Human Molecular Genetics, 2011, Vol. 20, No. 6 **1232–1240**
doi:10.1093/hmg/ddq552
Advance Access published on December 24, 2010

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

Irene Pichler[1,*,†], Cosetta Minelli[1,†], Serena Sanna[2,†], Toshiko Tanaka[3], Christine Schwienbacher[1,4], Silvia Naitza[2], Eleonora Porcu[2], Cristian Pattaro[1], Fabio Busonero[2], Alessandra Zanon[1], Andrea Maschio[2], Scott A. Melville[1,5], Maria Grazia Piras[2], Dan L. Longo[3], Jack Guralnik[6], Dena Hernandez[7], Stefania Bandinelli[8], Elmar Aigner[9], Anthony T. Murphy[10], Victor Wroblewski[10], Fabio Marroni[1,11], Igor Theurl[12], Carsten Gnewuch[13], Eric Schadt[14], Manfred Mitterer[15], David Schlessinger[16], Luigi Ferrucci[3], Derrick R. Witcher[10], Andrew A. Hicks[1], Günter Weiss[12], Manuela Uda[2] and Peter P. Pramstaller[1,17,18,*]

[1]Institute of Genetic Medicine, European Academy Bozen/Bolzano (EURAC), 39100 Bolzano, Italy; Affiliated Institute of the University of Lübeck, 23538 Lübeck, Germany, [2]Istituto di Neurogenetica e Neurofarmacologia del CNR, 09042 Monserrato, Cagliari, Italy, [3]Clinical Research Branch, National Institute on Aging, 21225 Baltimore, MD, USA, [4]Department of Experimental and Diagnostic Medicine, University of Ferrara, 44121 Ferrara, Italy, [5]Genetics Program, School of Medicine, Boston University, 02118 Boston, MA, USA, [6]Laboratory of Epidemiology, Demography and Biometry and [7]Laboratory of Neurogenetics, National Institute on Aging, 20892 Bethesda, MD, USA, [8]Geriatric Rehabilitation Unit, Azienda Sanitaria Firenze (ASF), 50100 Florence, Italy, [9]Zentrallabor, Molekularbiologie, Salzburger Landeskliniken, 5020 Salzburg, Austria, [10]Biotechnology Discovery Research, Lilly Research Laboratories, Lilly Corporate Center, 46201 Indianapolis, IN, USA, [11]Institute of Applied Genomics, IGA, 33100 Udine, Italy, [12]Department of Internal Medicine I, Clinical Immunology and Infectious Diseases, Medical University of Innsbruck, 6020 Innsbruck, Austria, [13]Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, 93047 Regensburg, Germany, [14]Pacific Biosciences, 94025 Menlo Park, CA, USA, [15]Department of Transfusion Medicine, Franz Tappeiner Hospital, 39012 Merano, Italy, [16]Laboratory of Genetics, National Institute on Aging, 21224 Baltimore, MD, USA, [17]Department of Neurology, University of Lübeck, 23538 Lübeck, Germany and [18]Department of Neurology, General Central Hospital, 39100 Bolzano, Italy

Received October 18, 2010; Revised December 9, 2010; Accepted December 16, 2010

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

*To whom correspondence should be addressed at: Institute of Genetic Medicine, European Academy of Bolzano, Viale Druso 1, 39100 Bozen/Bolzano, Italy. Tel: +39 471055512; Fax: +39 471055599; Email: irene.pichler@eurac.edu (I.P.); Tel: +39 471055501; Fax: +39 471055599; Email: peter.pramstaller@eurac.edu (P.P.P.)
†These authors contributed equally to this work.

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 anemia 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), $n \sim 1300$; SardiNIA study, $n \sim 4300$] and used a two-stage design to perform replication in an additional three cohorts [SardiNIA stage 2 study, $n \sim 1500$; InCHIANTI study, $n \sim 1200$; BLSA (Baltimore Longitudinal Study on Aging), $n \sim 500$], 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 $P = 5.0 \times 10^{-4}$) and confirmed previously described associations in the *TMPRSS6* (rs4820268, replication $P = 5.5 \times 10^{-7}$) 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 ($P = 1.4 \times 10^{-6}$ and $P = 1.5 \times 10^{-5}$, respectively), but these signals were not replicated (Supplementary Material, Table S2).

For ferritin, we identified an association in the *SLC17A1* gene (rs17342717, replication $P = 8.0 \times 10^{-6}$, 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

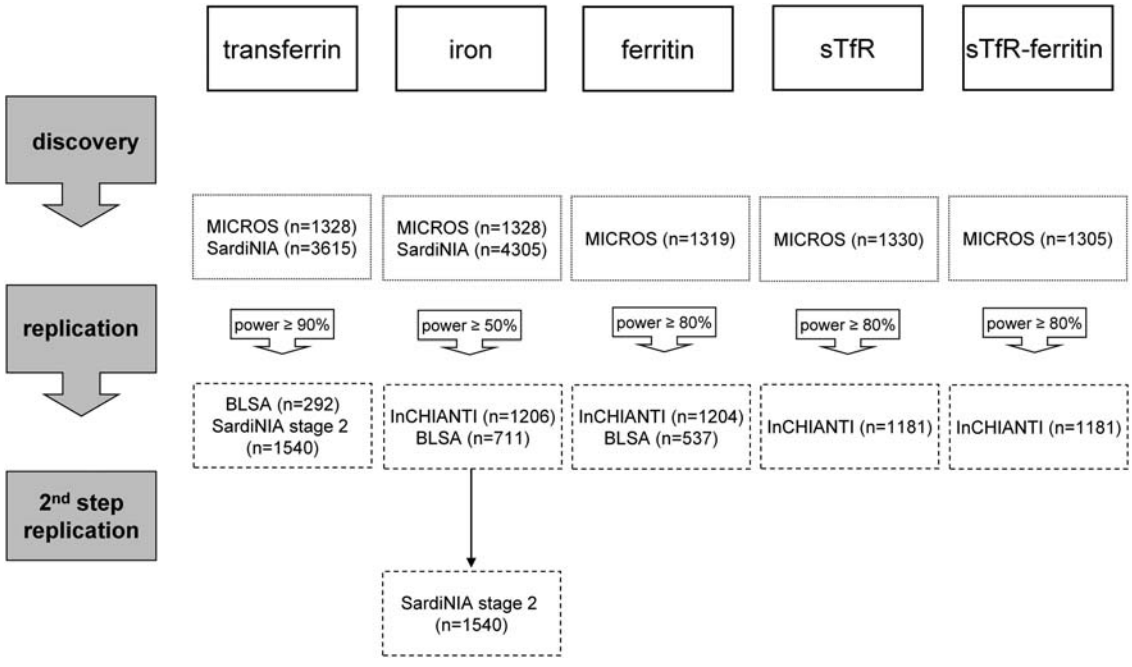

Figure 1. Overview of the discovery and replication stages of the study. Sequential criteria were used for the selection of SNPs to go through replication, based on expected power in addition to other criteria (see Materials and Methods).

(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: −4.2 μg/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

Table 1. Replicated findings for iron, ferritin and transferrin

SNP	Trait	Chr.	Gene annotation	Coded allele/ other	Frequency-coded allele	Discovery Effect (SE)	*P*-value	Replication Effect (SE)	*P*-value (one-sided)	Combined Effect (SE)	*P*-value	Previously described association with related traits	Reference number
Novel findings													
rs7385804[a]	Iron, μg/dl	7	*TFR2* (intron)	A/C	0.65	4.19 (0.94)	8.9×10^{-6}	2.55 (0.79)	5.0×10^{-4}	3.23 (0.60)	7.3×10^{-8}	Hematocrit, mean corpuscular volume, red blood cell count	(20,21)
rs17342717[a]	Ferritin, ng/ml	6	*SLC17A1* (intron)	C/T	0.93	−42.33 (10.56)	6.1×10^{-5}	−33.37 (7.73)	8.0×10^{-6}	−36.50 (6.24)	4.9×10^{-9}	Hemoglobin	(20)
Confirmation of previously reported findings													
rs3811647	Transferrin, mg/dl	3	*TF* (intron)	A/G	0.29	17.60 (1.38)	2.4×10^{-37}	11.33 (1.79)	1.2×10^{-10}	15.34 (1.06)	2.9×10^{-47}	Ferritin, transferrin saturation	(15,16)
rs4820268	Iron, μg/dl	22	*TMPRSS6* (exon)	G/A	0.47	−4.21 (0.95)	9.7×10^{-6}	−4.29 (0.88)	5.5×10^{-7}	−4.24 (0.64)	3.9×10^{-11}	Transferrin, transferrin saturation, hemoglobin	(15,16,19,20)
rs1799945[a]	Iron, μg/dl	6	*HFE* (exon)	C/G	0.83	−5.78 (1.13)	2.9×10^{-7}	−3.86 (1.27)	0.001	−4.95 (0.84)	3.2×10^{-9}	Hemoglobin	(19,20)

Chr., chromosome; SE, standard error.
[a]Imputed SNP.

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

SNP	Gene expression	*P*-value[a]
rs7385804	*TFR2*	0.018
rs17342717[b]	*SLC17A1*	0.016
rs3811647	*TF*	5.4×10^{-4}
rs4820268	*TMPRSS6*	0.020
rs1799945	*HFE*	0.015

[a]When multiple oligonucleotide probes were present on the array, the best *P*-value was chosen.
[b]In 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 ($P = 1.3 \times 10^{-9}$), hemoglobin ($P = 0.013$), MCV ($P = 5.2 \times 10^{-7}$) and MCH ($P = 3.6 \times 10^{-9}$), and an increase of transferrin ($P = 0.007$), sTfR ($P = 1 \times 10^{-4}$) 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). Transferrin 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

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

Expression levels	Association stratified by the *TMPRSS6* rs4820268 genotype			
	GG Mean (SD)	AG Mean (SD)	AA Mean (SD)	*P*-value
Hepcidin (*HAMP*) mRNA (blood)	0.91 (0.49)	NA	1.24 (1.06)	0.062
Hepcidin protein (urine)	96.60 (85.66)	87.65 (101.22)	114.00 (148.59)	0.05

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

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).

TMPRSS6 rs4820268 is not known to be a functional variant, but a strong differential allelic expression of the *TMPRSS6* mRNA has been reported for this variant (40). Furthermore, it is in strong LD with rs855791 (HapMap CEU $r^2 = 0.9$), a coding SNP for a common amino acid variation [valine/alanine (V736A)] in the serine protease domain of matriptase-2 (results for rs855791 in the discovery: $P = 3.5 \times 10^{-6}$; effect $= -4.5$; standard error $= 0.96$). Given the strong LD, genetic associations with either of the SNPs most likely represent the same signal. This supports the hypothesis that common variants in *TMPRSS6* may affect systemic iron homeostasis through mechanisms that include altered expression and/or activity of matriptase-2. Replication of our data in larger samples and further direct functional studies on the effect of *TMPRSS6* rs855791 on the signaling cascade involved in hepcidin regulation are necessary to confirm these findings. Nevertheless, it is reasonable to expect that an alteration in signaling efficiency could influence both iron mobilization from monocytes/macrophages and iron absorption, which could thus increase susceptibility to imbalances of iron homeostasis.

For all genetic associations identified and replicated in our study, we observed a high consistency of direction and magnitude of the effect across all study populations, which include both semi-isolated and outbred populations from Italy and the USA, supporting common regulatory mechanisms for iron status. However, this and previous studies show that the

Table 4. Association results of *TMPRSS6* rs4820268 with iron markers, by genotype group

Trait	AG versus AA				GG versus AA			
	MICROS	InCHIANTI	BLSA	M-A	MICROS	InCHIANTI	BLSA	M-A
Iron, μg/dl	−2.84 (2.88) [0.323]	−1.61 (1.73) [0.352]	−6.95 (2.65) [0.009]	−3.13 (1.29) [0.016]	−13.67 (3.33) [4.3×10^{-5}]	−6.32 (2.08) [0.002]	−15.10 (3.38) [<0.001]	−9.82 (1.56) [3.4×10^{-10}]
Transferrin, mg/dl	0.92 (2.85) [0.747]	NA	13.16 (8.35) [0.116]	2.20 (2.70) [0.415]	7.24 (3.29) [0.028]	NA	14.01 (10.62) [0.1882]	7.83 (3.14) [0.013]
(log)Ferritin, ng/ml	0.01 (0.06) [0.943]	−0.01 (0.03) [0.717]	−0.15 (0.08) [0.056]	−0.02 (0.03) [0.419]	−0.17 (0.07) [0.019]	0.02 (0.03) [0.476]	−0.16 (0.10) [0.115]	−0.03 (0.03) [0.277]
sTfR, mg/L	0.04 (0.03) [0.238]	0.01 (0.03) [0.734]	NA	0.03 (0.02) [0.239]	0.17 (0.04) [1.4×10^{-6}]	0.05 (0.04) [0.170]	ND	0.11 (0.03) [1.0×10^{-4}]
(log)sTfR−ferritin index	0.01 (0.01) [0.520]	0.01 (0.01) [0.714]	NA	0.01 (0.01) [0.157]	0.07 (0.02) [9.0×10^{-6}]	0.01 (0.02) [0.566]	ND	0.04 (0.01) [0.005]
Hemoglobin, g/dl	−0.07 (0.07) [0.350]	−0.14 (0.08) [0.083]	−0.08 (0.14) [0.557]	−0.10 (0.05) [0.047]	−0.11 (0.09) [0.184]	−0.21 (0.10) [0.027]	−0.23 (0.17) [0.181]	−0.17 (0.06) [0.008]
MCV, fl	−0.90 (0.33) [0.006]	−0.37 (0.32) [0.240]	−0.41 (0.38) [0.280]	−0.57 (0.20) [0.004]	−1.57 (0.38) [4.1×10^{-5}]	−0.90 (0.38) [0.019]	−1.17 (0.49) [0.016]	−1.22 (0.24) [2.2×10^{-7}]
MCH, pg	−0.31 (0.13) [0.017]	−0.17 (0.13) [0.186]	−0.08 (0.23) [0.747]	−0.22 (0.09) [0.011]	−0.65 (0.15) [2.0×10^{-5}]	−0.51 (0.15) [0.001]	−0.74 (0.31) [0.015]	−0.60 (0.10) [2.7×10^{-9}]

All models are adjusted for sex and age (+ village in MICROS). Reported are effect (SE) (*P*-value); (reference genotype = AA). M-A, meta-analyses; ND, not determined; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

variance of iron-related traits captured by these common variants is relatively low in the general population (16,18), suggesting the presence of additional, not yet identified genetic loci contributing to the regulation of body iron homeostasis. Further research with a large number of populations and thus higher power should reveal more genetic variants with modest effects and/or allele frequencies. Finally, it is important to note that the newly identified common variant in the *TFR2* gene associated with iron levels in the general population spaces *TFR2* alongside *TMPRSS6* and *HFE,* in which the common variants that associate with modest effects on iron markers have also been shown to coexist with known mutations associated with Mendelian diseases of iron metabolism.

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 (μg/dl), transferrin (mg/dl), ferritin (ng/ml), sTfR (mg/l) and the sTfR–ferritin index, defined as the ratio between sTfR and $\log_{10}$(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 ~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 μg 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 *TBP* (4326317E) and *GAPDH* (4326322E) genes as reference genes and evaluated using the Pfaffl modification of the ΔΔCt 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/NCBI2R/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 (λ) across all imputed SNPs prior to the meta-analysis.

SNPs to be tested in the replication studies were selected from those with $P < 10^{-5}$, or those with $P < 10^{-4}$ if two or more of them adjacent (see Supplementary Material), based on their power to replicate (power ≥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.

ACKNOWLEDGEMENTS

MICROS: We are grateful to the study participants for their participation and collaboration in this research project. We thank Christian Fuchsberger, Clemens Egger, Yuri D'Elia, Fabiola Del Greco and Veronika Lang for technical support.

SardiNIA: We thank all volunteers who participated in this study, Monsignore Piseddu, the Bishop of Ogliastra, the mayors and citizens of the Sardinian towns (Lanusei, Ilbono, Arzana and Elini), the head of the Public Health Unit ASL4 for their participation and cooperation, the team of physicians, nurses and the recruitment personnel and Professor Antonio Cao for his help and advice.

InCHIANTI and BLSA: We thank all volunteer study participants.

Conflict of Interest statement. None declared.

FUNDING

The work was supported by the Ministry of Health and Department of Educational Assistance, University and Research of the Autonomous Province of Bolzano; the South Tyrolean Sparkasse Foundation for the MICROS study; the National Institute on Aging [NO1-AG-1-2109 to the SardiNIA ('ProgeNIA') team] for the SardiNia study; the Italian Ministry of Health (ICS110.1/RF97.71); the US National Institute on Aging (263 MD 9164, 263 MD 821336) for the InCHIANTI study; the Intramural Research Program of the National Institute on Aging (Z01AG000015-50); and an R&D contract with MedStar Research Institute for the BLSA study.

REFERENCES

1. Hentze, M.W., Muckenthaler, M.U. and Andrews, N.C. (2004) Balancing acts: molecular control of mammalian iron metabolism. *Cell*, **117**, 285–297.
2. De Domenico, I., McVey Ward, D. and Kaplan, J. (2008) Regulation of iron acquisition and storage: consequences for iron-linked disorders. *Nat. Rev. Mol. Cell. Biol.*, **9**, 72–81.
3. Andrews, N.C. and Schmidt, P.J. (2007) Iron homeostasis. *Annu. Rev. Physiol.*, **69**, 69–85.
4. Jiang, R., Manson, J.E., Meigs, J.B., Ma, J., Rifai, N. and Hu, F.B. (2004) Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. *JAMA*, **291**, 711–717.
5. Theurl, I., Aigner, E., Theurl, M., Nairz, M., Seifert, M., Schroll, A., Sonnweber, T., Eberwein, L., Witcher, D.R., Murphy, A.T. *et al.* (2009) Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. *Blood*, **113**, 5277–5286.
6. Altamura, S. and Muckenthaler, M.U. (2009) Iron toxicity in diseases of aging: Alzheimer's disease, Parkinson's disease and atherosclerosis. *J. Alzheimers Dis.*, **16**, 879–895.
7. Silverberg, D.S., Wexler, D., Iaina, A. and Schwartz, D. (2008) The role of correction of anaemia in patients with congestive heart failure: a short review. *Eur. J. Heart Fail.*, **10**, 819–823.
8. Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T. and Kaplan, J. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*, **306**, 2090–2093.
9. Muckenthaler, M.U. (2008) Fine tuning of hepcidin expression by positive and negative regulators. *Cell Metab.*, **8**, 1–3.
10. Njajou, O.T., Alizadeh, B.Z., Aulchenko, Y., Zillikens, M.C., Pols, H.A., Oostra, B.A., Swinkels, D.W. and van Duijn, C.M. (2006) Heritability of serum iron, ferritin and transferrin saturation in a genetically isolated population, the Erasmus Rucphen Family (ERF) Study. *Hum. Hered.*, **61**, 222–228.
11. Whitfield, J.B., Cullen, L.M., Jazwinska, E.C., Powell, L.W., Heath, A.C., Zhu, G., Duffy, D.L. and Martin, N.G. (2000) Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am. J. Hum. Genet.*, **66**, 1246–1258.
12. Pilia, G., Chen, W.M., Scuteri, A., Orrú, M., Albai, G., Dei, M., Lai, S., Usala, G., Lai, M., Loi, P. *et al.* (2006) Heritability of cardiovascular and personality traits in 6,148 Sardinians. *PLoS Genet.*, **2**, e132.
13. Marroni, F., Grazio, D., Pattaro, C., Devoto, M. and Pramstaller, P. (2008) Estimates of genetic and environmental contribution to 43 quantitative traits support sharing of a homogeneous environment in an isolated population from South Tyrol, Italy. *Hum. Hered.*, **65**, 175–182.
14. Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R. Jr, Ellis, M.C., Fullan, A. *et al.* (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat. Genet.*, **13**, 399–408.
15. Benyamin, B., McRae, A.F., Zhu, G., Gordon, S., Henders, A.K., Palotie, A., Peltonen, L., Martin, N.G., Montgomery, G.W., Whitfield, J.B. and Visscher, P.M. (2009) Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. *Am. J. Hum. Genet.*, **84**, 60–65.
16. Benyamin, B., Ferreira, M.A., Willemsen, G., Gordon, S., Middelberg, R.P., McEvoy, B.P., Hottenga, J.J., Henders, A.K., Campbell, M.J., Wallace, L. *et al.* (2009) Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nat. Genet.*, **41**, 1173–1175.
17. Du, X., She, E., Gelbart, T., Truksa, J., Lee, P., Xia, Y., Khovananth, K., Mudd, S., Mann, N., Moresco, E.M. *et al.* (2008) The serine protease TMPRSS6 is required to sense iron deficiency. *Science*, **320**, 1099–1092.
18. Tanaka, T., Roy, C.N., Yao, W., Gordon, S., Middelberg, R.P., McEvoy, B.P., Hottenga, J.J., Henders, A.K., Campbell, M.J., Wallace, L. *et al.* (2010) A genome-wide association analysis of serum iron concentrations. *Blood*, **115**, 94–96.
19. Chambers, J.C., Zhang, W., Li, Y., Sehmi, J., Wass, M.N., Zabaneh, D., Hoggart, C., Bayele, H., McCarthy, M.I., Peltonen, L. *et al.* (2009) Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. *Nat. Genet.*, **41**, 1170–1172.
20. Ganesh, S.K., Zakai, N.A., van Rooij, F.J., Soranzo, N., Smith, A.V., Nalls, M.A., Chen, M.H., Kottgen, A., Glazer, N.L., Dehghan, A. *et al.*

(2009) Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. *Nat. Genet.*, **41**, 1191–1198.
21. Soranzo, N., Spector, T.D., Mangino, M., Kühnel, B., Rendon, A., Teumer, A., Willenborg, C., Wright, B., Chen, L., Li, M. *et al.* (2009) A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. *Nat. Genet.*, **41**, 1182–1190.
22. Zhong, H., Beaulaurier, J., Lum, P.Y., Molony, C., Yang, X., Macneil, D.J., Weingarth, D.T., Zhang, B., Greenawalt, D. and Dobrin, R. (2010) Liver and adipose expression associated SNPs are enriched for association to type 2 diabetes. *PLoS Genet.*, **6**, 1000932.
23. Kawabata, H., Yang, R., Hirama, T., Vuong, P.T., Kawano, S., Gombart, A.F. and Koeffler, H.P. (1999) Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *J. Biol. Chem.*, **274**, 20826–20832.
24. Ikuta, K., Yersin, A., Ikai, A., Aisen, P. and Kohgo, Y. (2010) Characterization of the interaction between diferric transferrin and transferrin receptor 2 by functional assays and atomic force microscopy. *J. Mol. Biol.*, **397**, 375–384.
25. Hentze, M.W., Muckenthaler, M.U., Galy, B. and Camaschella, C. (2010) Two to tango: regulation of Mammalian iron metabolism. *Cell*, **142**, 24–38.
26. Wallace, D.F., Summerville, L., Lusby, P.E. and Subramaniam, V.N. (2005) First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin. *Gut*, **54**, 980–986.
27. Weiss, G. (2010) Genetic mechanisms and modifying factors in hereditary hemochromatosis. *Nat. Rev. Gastroenterol. Hepatol.*, **7**, 50–58.
28. Camaschella, C., Roetto, A., Cali, A., De Gobbi, M., Garozzo, G., Carella, M., Majorano, N., Totaro, A. and Gasparini, P. (2000) The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nat. Genet.*, **25**, 14–15.
29. Fleming, R.E., Migas, M.C., Holden, C.C., Waheed, A., Britton, R.S., Tomatsu, S., Bacon, B.R. and Sly, W.S. (2000) Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. *Proc. Natl Acad. Sci. USA*, **97**, 2214–2219.
30. Waalen, J., Felitti, V.J., Gelbart, T. and Beutler, E. (2008) Screening for hemochromatosis by measuring ferritin levels: a more effective approach. *Blood*, **111**, 3373–3376.
31. Davies, P.S. and Enns, C.A. (2004) Expression of the hereditary hemochromatosis protein HFE increases ferritin levels by inhibiting iron export in HT29 cells. *J. Biol. Chem.*, **279**, 25085–25092.
32. Rosmorduc, O., Poupon, R., Nion, I., Wendum, D., Feder, J., Bériziat, G. and Hermelin, B. (2000) Differential HFE allele expression in hemochromatosis heterozygotes. *Gastroenterology*, **119**, 1075–1086.
33. Ramsay, A.J., Hooper, J.D., Folgueras, A.R., Velasco, G. and López-Otín, C. (2009) Matriptase-2 (TMPRSS6): a proteolytic regulator of iron homeostasis. *Haematologica*, **94**, 840–849.
34. Finberg, K.E., Heeney, M.M., Campagna, D.R., Aydinok, Y., Pearson, H.A., Hartman, K.R., Mayo, M.M., Samuel, S.M., Strouse, J.J., Markianos, K. *et al.* (2008) Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nat. Genet.*, **40**, 569–571.
35. Melis, M.A., Cau, M., Congiu, R., Sole, G., Barella, S., Cao, A., Westerman, M., Cazzola, M. and Galanello, R. (2008) A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production in familial iron deficiency anemia refractory to oral iron. *Haematologica*, **93**, 1473–1479.
36. Folgueras, A.R., de Lara, F.M., Pendás, A.M., Garabaya, C., Rodríguez, F., Astudillo, A., Bernal, T., Cabanillas, R., López-Otín, C. and Velasco, G. (2008) Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. *Blood*, **112**, 2539–1545.
37. Silvestri, L., Pagani, A., Nai, A., De Domenico, I., Kaplan, J. and Camaschella, C. (2008) The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell Metab.*, **8**, 502–511.
38. Ganz, T., Olbina, G., Girelli, D., Nemeth, E. and Westerman, M. (2008) Immunoassay for human serum hepcidin. *Blood*, **112**, 4292–4297.
39. Swinkels, D.W., Girelli, D., Laarakkers, C., Kroot, J., Campostrini, N., Kemna, E.H. and Tjalsma, H. (2008) Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PloS ONE*, **3**, e2706.
40. Serre, D., Gurd, S., Ge, B., Sladek, R., Sinnett, D., Harmsen, E., Bibikova, M., Chudin, E., Barker, D.L., Dickinson, T. *et al.* (2008) Differential allelic expression in the human genome: a robust approach to identify genetic and epigenetic *cis*-acting mechanisms regulating gene expression. *PLoS Genet.*, **4**, e1000006.
41. Pattaro, C., Marroni, F., Riegler, A., Mascalzoni, D., Pichler, I., Volpato, C.B., Dal Cero, U., De Grandi, A., Egger, C., Eisendle, A. *et al.* (2007) The genetic study of three population microisolates in South Tyrol (MICROS): study design and epidemiological perspectives. *BMC Med. Genet.*, **5**, 8–29.
42. Arnaud-Lopez, L., Usala, G., Ceresini, G., Mitchell, B.D., Pilia, M.G., Piras, M.G., Sestu, N., Maschio, A., Busonero, F., Albai, G. *et al.* (2008) Phosphodiesterase 8B gene variants are associated with serum TSH levels and thyroid function. *Am. J. Hum. Genet.*, **82**, 1270–1280.
43. Ferrucci, L., Bandinelli, S., Benvenuti, E., Di Iorio, A., Macchi, C., Harris, T.B. and Guralnik, J.M. (2000) Subsystems contributing to the decline in ability to walk: bridging the gap between epidemiology and geriatric practice in the InCHIANTI study. *J. Am. Geriatr. Soc.*, **48**, 1618–1625.
44. Shock, N.W., Greulich, R.C., Andres, R., Arenberg, D., Costa, P.T. Jr., Lakatta, E.G. and Tobin, J.D. (1984) *Normal Human Aging: The Baltimore Longitudinal Study of Aging*. US Government Printing Office, Washington DC, NIH Publication No. 84-2450.
45. Kupka, R., Msamanga, G.I., Mugusi, F., Petraro, P., Hunter, D.J. and Fawzi, W.W. (2007) Iron status is an important cause of anemia in HIV-infected Tanzanian women but is not related to accelerated HIV disease progression. *J. Nutr.*, **137**, 2317–2323.
46. Li, Y., Willer, C., Sanna, S. and Abecasis, G. (2009) Genotype imputation. *Annu. Rev. Genomics Hum. Genet.*, **10**, 387–406.
47. Ferrucci, L., Semba, R.D., Guralnik, J.M., Ershler, W.B., Bandinelli, S., Patel, K.V., Sun, K., Woodman, R.C., Andrews, N.C., Cotter, R.J. *et al.* (2010) Proinflammatory state, hepcidin, and anemia in older persons. *Blood*, **115**, 3810–3816.