantile plots of P-values, are reported in Supplementary Material, Figure S1.

Ten to 38 SNPs were selected for replication for each of the five traits, based on the estimated power to replicate (Supplementary Material, Table S2). Table 1 lists the results for replicated SNPs for iron, ferritin and transferrin, along with the results of the combined analyses of discovery and replication samples. For sTfR and sTfR–ferritin index, no SNP was replicated (Supplementary Material, Table S2).

For iron, we identified a novel association with a common variant in the type-2 transferrin receptor (TFR2) gene (rs7385804, replication P = 5.0 × 10^{-4}) and confirmed previously described associations in the TMPRSS6 (rs4820268, replication P = 5.5 × 10^{-5}) and HFE (rs1799945, replication P = 0.001) genes (15,16,18). Direction and magnitude of the effect sizes were confirmed in all studies, with P-values for the combined analyses smaller than those of the discovery analyses (Table 1). In the discovery phase, we detected the association of rs4820268 in TMPRSS6 also with sTfR and sTfR–ferritin index (P = 1.4 × 10^{-6} and P = 1.5 × 10^{-5}, respectively), but these signals were not replicated (Supplementary Material, Table S2).

For ferritin, we identified an association in the SLC17A1 gene (rs17342717, replication P = 8.0 × 10^{-6}, Table 1), although this signal likely reflects an association with the HFE gene. In fact, SLC17A1 rs17342717 correlates with the HFE variant rs1800562, associated with hemochromatosis.
(HapMap CEU $r^2 = 0.42$), and its observed effect disappeared after adjusting for $HFE$ rs1800562 ($P = 0.417$).

For transferrin, we confirmed the previously described association of rs3811647 in the TF gene (replication $P = 1.2 \times 10^{-10}$, Table 1) (15,16) and found association of two other variants in moderate linkage disequilibrium (LD) with this SNP. However, the effects of these two variants disappeared in conditional analyses adjusting for rs3811647 (Supplementary Material, Table S3).

Forest plots for the meta-analyses of all studies for the novel findings of an association between TFR2 and iron and between SLC17A1 (in LD with the HFE gene) and ferritin (Supplementary Material, Fig. S2) show that the estimates of the genetic effects are highly consistent across studies, with low $I^2$ values (percentage of the observed heterogeneity in excess of what can be explained by chance alone) of 8 and 0%, respectively.

The phenotypic variance explained by the replicated SNPs for iron varied across samples between 1.2 and 2.7% (TFR2 rs7385804, TMPRSS6 rs4820268 and HFE rs1799945); for ferritin between 0.9 and 1.5% (SLC17A1 rs17342717, in LD with HFE rs1800562); and for transferrin between 2.1 and 7.2% (TF rs3811647), in line with the value of 10% reported for other populations (15) (Supplementary Material, Table S4).

Expression analyses

The five replicated variants from the GWA analysis (Table 1) were tested for association with expression levels (eSNP analysis) of the corresponding genes using publicly available data on 707 human liver samples (22). Nominally statistically significant expression differences by genotype were observed for all genes, although only rs3811647 in the TF gene survived the Bonferroni correction for multiple testing (five tests, $P < 0.01$) (Table 2). In the regions of each of the genes reported here, additional SNPs showing no strong LD with the five replicated SNPs, showed more significant association with expression levels of the corresponding genes (data not shown). Characterizing the best eSNP in the same region of a GWA-significant SNP can help determine whether there are multiple independent variants capable of associating the gene expression with the trait of interest, or whether there may be a single underlying causal variant that influences the trait through an effect on gene expression. The presence of independent eSNP variants with stronger effects on the expression in this case indicates that although gene expression may contribute to changes in the iron traits measured, it is probably not the only mechanism.

Notably, all three genes identified as associated with serum iron levels (TFR2, TMPRSS6 and HFE) are known to be involved in the regulation of hepcidin expression in response to iron challenge (9,17). TMPRSS6 rs4820268 showed the strongest association with iron levels (effect size for each copy of the G allele from the combined analysis: $2.4.2 \mu$g/dl; 95% CI: $-5.5$ to $-3.0$). The serine protease TMPRSS6 functions as a negative regulator of hepcidin (HAMP) expression. Since probes to evaluate hepcidin (HAMP) mRNA expression were neither contained in the liver expression data set nor in more than 25 additional expression quantitative trait locus data sets (Andrew Johnson, personal communication), we evaluated its association with the TMPRSS6 rs4820268 genotype in a subsample of the MICROS study, consisting of individuals homozygous for either GG ($n = 40$) or AA ($n = 43$) genotype. Whole-blood-derived mRNA levels in these subjects showed a reduction of 28% in hepcidin mRNA in GG compared
Reference

Replicated findings for iron, ferritin and transferrin

Table 1.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Coded allele/other</th>
<th>Chr.</th>
<th>Gene</th>
<th>Coded annotation</th>
<th>Chr.</th>
<th>Gene annotation</th>
<th>Coded allele/other</th>
<th>Effect (SE)</th>
<th>P-value</th>
<th>Replication Effect (SE)</th>
<th>Combined Effect (SE)</th>
<th>P-value</th>
<th>Previous association with related traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs735804</td>
<td></td>
<td>7</td>
<td>TFR2</td>
<td></td>
<td>6</td>
<td>TFR2 (intron)</td>
<td>A/C</td>
<td>4.19 (0.84)</td>
<td>8.9×10^-6</td>
<td>2.55 (0.79) 5.0×10^-1</td>
<td>3.23 (0.60) 7.3×10^-4</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>rs17342717^a</td>
<td></td>
<td>6</td>
<td>SLC17A1</td>
<td></td>
<td>2</td>
<td>SLC17A1 (intron)</td>
<td>CT</td>
<td>-4.23 (0.50)</td>
<td>6.1×10^-5</td>
<td>-3.37 (0.72) 8.0×10^-4</td>
<td>-36.50 (6.24) 4.9×10^-9</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>rs3811647</td>
<td></td>
<td>3</td>
<td>TF</td>
<td></td>
<td>2</td>
<td>TF (exon)</td>
<td>G/A</td>
<td>17.60 (1.38)</td>
<td>2.4×10^-37</td>
<td>11.00 (1.79) 1.2×10^-10</td>
<td>15.34 (1.66) 2.9×10^-14</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>rs4820268</td>
<td></td>
<td>22</td>
<td>TFR2</td>
<td></td>
<td>6</td>
<td>TFR2 (exon)</td>
<td>A/G</td>
<td>-4.21 (0.95)</td>
<td>9.7×10^-6</td>
<td>-4.29 (0.88) 5.5×10^-7</td>
<td>-4.24 (0.64) 3.9×10^-11</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>rs1799945</td>
<td></td>
<td>6</td>
<td>HFE</td>
<td></td>
<td>6</td>
<td>HFE (exon)</td>
<td>C/G</td>
<td>-3.67 (1.27)</td>
<td>0.001</td>
<td>-3.86 (1.27) 0.001</td>
<td>-3.85 (0.74) 3.2×10^-9</td>
<td>8.9</td>
<td></td>
</tr>
</tbody>
</table>

^aWhen multiple oligonucleotide probes were present on the array, the best P-value was chosen.

In LD with HFE rs1800562.

Table 2. Association of the identified variants in the TFR2, SLC17A1, TF, TMPRSS6 and HFE genes with mRNA expression levels of the corresponding genes in liver samples

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene expression</th>
<th>P-value^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs735804</td>
<td>TFR2</td>
<td>0.018</td>
</tr>
<tr>
<td>rs17342717^b</td>
<td>SLC17A1</td>
<td>0.016</td>
</tr>
<tr>
<td>rs3811647</td>
<td>TF</td>
<td>5.4×10^-4</td>
</tr>
<tr>
<td>rs4820268</td>
<td>TMPRSS6</td>
<td>0.020</td>
</tr>
<tr>
<td>rs1799945</td>
<td>HFE</td>
<td>0.015</td>
</tr>
</tbody>
</table>

^bIn LD with HFE rs1800562.

with AA homozygotes, which was of borderline statistical significance (P = 0.062) (Table 3).

In addition, in a subsample of the InCHIANTI study (n = 529 individuals), data on urinary hepcidin were available, and we investigated its association with the TMPRSS6 rs4820268 genotype. We again found a borderline significant association (P = 0.05), with effect direction consistent with that observed for blood mRNA expression.

The observed differences in hepcidin mRNA and protein levels by the TMPRSS6 rs4820268 genotype are paralleled by significant differences of markers of iron status between the two homozygous groups in the entire sample, comprising all discovery and replication populations except for SardiNIA (in which the SNP was imputed rather than genotyped) (Table 4). Compared with the AA genotype, the GG genotype was associated with a decrease of iron (P = 1.3×10^-9), hemoglobin (P = 0.013), MCV (P = 5.2×10^-7) and MCH (P = 3.6×10^-9), and an increase of transferrin (P = 0.007), sTfR (P = 1×10^-4) and sTfR–ferritin index (P = 0.005).

DISCUSSION

Our findings identify genetic variants associated with serum levels of iron markers in the general population from five population-based studies. We found a novel association with a common variant in TFR2, which influences the regulation of iron levels in individuals not affected by overt disorders of iron metabolism. We also report the first noted association of the HFE locus with ferritin levels and confirm previously reported associations of the TF gene with transferrin and of the TMPRSS6 and HFE genes with iron.

TFR2 is a homologue of the type-1 transferrin receptor (TfR1) (23). TfR2 binds to TFR2 with a lower affinity than to TfR1 (24), but TfR2 may nevertheless participate in cellular iron uptake. In addition, TfR2 helps sense and regulate iron levels in the body by contributing to hepcidin activation (9). For this pathway, a model has been suggested, in which high concentrations of diferric transferrin displace HFE from TfR1 to promote its interaction with TfR2, and the interaction is further stabilized by increased binding of diferric transferrin to TfR2; the HFE–TFR2 complex then activates hepcidin transcription (25).

Targeted deletion of the TFR2 gene in mice causes iron overload with low basal hepcidin levels (26), and similar observations have been reported in humans, where a variety
of mutations in this gene lead to autosomal recessive hemochromatosis type 3 (MIM 604250) (27,28). We now demonstrate, for the first time, that common variants in TFR2 are also associated with altered iron levels in clinically unaffected individuals. Furthermore, partially explicating its action, the gene variant identified in our study (rs7385804) was associated with TFR2 mRNA expression levels in human liver samples, where TFR2 is predominantly expressed (in contrast to the ubiquitously expressed Tfr1) (29). The same genetic variant has also been recently reported as associated with hematological parameters, including red blood cell count and mean corpuscular volume (20,21); those associations can be rationalized by a direct effect on iron levels.

The association of the HFE gene with iron, transferrin, transferrin saturation and other hematological parameters is well established (15,16,19–21), and our study shows that this locus is additionally associated with ferritin levels. Mutations in the HFE gene are responsible for hereditary hemochromatosis type 1 (MIM 235200), the most common form of this disorder, which is inherited in an autosomal recessive pattern. Measurement of the serum ferritin levels in hemochromatosis patients can predict the risk of cirrhosis, the main clinical manifestation of the disorder (30). Cell models suggest that the expression of HFE protein alters ferritin levels, dependent on the expression of iron transport proteins (31). The most significant association signal in HFE for iron levels was at the H63D mutation (rs1799945), the second most common mutation in hereditary hemochromatosis type 1. This variant is independent of the effect of the C282Y mutation (rs1800562), the main mutation in this disorder (HapMap CEU $r^2 = 0.007$), and has been recently found to be involved in the regulation of hemoglobin levels (19).

From the expression analysis in human liver samples, differential expression was detected in the presence of the H63D mutation (rs1799945), in line with a previous study in peripheral blood mononuclear cells (32).

Our study also confirms recently reported associations of common variants in TMPRSS6, including rs4820268, with alterations in iron levels (15,16,18). Mutations in this gene, which predominantly result in matriptase-2 protein lacking protease activity (33), have been identified in individuals with autosomal recessive iron-refractory iron deficiency anemia (MIM 206200) (34,35). TMPRSS6 has been shown to be essential for adequate iron uptake to prevent iron deficiency (17,36), and it suppresses hepcidin expression in iron deficiency (9,37). Hepcidin in turn is a key iron regulator that governs systemic iron homeostasis by binding to ferroportin on the surface of macrophages, enterocytes and hepatocytes (9,26), inducing the degradation of ferroportin and thereby preventing the efflux of iron into the blood (8). Hepcidin formation is repressed by increased erythropoiesis in the bone marrow, as well as iron deficiency, and is induced by iron overload and inflammation. In fact, chronically elevated levels of hepcidin cause systemic iron deficiency, whereas low hepcidin levels lead to iron overload (1,5,8,9).

In our study, the TMPRSS6 rs4820268 variant is associated with iron levels and shows borderline-significant association with levels of hepcidin (HAMP) mRNA in white blood cells and urinary hepcidin levels. Although hepcidin is predominantly produced in the liver and secreted into serum, and therefore the relevance of hepcidin mRNA levels in white blood cells is unclear, we found an association of the variant in the same direction as that observed with levels of the protein in urine, which is known to correlate well with serum levels (38,39). We also attempted to quantify hepcidin mRNA levels directly in the liver and to correlate these with genotype. Whereas we observed an association in the same direction as that observed in blood mRNA and urine protein, the results were not statistically significant, likely due to the small number ($n = 61$) of liver samples available (data not shown).

**Table 3.** Association of the rs4820268 genotype in TMPRSS6 (GG versus AA) with expression levels of hepcidin mRNA in white blood cells and protein in urine

<table>
<thead>
<tr>
<th>Expression levels</th>
<th>Association stratified by the TMPRSS6 rs4820268 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG Mean (SD)</td>
</tr>
<tr>
<td>Hepcidin (HAMP) mRNA (blood)</td>
<td>0.91 (0.49)</td>
</tr>
<tr>
<td>Hepcidin protein (urine)</td>
<td>96.60 (85.66)</td>
</tr>
</tbody>
</table>

SD, standard deviation; NA, not available (HAMP mRNA was measured only in GG and AA homozygous individuals).
### Table 4.

Association results of TMPRSS6 rs4820268 with iron markers, by genotype group

<table>
<thead>
<tr>
<th>Trait</th>
<th>AG vs AA (MICROS BLSA)</th>
<th>GG vs AA (MICROS BLSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dl</td>
<td>0.17 (0.10)</td>
<td>0.15 (0.08)</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>0.57 (0.20)</td>
<td>0.54 (0.18)</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>0.60 (0.10)</td>
<td>0.58 (0.09)</td>
</tr>
<tr>
<td>Ferritin, ng/ml</td>
<td>0.60 (0.10)</td>
<td>0.58 (0.09)</td>
</tr>
<tr>
<td>iron, mg/dl</td>
<td>0.92 (0.28)</td>
<td>0.92 (0.28)</td>
</tr>
<tr>
<td>transferrin, mg/L</td>
<td>0.04 (0.03)</td>
<td>0.04 (0.03)</td>
</tr>
<tr>
<td>(log)sTfR–ferritin</td>
<td>0.01 (0.01)</td>
<td>0.01 (0.01)</td>
</tr>
</tbody>
</table>

All models are adjusted for sex and age (reference genotype = AA). M-A, meta-analyses; ND, not determined; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

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In conclusion, our data identify TFR2 as a novel candidate gene in the regulation of iron levels in clinically unaffected individuals. Our study also shows association of the HFE locus with ferritin levels and confirms the involvement of TF, TMPRSS6 and HFE genes in the maintenance of iron homeostasis. Finally, our study provides additional support for the hypothesis that common variants in TMPRSS6 may affect hepcidin expression, which could modify dietary iron absorption and recycling from macrophages even in the absence of iron challenge or inflammation (5).

### MATERIALS AND METHODS

For all studies, participants gave written informed consent and the study protocol was approved by the appropriate Research Ethics Committee.

#### Study populations

**MICROS study (discovery).** MICROS is a cross-sectional genetic study and was carried out in three semi-isolated Alpine villages of the Val Venosta, South Tyrol, Italy, in 2001–2003, as part of the genomic health care research program GenNova. A detailed description of the MICROS study is available elsewhere (41). Information on participants’ health status was collected through a standardized questionnaire. Laboratory data, including data on iron traits, were obtained from fasting blood samples, using standard blood analyses. Measurements of iron parameters were performed for all study participants from the same visit.

**SardiNIA (discovery) and SardiNIA stage 2 (replication) studies.** The SardiNIA study consists of 6148 volunteers from the Ogliastra region in Sardinia (12). All subjects underwent extensive phenotyping, which included assessment of several quantitative traits measurable in blood. For replication purposes, a set of samples (SardiNIA stage 2 study, n = 1540), consisting of volunteers who were not related to the individuals of the SardiNIA study (kinship coefficient = 0) and for whom iron and transferrin were available, was included (42).

**InCHIANTI study (replication).** The InCHIANTI study is a population-based study aimed at evaluating the factors that influence mobility in the older population living in the Chianti region in Tuscany, Italy. The details of the study...
have been reported previously (43). Briefly, 1616 residents were selected from the population registry of Greve in Chianti and Bagno a Ripoli. The participation rate was 90% \((n = 1453)\), and the subjects ranged between 21 and 102 years of age.

**BLSA study (replication).** BLSA is a population-based study aimed at evaluating contributors of healthy aging in the older population residing predominantly in the Baltimore–Washington DC area (44). Starting in 1958, participants have been examined every 1–4 years depending on their age. Currently, there are approximately 1100 active participants enrolled. Measurements of iron parameters were considered for all study participants from the same visit. African-American individuals were excluded from the present analysis.

**Iron markers determination**

Iron status was determined by measuring the level of serum iron \((\mu g/dl)\), transferrin \((mg/dl)\), ferritin \((ng/ml)\), sTfR \((mg/l)\) and the sTfR–ferritin index, defined as the ratio between sTfR and \(\log_{10}(\text{ferritin})\) (45). Methods used in each study are reported in Supplementary Material, Table S5.

**Genotyping platforms and imputation**

Genotyping was performed using Affymetrix (SardiNIA) and Illumina (MICROS, BLSA, InCHIANTI) platforms. Details regarding quality control procedures, imputation and software used for statistical analyses for both discovery and replication studies are summarized in Supplementary Material, Table S6. All studies conducted quality control procedures on genotyped SNPs, and all but one (SardiNIA stage 2) used imputation methods to test \(\sim 2.5\) M HapMap SNPs, based on HapMap Phase II CEU samples (46).

**mRNA quantification**

**eSNP analysis in a human liver cohort.** To test for the association of the identified variants with expression (eSNPs) of the corresponding genes, a cohort of liver samples, comprised of patients who underwent RXY gastric bypass surgery, was analyzed. RNA samples were profiled on a custom Agilent microarray with 39 280 oligonucleotide probes targeting transcripts representing 34 266 known and predicted genes; successful gene expression profiling results were obtained from 707 liver samples (22). Ratios of transcript abundance (experimental to control) were obtained following normalization and correction of the array intensity data. In addition, the strongest eSNP in the region of each gene was identified and the relationship between the top eSNP and the top GWA SNP tested by conditional analysis.

**Quantitative real-time PCR.** Hepcidin \((HAMP)\) expression data are absent from the liver expression data set examined and therefore \(HAMP\) mRNA expression was manually evaluated in a subsample of the MICROS study [83 individuals, homozygous for either GG \((n = 40)\) or AA \((n = 43)\) genotype] and a small set of human liver samples \((n = 61)\). Total RNA was extracted from peripheral blood and liver using TRIzol reagent (Invitrogen) according to manufacturer’s instructions. Single-stranded cDNA synthesis was performed from 1 \(\mu g\) RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using the TaqMan method (Applied Biosystems) with probes and primers designed to recognize all potential isoforms \((HAMP;\) HS 01057160_g1HAMP\). PCR was carried out in triplicate on each sample and fluorescent signals were captured using ABI7300 Real Time PCR System (Applied Biosystems). Relative quantification of expression levels was performed using the human \(TB\) \((4326317E)\) and \(GAPDH\) \((4326322E)\) genes as reference genes and evaluated using the Pfaffl modification of the \(\Delta\Delta CT\) equation (Gene Expression Macro™ version 1.1, BioRad).

**Hepcidin determination**

Urinary hepcidin levels were determined by immunodot assay in 24 h urine samples in a subsample of the InCHIANTI study consisting of 529 individuals (47). Urinary hepcidin concentrations were normalized by urinary creatinine concentration and expressed as nanograms per milligram of creatinine. Assays of hepcidin performed in the same urinary samples 1 year apart yielded values that were highly correlated \((r = 0.988)\), with no evidence for systematic trends toward lower or higher levels with time.

**Statistical methods**

**Discovery analyses.** All iron traits were analyzed without transformation, with the exception of the sTfR–ferritin index, which was \(\log_{10}\)-transformed. Analyses were performed using linear regression and assuming an additive genetic effect model. All models were adjusted for sex and age (and study location in the MICROS study), accounting for relatedness whenever necessary (Supplementary Material, Study-specific methods). Annotation of the GWA results was performed using an R script, available at http://cran.r-project.org/web/packages/NCI2R/index.html. An inverse-variance weighted fixed-effect meta-analysis of the MICROS and SardiNIA studies was performed for transferrin and iron using METAL (http://www.sph.umich.edu/csg/abecasis/metal/). Results of the two GWA studies were adjusted for the genomic control inflation factor \((\lambda)\) across all imputed SNPs prior to the meta-analysis.

SNPs to be tested in the replication studies were selected from those with \(P < 10^{-3}\), or those with \(P < 10^{-7}\) if two or more of them adjacent (see Supplementary Material), based on their power to replicate \((\text{power} \geq 80\%)\), with only one SNP being selected for each LD block to avoid redundancy and over adjustment for multiple testing. Power to replicate was calculated assuming similar effect and variance as observed in the discovery sample and adjusting for multiple testing using the Bonferroni correction.

**Replication analyses.** For iron, transferrin and ferritin, inverse-variance weighted fixed-effect meta-analyses of the replication samples were performed using Stata 10.1 software (StataCorp, College Station, TX, USA). A GWA result was considered
replicated if the effect estimate was in the same direction for discovery and replication, and if the replication result was statistically significant after Bonferroni correction (adjustment for the number of SNPs selected), using a one-sided test. A combined analysis of the discovery and replication samples was also performed using a two-sided test.

eSNP analysis. Gene expression traits were adjusted for age and sex. All expression traits (residuals) were tested for association with the identified SNPs using a linear model (lm procedure in the R statistical computing package), where the expression trait was treated as an independent variable and the genotypes for the SNP of interest were treated as a dependent categorical variable.

Hepcidin mRNA and protein expression analyses. Comparison of hepcidin (HAMP) mRNA levels between the two homozygous groups was performed using a t-test on log-transformed data, in Stata 10.1 software (StataCorp). Comparison of urine hepcidin levels across the three genotype groups was performed using a general linear model procedure implemented in SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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