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XXVII CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

INDIRIZZO GENETICO MOLECOLARE

TESI DI DOTTORATO DI RICERCA

IDENTIFICATION OF NOVEL LOCI AFFECTING HUMAN DISORDERS OF IRON HOMEOSTASIS AND THEIR EFFECT ON LIPID METABOLISM

Settore scientifico-disciplinare: MED/03

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Chapter 1

General introduction

Iron plays a key role in cellular processes of mammals

Iron is an essential element for metabolism of mammals acting as cofactor for several redox reaction. Body iron is imported in ferrous (Fe^{+2}) and ferric (Fe^{+3}) status and its ability to change ionic form can cause the production of oxygen free radicals[1]. Therefore iron is reactive and potentially toxic for cells and tissues and it can damage various cellular components. It is necessary a tight regulation of iron absorption, transport, storage and adequate distribution in bloodstream to maintain iron homeostasis of living organisms.

In plasma, ferric iron (Fe^{+3}) binds two high-affinity binding sites on the glycoprotein transferrin that maintains iron in a soluble form, limits the generation of free radicals and transports iron into the cells. Levels of transferrin saturation indicate the levels of plasma iron: in normal status transferrin saturation level measures about 30%, in iron deficiency transferrin saturation is below 16% and in iron overload transferrin exceed 45% of saturation till 60% that provokes the toxic accumulation of iron in bloodstream[2].

At physiological levels circulating iron is regulated by signals from several pathways that use iron and from cells that supply iron[2] so any disorder that affects the components of the tightly regulated pathway of iron distribution and storage could cause severe pathologies in humans.

How much iron do humans need?

Adult humans contain about 3-4 g of iron on average, most of which is bound to heme in haemoglobin. The remaining iron is stored in macrophages and hepatocytes in spleen and liver bound to the cytoplasmic protein ferritin and in muscle as myoglobin, an oxygen storage protein. Moreover iron is fundamental for energy production and synthetic metabolism of all the cells that contain iron in small concentrations[3].

The most relevant source of iron in human body is duodenal enterocytes but only a small proportion of total iron is released into bloodstream from duodenal enterocytes. Every day, 15-25 mg of iron in bloodstream come from aged erythrocytes that are recycled by macrophages in the spleen. Iron from enterocytes and macrophages is bound to the glycoprotein transferrin and reaches all the tissues where it is required in response to tissue oxygenation. The majority will be utilized for haemoglobin synthesis.

These observations and the lack of a regulated excretion of iron from organism suggest that the mechanism regulating the total amount of body iron requires a tight control of dietary iron absorption and of iron recycling from macrophages. Indeed, several studies focused on the molecules and biological processes involved in iron homeostasis and many have been identified.

Iron naturally occurs in low soluble oxidised form. Human diet is rich of ferritin, ferric iron and heme. Heme iron from meat and fish is efficiently absorbed but their consumption is evolutionary recent and geographically limited. Human populations had a vegetarian diet throughout most of their evolution and the efficient conservation and internal recycling of iron highlights how

mammalian evolution occurred in iron poor environment. Today, human populations from developed countries have access to iron rich food and most of them have to be able to limit iron uptake to avoid excessive toxic iron accumulation[3].

The transport of inorganic iron has been studied in details. Duodenal enterocytes absorbe 1-2 mg of inorganic dietary iron per day via a divalent metal transporter 1 (DMT1/SLC11A2 solute carrier family 11, member 2) expressed on the brush-border membrane as shown in Figure 1[2]. This mechanism is important to balance the iron physiological loss due to the desquamation of epithelial surfaces, sloughing of intestinal epithelial cells, urinary cells, blood loss and sweat[2,3].

Oxidized ferric iron is reduced by a membrane reductase DcytB (Duodenal cytochrome B) or Cybrd1. Heme iron is independently absorbed likely through HCP1, Heme carrier protein 1, but its mechanism of transport remains uncertain. The hemoxygenase 1 (HOX1) releases heme iron into the enterocytes.

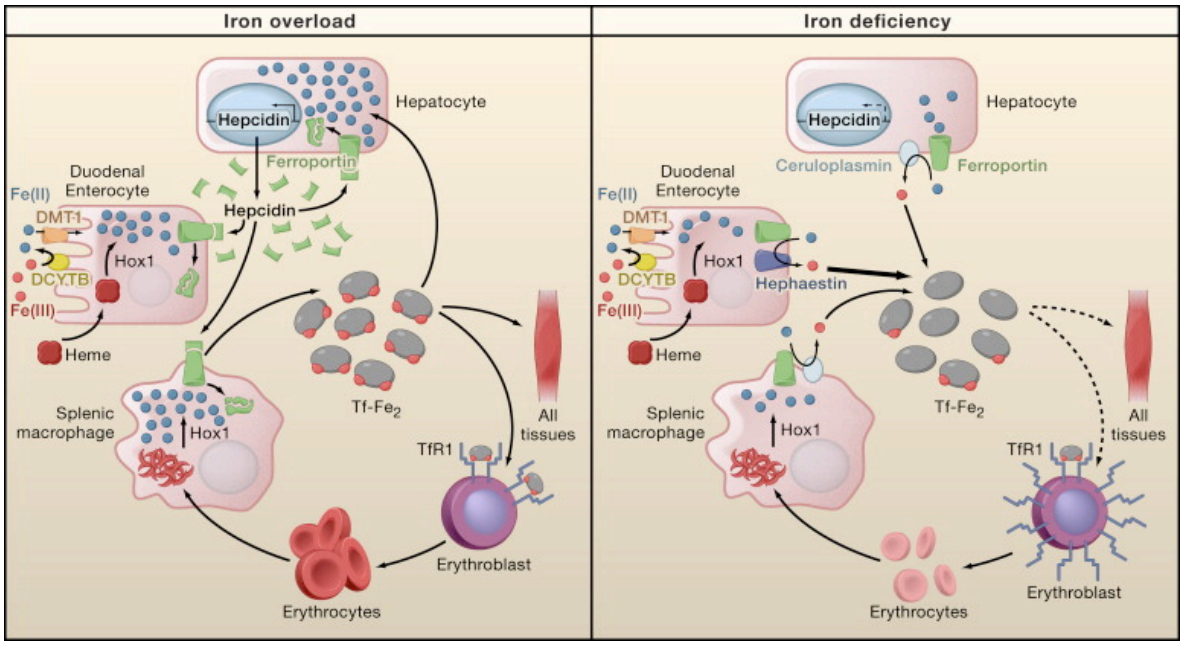


Figure 1. Regulation of systemic iron homeostasis. Divalent metal transporter 1 (DMT1) at the apical membrane of enterocytes takes up iron from the lumen of the duodenum after DCYTB reduces Fe^{+3} to Fe^{+2}. Ferroportin at the basolateral membrane cooperates with hephaestin that oxidizes Fe^{+2} to Fe^{+3}. Iron-loaded (diferric) transferrin (Tf-Fe2), indicated by red dots, supplies iron to all cells by binding to the transferrin receptor 1 (TfR1) and subsequent endocytosis. TfR1 is highly expressed on hemoglobin-synthesizing erythroblasts. Hepatocytes sense transferrin saturation/iron stores and release hepcidin accordingly. Red cell iron is recycled by macrophages via ferroportin and the ferroxidase ceruloplasmin. In iron overload (left), high hepcidin levels inhibit ferroportin-mediated iron export by triggering internalization and degradation of the complex to reduce transferrin saturation. Hepcidin expression is high. In iron deficiency (right), iron is released by ferroportin into the circulation. Hemoglobin-derived heme is catabolized in macrophages by hemoxygenase-1 (HOX1). Hepcidin expression is low. doi 10.1016/j.cell.2010.06.028

How does the body store and distribute the iron?

Hepatocytes represent the main storage site of iron in mammals where iron is bound to ferritin. Ferritin is composed of 24 chains of heavy (H) and light (L) type. The H subunits act as

ferroxidases to facilitate the conversion of cytosolic Fe^{+2} to the oxidized form for storage[3] and, according to body needs, hepatocytes release stored iron into the circulation. A soluble form of ferritin is present in blood plasma. This form is a 24-subunit polymer containing a low quantity of iron and mostly L-ferritin. Serum concentrations of ferritin are a clinically useful indication of iron storage status.

As shown in Figure 1 cytosolic iron can be exported from hepatocytes, macrophages and enterocytes by the basolateral iron exporter ferroportin SLC40A1 (solute carrier family 40, member 1 or IREG1 iron-regulated gene 1 or MTP1 metal transporter protein 1) expressed on the surface of iron-releasing cells[2]. To be loaded onto the 75-80-kDa iron carrier transferrin (TF), iron needs to be converted to Fe^{+3} by the oxygen-dependent ferroxidases hephaestin and ceruloplasmin. Hephaestin (HEPH) is a 130-kDa transmembrane protein expressed predominantly in enterocytes and the placenta, ceruloplasmin (CP) is a 130-kDa copper-containing protein highly expressed in the liver and the retina. Both are expressed in brain[3].

Transferrin is a glycoprotein that binds two ferric ions and delivers them to target tissues for uptake by transferrin receptor-1 (TFR1). TFR1 is composed by two identical monomeric subunits that link two transferrin molecules in a complex. It is highly expressed in erythroid precursors to promote the haemoglobin synthesis. Several years ago its homologous was cloned and mapped. TfR2 is a transmembrane gylocoprotein encoded by the gene TFR2 comprising 18 exons located on chromosome 7q22 in close proximity to the erythropoietin gene (EPO) and interacts with transferrin with lower affinity than TfR1[4]. The homeostatic system must maintain transferrin saturation at physiological levels, responding to signals from pathways that consume iron (such as erythropoiesis) and sending signals to the cells that supply iron to the bloodstream[2].

The erythropoiesis uses about 25 mg per day of recycled iron through the acquisition of iron by TfR1 that induces the maturation of erythroid precursors. The lack of TfR1 in mouse embryos provokes death due to severe anemia and the dysfunction of the other components like DMT1 in humans and mice cause similar phenotypes and liver iron accumulation[2].

The production of heme requires that iron is imported in the mithocondria through the membrane protein mitoferrin 1 (Mfrn1/SLC25A37, colute carrier family 25, member 37)[5]. The erythroid-specific enzyme for the heme precursor protoporphyrin IX, ALAS2 (gamma-aminolevulinic acid synthase 2) must coordinate the synthesis with the available iron and is regulated by IRE/IRP system (iron–responsive element / iron regulatory protein). The cytoplasmic Iron Regulatory Proteins (IRPs) interact with the sequences of nucleotides called Iron esponsive elements (IREs), about 30 nucleotides located on 5' or 3' of mRNA of iron regulatory genes as ferritin and TfR[6]: in iron deficiency condition IRP-IRE downregulates ferritin transcription and upregulates TfR1 transcription, viceversa in iron overload. Based on iron condition several mRNA as DMT1, transferrin or ALAS2 are simultaneously regulated.

These observations on iron homeostasis have led to the expectation that one or more systemically

acting hormones regulate the major flows of iron, the absorption by intestine, its utilization in erythropoiesis, the recycle of erythrocytes and the storage by hepatocytes and are in turn regulated by iron[3]. Accordingly in 2001 it was shown that iron export by ferroportin is strictly regulated by the liver hormone hepcidin (HAMP) that binds ferroportin and according to iron body needs induces its endocytosis and proteolysis in lysosomes in order to reduce the release of iron into circulation[7].

The role of hepcidin in iron metabolism has been suggested by the stimulation of hepcidin synthesis in iron-rich diets and observed by chance in a knockout mouse for the gene *usf2* next to *hamp*[8]. Knockout mouse showed a hereditary affection of iron homeostasis known as hemochromatosis.

In 2001 in a family study[9], subjects affected by a juvenile condition of hemochromatosis showed homozygous deleterious mutations in HAMP, confirming its essential role.

Hepcidin is the main regulatory molecule of iron homeostasis through a mechanism of feedback by plasma iron concentration. It is encoded by the single three exons gene HAMP on chromosome 19q13. Its mature form is a 2.7-kDa (25 amino acid) peptide generated from a 84 amino acid prepropeptide by furin cleavage. It is secreted by hepatocytes and circulates in blood plasma mostly free except for weak binding to albumin and α2-macroglobulin and is filtered by the kidneys[5]. As shown in Figure 2 hepcidin forms a hairpin structure with four intramolecular disulfide bonds[10], the NH_2-terminal contains six amino acids highly conserved and essential for the iron-regulatory function of hepcidin because of the interaction with receptor ferroportin[11]. In human urine two different NH_2-terminally truncated shorter forms (22 and 20 amino acids) were found. In human plasma a 20-amino acid form is present at much lower concentrations than the full-length 25-amino acid hepcidin[5].

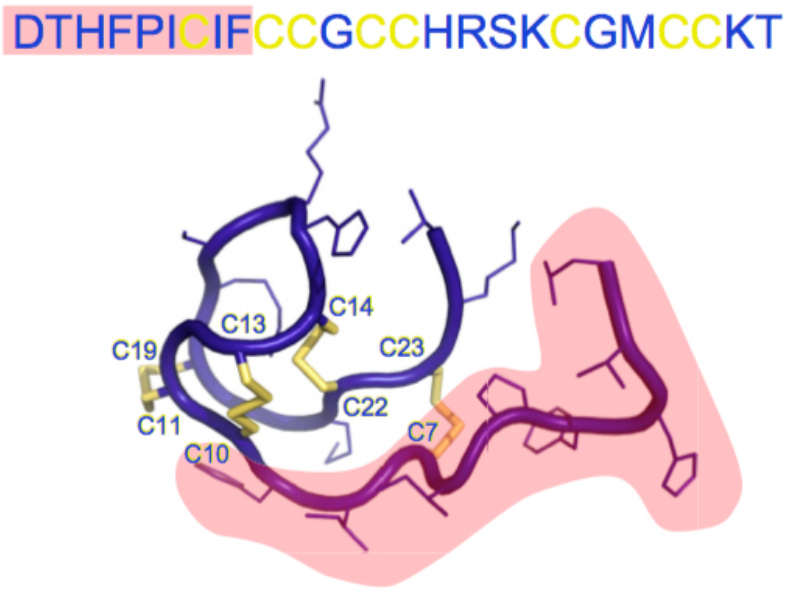


Figure 2. Hepcidin amino acid sequence and structure. The NH_2-terminal segment known to interact with ferroportin is shaded in light red. The characteristic cysteines and their disulfide bonds are shown in yellow. doi:10.1152/physrev.00008.2013

The study of molecular mechanisms underling hereditary hemochromatosis in humans and in mice has been fundamental to clarify hepcidin regulation processes in dysregulation of iron distribution conditions.

The interaction hepcidin-ferroportin effectively controls the flux of iron into plasma and the iron supply available to the iron-consuming tissues. Inherited and acquired disorders that perturb hepcidin production cause alteration of iron level. Overexpression of hepcidin causes iron deficiency anemia[12,13] both by inhibiting iron absorption and restricting the release of stored iron. On the contrary, hepcidin deficiency in humans causes iron overload in parenchymal organs including the liver, pancreas, and the heart, coupled with the paradoxical loss of macrophage iron stores[9,14,15]. The consequences of excess or deficiency of hepcidin highlight its fundamental role in the control of iron absorption and in the release of recycled iron from macrophages. In addition, heterozygous human ferroportin mutations interfere with hepcidin binding[16,17], confirming the critical role of the hepcidin-ferroportin interaction and suggesting that ferroportin may be the sole target of hepcidin[3].

Iron overload and iron deficiency disorders

Inherited disorders resulting from mutations of genes involved in regulating iron metabolism cause unbalanced iron levels and opposite clinical conditions: iron deficiency and iron accumulation in organs and tissues.

Low expressed hepcidin by hepatocytes or ferroportin resistance to the endocytic effect of hepcidin results in hereditary hemochromatosis (HH) that leads to iron overload of the liver and other organs causing cirrhosis and liver cancer, heart failure, diabetes and arthritis.

Hereditary hemochromatosis exists in four recessive and one dominant forms. A homozygous missense mutation of the HFE gene (C282Y) is the causative variant responsible for the most common recessive hereditary hemochromatosis[18]. At least 5-10% of Caucasians are heterozygous for this mutation that has a low penetrance and it is more common in affected older males. HFE (Human Hemochromatosis Protein) on chromosome 6 encodes a ubiquitously expressed major histocompatibility complex class 1-like molecule and the C282Y mutation affects β 2-microglobulin binding. The levels of hepcidin in affected cases are inadequately low for the degree of iron loading.

Other two forms of juvenile hereditary hemochromatosis are less common and characterized by undetectable levels of hepcidin. They are often associated to hypogonadism, refractory heart failure, and even premature death. They are caused by mutations of hemojuvelin (HJV) or of HAMP gene.

HJV is a glycophosphatidlyinositol-linked protein mostly expressed in liver, skeletal muscle, and heart. HJV is present in a membrane and soluble forms (mHJV and sHJV) and has been discovered as a bone morphogenetic protein (BMP) coreceptor[19].

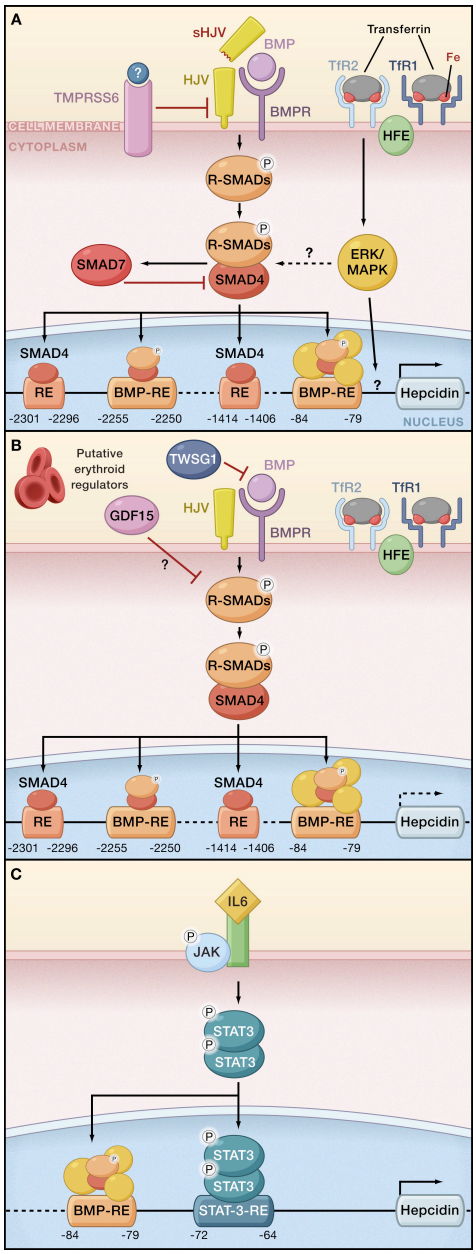


Figure 3. Regulation of Hepcidin Expression

(A) Hepcidin regulation by systemic iron availability. High concentrations of Tf-Fe2 displace HFE from TfR1 to promote its interaction with transferrin receptor 2 (TfR2). The HFE-TfR2 complex then activates hepcidin transcription via ERK/MAPK and bone morphogenetic protein (BMP)/SMAD signaling. The BMP coreceptor hemojuvelin (HJV) interacts with type I and type II BMP receptors (BMPR) at the plasma membrane to induce phosphorylation of receptor-activated SMAD (R-SMAD) proteins, and subsequent formation of active transcriptional complexes involving the co-SMAD factor SMAD4. This signaling is inhibited by soluble HJV (sHJV). TMPRSS6 physically interacts with HJV and causes HJV fragmentation. SMAD7 interferes with SMAD4-controlled hepcidin activation. Sequence motifs critical for SMAD- mediated control of the hepcidin promoter are shown. **(B)** Hepcidin regulation by erythropoietic signals. GDF15 and TWSG 1 are released by erythroid precursors to inhibit BMP/SMAD activation of hepcidin. This situation characterizes iron-loading anemias. **(C)** Hepcidin regulation by inflammatory stimuli. Interleukin 6 (IL6) activates the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) signaling pathway and stimulates the hepcidin promoter via a STAT-binding motif close to the transcription start site. The BMP signaling pathway also contributes to the inflammatory response via SMAD4. Doi 10.1016/j.cell.2010.06.028

As shown in figure 3A, BMPs belong to the Transforming Growth Factor β (TGFβ) family, soluble proteins that interact with specific receptors (BMPR) on cellular membrane. BMP6 binding to the cofactor HJV and to BMPR is involved in hepcidin transcription control via SMAD proteins signalling. Mutations in membrane HJV cause low hepcidin levels and iron overload suggesting an essential role in hepcidin regulation.

Hemochromatosis forms due to HAMP mutations are extremely rare.

The fourth hereditary hemochromatosis is due to TfR2 mutations but presents a less severe phenotype than the juvenile form[20]. As previously shown, TfR2 is a type II transmembrane protein that binds transferrin with lower affinity than TfR1.

A dominant form of hereditary hemochromatosis is caused by missense mutations in ferroportin and is called 'ferroportin disease'[21]. Mutations can reduce membrane localization or the ability of ferroportin to export iron. That causes macrophage iron retention, normal or low plasma iron levels, and in some cases iron-restricted erythropoiesis. The hepcidin-resistant ferroportin mutations cause high plasma iron and hepatocyte iron accumulation because hepcidin fails to bind ferroportin or its internalization and degradation.

Other imbalanced hepcidin levels are due to iron-loading anemias in which erythropoietic signals suppress hepcidin transcription in condition of high systemic iron load as reported for beta-thalassemia intermedia[3].

On the opposite in some condition the production and blood concentrations of hepcidin are inappropriately high or the membrane concentration or iron-transporting capacity of ferroportin is decreased. Anemia is caused by high hepcidin expression and low plasma iron levels due to lower iron release by macrophages and lower iron absorption. The most common pathologies are the acquired anemia of chronic diseases (ACD) and the genetic iron-refractory iron deficiency anemia (IRIDA).

Related to its evolutionary origin, hepcidin transcription is activated by inflammatory cytokines, especially interleukin 6 as described below and in Figure 3C. Excessive hepcidin production is also seen in patients with infections, malignancies, chronic kidney diseases, or any type of inflammation.

The genetic affection IRIDA is caused by mutations in a liver-expressed transmembrane serine protease of type II TMPRSS6 (matriptase-2), a gene that encodes a protease that negatively regulates hepcidin expression[22]**.** Genetic causative variant (rs855791) in TMPRSS6 is common in general population (about 45%) and may modulate the ability to absorb iron and to synthesize hemoglobin for maturing erythroid cells[23]. The structural features of matriptase-2 are highly conserved across mammalian species, including human, macaque monkey, dog, cow, mouse and rat. The extracellular domain contains multiple motifs and domains including bone morphogenetic

protein 1 (CUB) domain and a prodomain region that contains the cleavage site for protease fundamental for the activation of the signalling of HAMP transcription[24].

Pathway of up-regulation and down-regulation of hepcidin

After the discovery of the biological role of hepcidin, several progresses have highlighted the molecules and pathways that control hepcidin expression in response to concentration of iron and the role of the membrane proteins mutated in hereditary hemochromatosis (HFE, HJV, and TfR2) in this process.

Bone Morphogenetic Protein (BMP) receptor-hemojuvelin (HJV)-Son of Mothers Against Decapentaplegic (SMAD) pathway are the core of hepcidin regulation pathway as indicated by the presence of several functional BMP-response elements (BREs) in the hepcidin promoter. BMP6 is specific for BMP receptor (BMPR) and essential in mouse: knockout mice show low hepcidin levels and iron overload[3].

As previously indicated, HJV is an essential co-receptor of BMP receptor for the activation of iron signalling. HJV can bind BMP2 and BMP4[25,26] but the binding to BMP6 with BMPR can induce SMAD cascade. In humans and mice the absence of HJV leads to the same severe phenotype as the absence of BMP. But it is unclear how BMP6 mRNA expression is activated by increased iron levels and repressed by iron deficiency. The BMP/HJV complex joins the type I (Alk2 and Alk3) and the type II (ACTRIIA) BMP receptors to induce phosphorylation of receptor activated SMAD (R-SMAD) proteins and subsequent formation of active transcriptional complexes involving the co-SMAD factor, SMAD4[27] (Figure 3A).

Other extracellular sensors bind BMP pathway in an uncertain way.

On the plasma membrane of hepatocytes TfR1 and TfR2 act as sensors of the concentration of Tf-Fe_2: the hemachromatosis-related membrane protein HFE binds TfR1 at a site that overlaps the transferrin binding domain and it competes with the binding of Tf-Fe_2. TfR2 can bind HFE and Tf-Fe_2 simultaneously[28]. Mice with mutations that increase the binding of HFE to TfR1 show a similar phenotype of HFE-deficient mice: low hepcidin expression and iron overload. Mutations that abolish HFE-TfR1 interaction show high levels of hepcidin expression and iron deficiency.

These findings suggest that high concentration of Tf-Fe_2 remove HFE from TfR1 to promove the interaction with TfR2, which is further stabilized by increased Tf-Fe2 binding to the lower-affinity TfR2. Afterwards HFE-TfR2 complex activates hepcidin transcription.

As shown in Silvestri et al 2008[29], the transmembrane serine protease maptriptase-2 TMPRSS6 that causes the severe genetic disorders iron-refractory iron deficiency anemia (IRIDA) in mice and humans, has a strong effect on hepcidin suppression as shown by the *Mask* and *Tmprss6* deficient mouse model, which results from a deletion of the serine protease domain in inappropriately high levels of *Hamp* mRNA expression and are unable to absorb oral iron.

TMPRSS6 function *in vitro* as negative regulator of hecpdin-BMP signalling cleaving the BMP

agonist hemojuvelin switching off BMP signaling and thereby decreasing hepcidin transcription suggesting that HJV is the major TMPRSS6 target for iron regulation. Genetically, the combined deficiency of HJV and TMPRSS6 causes iron overload, suggesting that TMPRSS6 acts upstream of HJV.

It is unknown whether, *in vivo*, TMPRSS6 cleaves other substrates. In iron deficiency, the function of TMPRSS6 is essential to suppress hepcidin and to allow iron absorption. *In vitro*, the expression of TMPRSS6 is up-regulated by hypoxia and iron deficiency and its proteolytic activity is inhibited by hepatocyte growth factor activator inhibitor type-2 (HAI-2), an inhibitor of the homologous protease matriptase-1[30]. The regulation of TMPRSS6 *in vivo* is largely unknown.

As shown in Figure 4 the expression of hepcidin in hepatocytes is transcriptionally feedback-regulated also by hepatic iron storage, hypoxia, erythropoietic activity and inflammatory states.

Iron stored in liver acts as a regulator of hepcidin transcription in an uncertain way. It seems that the expression of BMP6 is regulated by iron storage. The ablation of BMP6 or hemojuvelin interferes partially with the hepcidin response to increased iron stores.

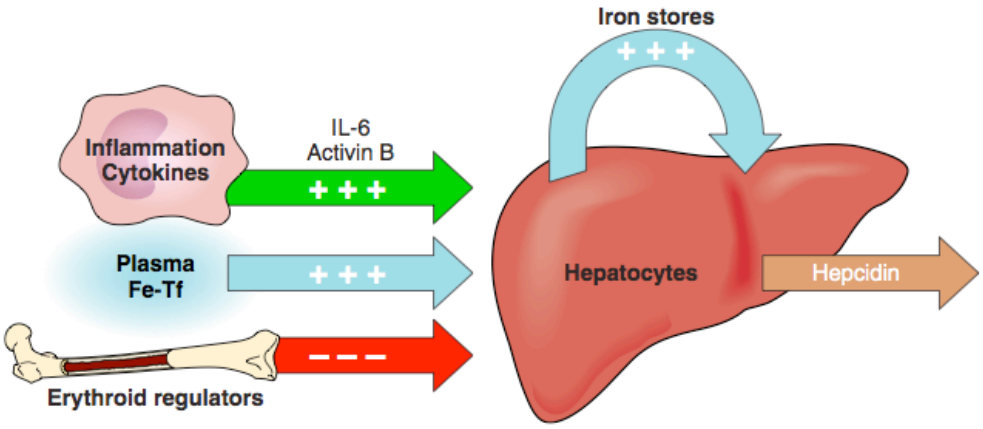


Figure 4. Regulation of hepcidin synthesis in hepatocytes. The major regulatory influences include iron-transferrin and iron stores (blue), inflammation (green), and erythroid activity (red). doi:10.1152/physrev.00008.2013

Hypoxia is a condition of significant lower oxygen concentration in cells and tissues that constitutes a stress for all mammals cells in different conditions: high altitudes, during prenatal development and in pathological conditions as cardiovascular diseases and cancer. Mammals cells have evolved mechanisms sensible to changes in oxygen concentration and adaptive responses to reach the homeostasis[31].

The adaptions include the increased oxygen-carrying capacity of blood flow to hypoxic organs and tissues thanks to the activation of genes and proteins responsible for erythropoiesis as erythropoietin EPO, iron transport as TF, TfR, CP, MFRN, ALAS2, ferritin genes and the switching from aerobic to anaerobic metabolic pathways.

The main mediators of the adaptions are the key regulatory proteins of iron metabolism hypoxia-inducible factor 1 HIF1 and HIF2 and Iron responsive protein IRP1 and IRP2 that act as oxygen

sensors.

HIFs are heterodimeric transcription factors composed of α and ß subunits with three isoforms each one. In normal condition HIF α subunit is hydroxylated and proteolytically degraded viceversa in oxygen deficiency it traslocates in the nucleus and with HIF ß bind to the hypoxic-responsive enhancer element (HRE) to activate the hypoxia-inducible gene expression.

Hepcidin is suppressed by both anemia and hypoxia and iron deprivation and hypoferric anemia lead to poor tissue oxygenation. The downregulation of hepcidin when HIF levels are elevated suggests that HIF may be one of the missing links between iron homeostasis and hepcidin regulation. The expression of HAMP is downregulated in hypoxia condition probably through the HIF1 and HIF2 however the physiologic relevance and the mechanisms of hepcidin regulation by hypoxia are still uncertain and conflicting.

The IRP1 and IRP2 react in different way to hypoxia. They are regulated by oxygen at a posttranscriptional level but the total amount of mRNA of IRP1 and IRP2 does not change in hypoxic condition. The IRP1 gene expression is HIF independent while the levels of IRP2 protein increase under hypoxic condition because they need sufficient iron to be degraded. Under hypoxia IRP2 is predominant and mainly binds to IREs and regulates HIF2 α .

In hypoxic condition the production of erythrocytes is enhanced. Erythropoiesis utilizes haemoglobin and relies on the heme synthesis. The increased heme synthesis requires greater iron availability to erythorid precursor cells. EPO production has been shown to increase 1000-fold in response to hypoxia because of the binding of HIF to a HRE found in *epo* gene.

EPO injection into mice reduces hepcidin levels in a dose-dependent manner and can override signals that activate hepcidin expression whereas in human injections decrease urinary excretion of hepcidin. These findings suggest that EPO likely suppresses hepcidin by stimulation of erythropoiesis rather than more directly.

The regulation of hepcidin occurs also by erythropoiesis that needs large quantities of iron. In case of haemorrhage or erythropoietin, the iron absorption by intestine greatly increases in response to the need of iron for accelerated erythropoiesis.

In response to erythropoietin, bone marrow produces a hepcidin suppressor that could act in similar way in anemias with ineffective erythropoiesis where hepcidin is decreased despite iron overload and even in the absence of transfusions[32,33,34,35]. In Figure 3B GDF15 (Growth differentiation factor 15) and TWSG1 (Twisted gastrulation protein homolog 1) released by erythroid precursors, are proposed as possible suppressor of BMP-dependent activation of hepcidin transcription. High levels of GDF15, especially present in thalassemia cases, can suppress hepcidin transcription but their physiologic or pathological role is uncertain.

TWSG1 expression is increased in thalassemic mice, where it is produced during early erythroblast maturation. In cellular models, the BMP-binding protein TWSG1 inhibits BMP-dependent

activation of Smad-mediated signal transduction that leads to hepcidin activation.
The dentification of the physiological and pathological erythroid regulators of hepcidin is crucial.

Finally, as shown in Figure 3C, hepatocyte synthesis of hepcidin is regulated also by inflammatory cytokines, especially interleukin 6 (IL6) through the STAT-3 signalling pathway.
Inflammation-induced hepcidin increase causes the hypoferremia that develops early during infections or inflammatory diseases likely to limit the multiplication of iron-dependent extracellular microbes. On the other side, the hypoferremia can limit the availability of iron for erythropoiesis and contribute to anemia of inflammation or anemia of chronic disease[36].

Genetic studies of human variation of hepcidin and iron parameters levels

As previously shown, variation in body iron level is associated with pathological processes and rare monogenic disorders. It is however likely that common and multiple genetic factors may also cause variation in iron homeostasis.
One of the aims of my work was to analyze the genetic and environmental factors that determine the quantitative levels of hepcidin and iron parameters in humans. Today human genome wide association studies (GWAS) highlighted the significance of matriptase-2 in regulation of iron homeostasis by identifying common TMPRSS6 variants associated with pathological hematological parameters, including hemoglobin (Hb), transferrin saturation (TfSat), erythrocyte mean cell volume (MCV) and serum iron concentrations[37,38]. Ganesh et al in 2009[39] identified genome-wide significant association of SNPs within the HFE gene associated to Hb, MCV, hematocrit (PCV) and mean corpuscular hemoglobin (MCH). Additionally, the mutation in HFE encoding the C282Y substitution was associated with increased MCV and Hb concentrations in a study of individuals drawn from a screening study for hemochromatosis and iron overload. Individuals heterozygous for either allele do not manifest clinical iron overload but may have an increased iron uptake and resistance to anemia, and the C282Y-encoding mutation may increase the risk of coronary heart disease by increasing iron stores and lipid oxidation. The transferrin receptor (TFR1) and transferrin receptor 2 (TFR2) SNPs were associated with MCH and MCV, and SNPs within TFR2 were associated with PCV and MCV.
The identification of novel loci that affect iron metabolism can clarify many clinical or subclinical conditions and offer novel potential targets for the diagnosis and treatment of iron overload and anemias.

A large population study: Val Borbera Project

The prediction of the risk for disease in healthy individuals requires a large sample size, detailed knowledge of the risk factors, their effect size and how they interact. The large genome wide association studies (GWAS) performed to date have provided initial information on the genetic

architecture of many diseases, but they have identified variants that individually explain a very small fraction of the genetic variance, to be used for an accurate prediction of the genetic risks. Isolated founder populations provide an attractive alternative for the study of complex traits as they typically exhibit greater genetic and environmental homogeneity and present enrichment in low frequency and rare variants respect to mixed outbred populations[40].

The origin of isolated populations from relatively recent common ancestors has increased linkage disequilibrium (LD) making possible to reconstruct extended and conserved haplotypes and long stretches of consecutive homozygous genotypes at adjacent single nucleotide polymorphisms (SNPs) loci called ROHs[41] as shown in Figure 5. Knowledge of the underlying genetic and population structure is essential to carefully design association studies, including choice of the most appropriate analysis approach that may depend from the degree of isolation, the length of the time the population has remained isolated and the size of the funding group.

Val Borbera Project started in 2005 with the aim to investigate the genetic factors responsible for human complex diseases. The population recruited is located in Val Borbera, a large valley in Northern-Western Italy isolated by the surrounding mountains and by a deep canyon.

In the past centuries the population that lived in the seven villages of the valley increased till 10,000 individuals but in the last century decreased due to the emigration the population and now includes about 3,000 individuals that live in the valley and in the surrounding areas.

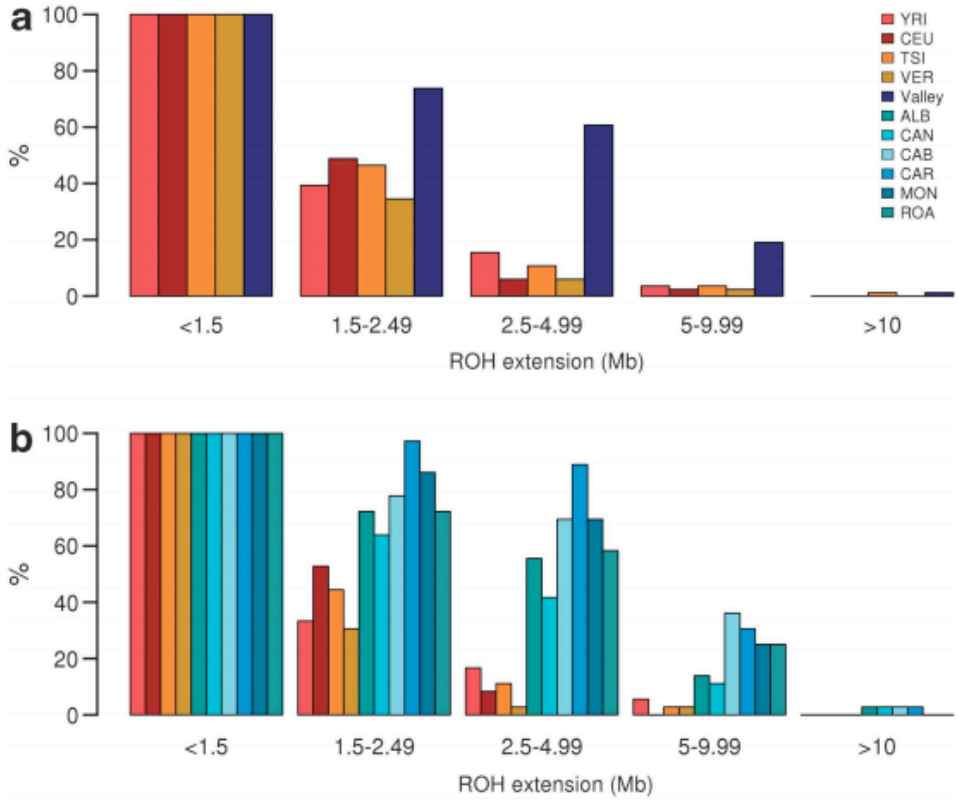


Figure 5. Statistics on the extension of the ROHs in the valley (a) and villages (b) with respect to other populations. (A) ROHs were binned according to length and per each population the percentage of individual having at least one ROH

of a given length is indicated on the y axis. As the length of the ROHs increases different trends are visible for the isolate and the reference populations. Villages behave similarly among them (B). doi: 10.1038/ejhg.2012.113

To reconstruct a complete genealogical pedigree from birth, marriage and death records extracted from city and parish church archives, a powerful algorithm has been implemented in our laboratory[42]. As we showed in 2009 in Traglia et al[43] most of the population (89.5%) was included in a large genealogical tree of about 50,000 people tracing back up to 16 generations. As shown in Figure 6 endogamy was 70% in the 17th century, indicating some immigration. It increased and reached 80% with peaks of 90% in 1800 and started to decrease in the middle of the 1900.

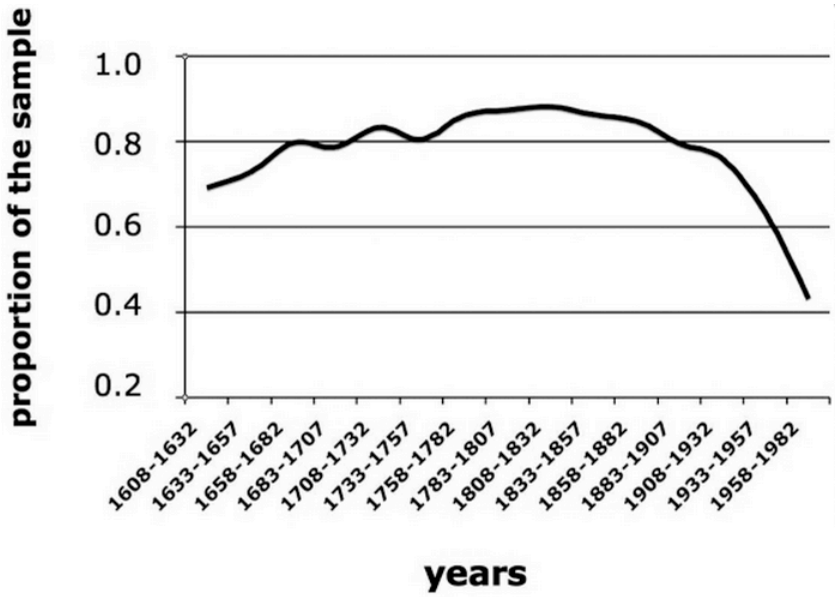


Figure 6. Percentage of endogamic marriages, over the centuries. 25 years periods were considered and are indicated along the X-axis. Marriages were counted from the marriage registers. doi: 10.1371/journal.pone.0007554

The healthy subjects enrolled in the study are 1,803 in 18-102 years range of age (Figure 7). This sample was enriched in females (56%) and in older people (mean age 55 years). There is an enrichment in older individuals: 34.4% are older than 65 years and 8.6% are older than 80 years.
About 50% of the participants were born in Val Borbera, 90% of the rest were born in the nearby area but 80% of the parents and 90% of the four grandparents of the participants were born in Val Borbera.
Thanks to the reconstructed full genealogy, the kinship coefficient (kc) of the living descendants of the original population has been calculated: the average kinship is 0.000373 with 3.5% of the entire population presenting a kinship=0. The average inbreeding in the population is 0.000746.

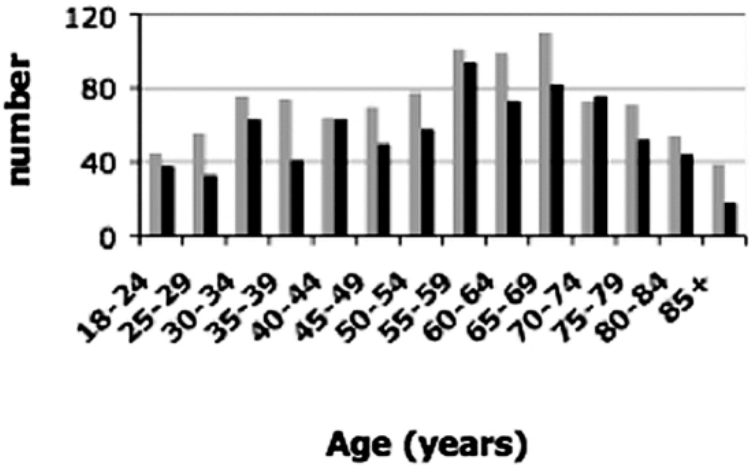


Figure 7. Age and sex distribution of the participants to the study. 5 years periods were considered. In black are the males, in grey are the females.

Genetic study of risk factor for iron-related diseases

Val Borbera study (VB) collected a large set of quantitative traits risk factor for iron-related disorders, obesity, cardiovascular diseases as iron homeostasis parameters, hematological and lipid metabolism traits: serum iron, transferrin, transferrin saturation, ferritin and hepcidin, hemoglobin (Hb), hematocrit (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red blood cell (RBC), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG). The genetic study of this set of quantitative traits is useful to predict the risk of disorders that affect iron stable status in healthy individuals and to understand the genetic architecture of diseases and biological processes of the onset of different clinical conditions.

To assess a set of Val Borbera genotypes and perform genome-wide association analysis (GWAS), most of individuals (n=1,664) has been genotyped with Illumina 370K Quad v3 Array and n=121 individuals with Illumina 700k Array, commercial chips enriched in variants that are common in the general population (Minor allele frequency - MAF >=5%).

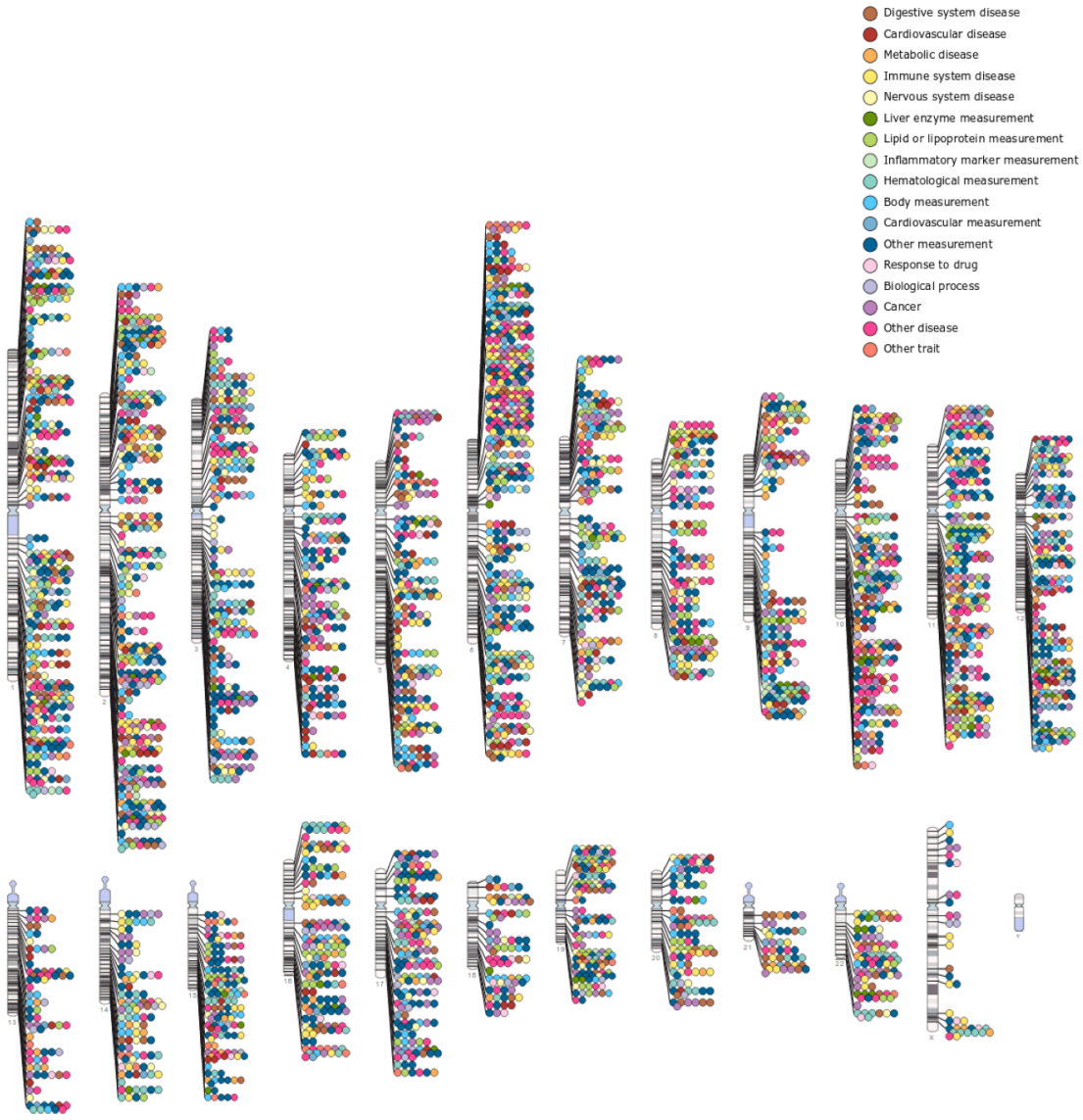
HapMap Project[44] is an international consortium born in 2002 with the aim to obtain the most complete set of common variants and haplotypes representative of the general population. Several groups collected 270 samples from four different Asian, African and European populations and calculacteed the linkage disequilibrium (LD) between the variants and their haplotypes. In each genomic region were extracted tag-SNPs representative of the total SNPs in each haplotypes. The results represent a useful genetic map of the human genome published in 2005-2009 and available in public database to be used as reference for genetic studies.

Thanks to this improvement most of published GWAS results showed common tag SNPs associated to complex traits and diseases as reported in GWA catalogue by National Human Genome Research Institute (NHGRI) (www.genome.gov/gwastudies/). To date GWAS studies

have examined more than 15 categories of common diseases and traits and discovered about 12,000 SNP-trait whole-genome associations as shown in Figure 8.

To improve the number of variants and their accuracy Val Borbera study firstly inferred the genotypes of the total sample of 1,785 individuals to 2.5M markers of HapMap2 Project release and to 3.2M markers of HapMap3 Project release[31] based on common haplotypes and allele frequencies.

A.



B.

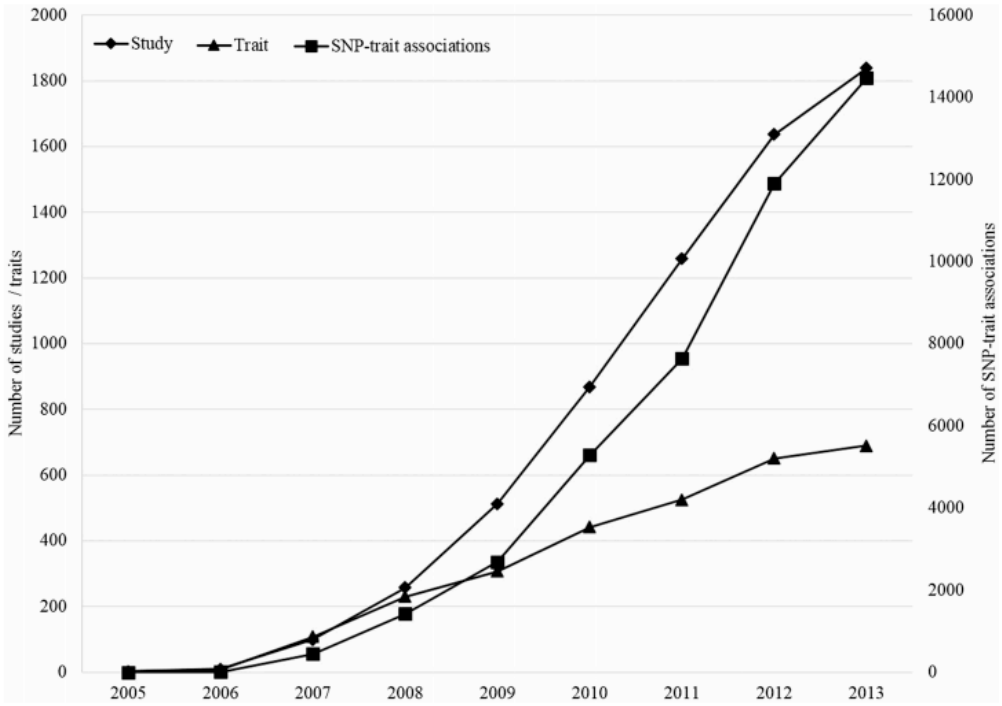


Figure 8. Genome-wide association study results for complex diseases and traits. (A) GWAS findings by categories reported in www.genome.gov/gwastudies/ and adapted from http://www.ebi.ac.uk/fgpt/gwas/. Last update Dec 2013. (B) Studies, traits and SNP-trait associations from 2005–2013 reveal the growth in eligible studies. doi:10.1093/nar/gkt1229

The associated common variants explained only a small fraction of phenotypic variability as expected by literature[45]. The low frequency (MAF 1-5%) and rare variants (MAF<1%) are known to have higher impact on the phenotypes and potentially explain a large percentage of the total variance but they are not included in the widespread genotyping arrays.

Thanks to the improvement of high throughput sequencing techniques and the concomitant fall in costs, several international projects had sequenced samples from different ethnic groups around the world to improve the knowledge about genetic variations in particular about low frequency variants. The first project that sequences a large number of people was 1000 Genomes Project[46] that analysed different ancestries individuals to provide a comprehensive resource on human genetic variants that have frequencies of at least 1% in the populations studied.

The project started in 2008 as a Pilot phase and the publication in 2010[47] of the results obtained from low-coverage whole-genome sequences of 179 individuals from four populations; high-coverage sequences of two mother–father–child trios; and exon-targeted sequencing of 697 individuals from seven populations. The final phase 3 data collect the low coverage (4x) sequences of 2,577 individuals: 523 Eastern Asians, 494 Southern Asians, 691 Africans, 514 Europeans and

355 Americans spread in 25 populations. The data are fully available for autosomes, chromosome X and Y in October 2014.

To focus on the rare variants effects most recently Val Borbera genotypes were inferred to about 38M of markers released by 1000 Genomes Project[46] and used in the current largest international meta-analyses for the genetic study of complex traits.

The powerful contribution of genetic isolates to human variation knowledge

Italian network of genetic isolates (INGI)

The Italian isolated populations joined together in a consortium, the Italian Network of Genetic Isolates (INGI) to enlarge the samples size and perform large genetic meta-analysis in order to better exploit the typical enrichment in rare variants and the homogeneity of isolated cohorts in Italy.

Population genetic studies on European populations have highlighted Italy as one of genetically most diverse regions, reflecting its demographic and geographic history[48]. Figure 9 showes the genetic diversity of Italian isolates respect to Europeans.

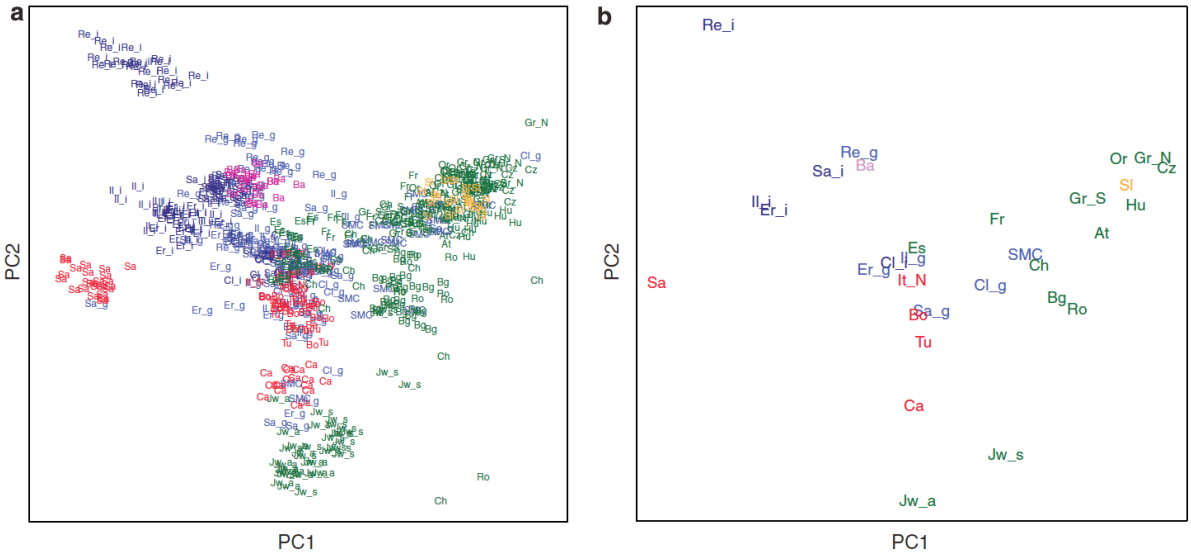


Figure 9. Principal component analysis of genetic diversity in Europeans. Label position indicates the (a) specific PC1 and PC2 coordinate values for each individual and (b) the mean PC1 and PC2 coordinate values for each population. For (a, b), the colors have a following meaning: (1) dark blue color: a homogeneous fraction of the FVG population; a blue color: more general fraction of the FVG population; a red color: other Italian samples; a violet color: Basques; an orange color: Slovenians; and green color: all other populations. For (a, b), the following population abbreviation labels are used: AT, Austrians; BA, French Basques; BG, Bulgarians; BO, Borbera; CA, Carlantino; CL, Clauzetto; CH, Swiss; CZ, Czechs; GR, Germans; ER, Erto; ES, Spaniards; FR, French; HU, Hungarians; IL, Illegio; IT, Italians; JW_A, Ashkenazy Jews; JW_S, Sephardic Jews; OR, Orcadians; RE, Resia; RO, Romanians; SA, Sardinians; SA_, Sauris; SMC, San Martino del Carso; SI, Slovenians; TU, Tuscans. The extra abbreviations: N, northern; S, southern; I, a more homogeneous sub-population; G, a more general sub-population. doi:10.1038/ejhg.2012.229

To date INGI collects four main projects: Val Borbera Project (VB)[43], the East-Northern villages of Genetic Park of Friuli Venezia Giulia (FVG)[49], the South village Carlantino (CARL)[50] and

Campora and Gioi-Cardile in Cilento Genetic Park (CILENTO)[51] and a total of more than 6,000 genotyped and phenotyped individuals.

Several studies published the results of INGI collaborations as the association of TAF3 to MCHC[52], the study of the bitter receptor genes associated to food preferences[53], the variation in platelet count in Italy[54], the hearing function study[49] and the meta-analysis on Anti Mullerian hormone for study of fertility[55].

INGI cohorts joined international consortia for the genetic study of many risk factors for common diseases in inbred and outbred populations: Genetic Iron Status (GIS) consortium for iron parameters study that identified a correlation between iron and lipid metabolism as shown in Chapter 8[56], HaemGen consortium that discovered seventy novel loci for hemoglobin and haematological traits[57], Global Urate Genetics Consortium (GUGC) for the study of serum urate[58] and a large meta-analysis for the study of uromodulin in urinary traits[59], Platelet consortium[60] that identified eleven novel regulators of blood cell formation, Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium for the study of age of menarche, age of menopause[61], thyroids diseases[62] and CDKGen for renal traits[63].

Further population specific analyses have been performed on phenotypes collected by single INGI cohorts. As shown in Chapter 7 Val Borbera organized the first international large meta-analysis on hepcidin hormone[64] in collaboration with other two Dutch outbred cohorts to dissect the loci that regulate hepcidin and iron pathway.

How to uncover the missing disease-causing variants

Whole-genome sequencing (WGS)

Therefore to date INGI have been used in large meta-analyses to highlight genetic association of common variants with several complex traits. Thanks to next-generation sequencing approaches we are now able to map population-specific low-frequency variants.

In 2012, INGI cohorts joined the 'European Sequencing and Genotyping Infrastructure Project' (ESGI) and 'UK10k Project[65]' coordinated by Wellcome Trust Sanger Institute, Hinxton, UK to focus on powerful enrichment in low-frequency variants of isolated populations and figure out most high-impact and causative variants associated to complex diseases in known and novel loci.

UK10k Project[65] collects 10,000 British individuals coming from Alspac cohort and Twins UK cohort and sequenced at low coverage (6x) about 4,000 people from both cohorts.

The large-scale genome-wide studies of 4,000 DNA sequences allowed the exploration of rare variants of an order of magnitude greater depth (down to 0.1% allele frequency) than the 1000 Genomes Project[46] in different categories of phenotypes: hematological, cardiovascular, lipid metabolism, renal and anthropometric traits.

The whole Italian cohort INGI genotypes dataset has been imputed using as reference the set of UK10k sequences to highlight associations in rare variants for about 30 traits shared between VB

and UK10k projects that affect metabolism: the results show several population-specific associated loci enriched in rare variants, other suggestive loci shared in two or more cohorts and most of UK10k outcomes replicated by Italian cohorts. These preliminary results highlight the power of genetic isolates to find out causative rare variants in novel and known loci.

Reference panel of Italian sequences

Recently several population studies used high-throughput sequencing resources to improve the power of their cohorts of discoverying rare variants associated to complex traits as the association of a very low frequency variant in APOC3 locus to plasma triglycerides in UK[66] or gene-based association to psychophysiological phenotypes[67] and to characterize the structure and genetic variation content of their population as Genome of Netherlands consortium[68] and Finnish founder populations[69].

For the same purpose, about 1,000 samples were randomly selected from each isolated cohort INGI and are being typed by low-coverage whole-genome sequencing and high-coverage exome-sequencing. The main aim is to further enrich in lower frequency variants and design an Italian reference panel to be used in genome-wide association analyses on iron-related diseases and other common diseases and secondly to quantify the genetic drifting events occourred in the history of Italian isolates and their evolutionary processes[70].

Afterwards it would be interesting to improve the INGI reference panel with additional sequences from South-Europe populations as Greek[71] and other Mediterranean isolates as Croatian isles[72] and to compare lower frequency to other general Italian genotyped populations as renal-diseases cohort study in 'INCIPE Project' at University of Verona.

Aims and outline of the thesis

The aim of this thesis is the identification of novel loci involved in regulation and disorders of iron homeostasis through epidemiological and genetic characterization of quantitative levels of hepcidin hormone and serum iron homeostasis parameters ferritin, iron, transferrin and transferrin saturation in a healthy Italian isolated population.

The studies in this thesis consist of three major parts. The **first part** reported the study of hepcidin levels variability in the Italian cohort Val Borbera. In **Chapter 2** a characterization of hepcidin-20 isoform, a truncated isoform found in elevated levels in heterogeneous pathological conditions like acute myocardial infarction, anemia of chronic disease (ACD), and, particularly, in chronic kidney disease (CKD) has described and in **Chapter 3,** to better understand the unclear link between iron levels and metabolic syndrome (MetS) we tried to assess if the levels of hepcidin-25 isoform in individuals affected by metabolic syndrome correlate with their high levels of ferritin.

The **second part** of this thesis focused on the effect in Val Borbera of the causative variant rs855791 in serine protease TMPRSS6 locus, the negative regulator of hepcidin. The effects in

vitro of 736A and 736V mutations on the inhibition of hepcidin and the levels of hepcidin and iron in homozygotes 736A respect to 736V in Val Borbera healthy individuals have been studied (**Chapter 4**). In **Chapter 5** we evaluated whether the A736V TMPRSS6 polymorphism influences hepcidin levels and erythropoiesis in chronic hemodialysis cases respect to controls.

In the **third part** of this thesis are reported several studies aimed to assess the genetic architecture of iron parameters and hepcidin. In particular **Chapter 6** explains the association of HFE and TMPRSS6 to hepcidin, iron and hematological traits. The effects of inflammation and acquired iron deficiency on iron homeostasis were considered to to highlight the genetic effects. Due to the low statistical power of Val Borbera cohort to find out novel loci for hepcdin, we collaborated with two other Dutch groups and measured hepcidin levels for about 6,000 individuals in a large meta-analysis (**Chapter 7**). Two novel candidate genes on chromosome 2 and 10 are reported. At the same time iron and the correlated parameters ferritin, transferrin and transferrin saturation have been studied in a large international meta-analysis planned by Genetic Iron Status (GIS) consortium from 2010 on 11 European cohorts that collect 48,000 individuals. Significative associations of known loci are confirmed and two novel loci for ferritin and three loci for transferrin discovered. Some loci have a high pleiotropic effect with lipid metabolism connecting iron homeostasis with cardiovascular risk (**Chapter 8**).

The large dataset has been used in a Mendelian Randomization study (MR) to assess the effect of the increase of levels of iron in the brains of patients with Parkinson disease (PD) where genes known to modify iron levels were used to estimate the effect of iron on PD risk (**Chapter 9**).

Chapter 10 debates the future perspectives to exploit the enrichment in rare and low-frequency variants of isolates INGI through the use of the innovative approach of whole-genome sequencing (WGS). A selected random sample of individulas from Val Borbera and from the villages of Genetic Park of Friuli Venezia Giulia has been analysed in order to improve the set of enriched rare and population specific variants. The INGI sequences will be combined in a haplotype enriched panel to be used as reference for the imputation of INGI cohorts and other Italian cohorts to improve the statistical power of GWAS and to better assess the biological processes of iron genetic disorders and other correlated metabolic parameters.

REFERENCES

1. Rochette L, Gudjoncik A, Guenancia C, Zeller M, Cottin Y, Vergely C. 2014 The iron-regulatory hormone hepcidin: A possible therapeutic target?. Pharmacol Ther. 2014 Sep 7. pii: S0163-7258(14)00166-1

2. Hentze MW, Muckenthaler MU, Galy B, Camaschella C 2010 Two to Tango: Regulation of Mammalian Iron Metabolism. Cell. 2010 Jul 9;142(1):24-38.

3. Ganz T 2013 Systemic iron homeostasis. Physiol Rev 93: 1721–1741

4. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. 2004 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004 Dec 17;306(5704):2090-3.

5. Park CH, Valore EV, Waring AJ, Ganz T. 2201 Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276: 7806 –7810

6. Rouault T. 2002 Post-transcriptional regulation of human iron metabolism by iron regulatory proteins. Blood Cell Mol Dis. 2002;29(3):309-14

7. Pigeon C, Ilyin G, Courselaud B, et al. 2001 A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276:7811–7819.

8. Nicolas G, Bennoun M, Devaux I, et al. 2001 Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci USA 2001; 98: 8780–8785

9. Roetto A, Papanikolaou G, Politou M, et al. 2003 Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003; 33: 21–22.

10. Jordan, J.B., Poppe, L., Haniu, M., Arvedson, T., Syed, R., Li, V., Koh- no, H., Kim, H., Schnier, P.D., Harvey, T.S., et al. 2009. Hepcidin revisited, disulfide connectivity, dynamics, and structure. J. Biol. Chem. 284, 24155–24167.

11. Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. 2006 The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. Blood 107: 328 –333

12. Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirito M, Sawadogo M, Kahn A, Vaulont S. 2002 Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci USA 99: 4596 – 4601

13. Roy CN, Mak HH, Akpan I, Losyev G, Zurakowski D, Andrews NC. 2007 Hepcidin anti- microbial peptide transgenic mice exhibit features of the anemia of inflammation. Blood 109: 4038 – 4044.

14. Lesbordes-Brion JC, Viatte L, Bennoun M, Lou DQ, Ramey G, Houbron C, Hamard G, Kahn A, Vaulont S. 2006 Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis. Blood 108: 1402–1405

15. Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S. 2001 Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci USA 98: 8780 – 8785

16. Sham RL, Phatak PD, Nemeth E, Ganz T. 2009 Hereditary hemochromatosis due to resistance to hepcidin: high hepcidin concentrations in a family with C326S ferroportin mutation. Blood 114: 493– 494

17. Sham RL, Phatak PD, West C, Lee P, Andrews C, Beutler E. 2005 Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features. Blood Cells

Molecules Diseases 34: 157–161

18. Feder JN, Gnirke A, Thomas W, et al. 1996 A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996;13:399-408.

19. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ et al. 2006 Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat. Genet. 38, 531–539

20. Camaschella, C. 2005 Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. Blood 106, 3710–3717.

21. Pietrangelo A. 2004 The ferroportin disease. Blood Cells Molecules Diseases 32: 131–138

22. 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, 1088–1092.

23. Andrews, N.C. 2009 Genes determining blood cell traits. Nat. Genet. 41, 1161–1162.

24. Camaschella C and Silvestri L. 2008 New and old players in the hepcidin pathway. Haematologica 2008.

25. Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY. 2008 Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. Blood 2008;111:5195-204.

26. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, et al. 2006 Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 2006; 38:531-9.

27. Wang, RH, Li, C, Xu, X, Zheng, Y, Xiao, C, Zerfas, P, Cooperman, S, Eckhaus, M, Rouault, T, Mishra, L, and Deng, CX. 2005 A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab. 2, 399–409.

28. Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, and Enns CA. 2009 Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab. 9, 217–227.

29. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. 2008 The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell metabolism 2008

30. Wang CY, Meynard D, Lin HY. 2014 The role of TMPRSS6/matriptase-2 in iron regulation and anemia. Front Pharmacol. 2014 May 19;5:114

31. Nikolai L. Chepelev and William G. Willmore 2011 Regulation of iron pathways in response to hypoxia. Free Radical Biology & Medicine 50 (2011) 645–666

32. Gardenghi S,Marongiu MF,Ramos P,Guy E et al. 2007 Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood 109: 5027–5035

33. Origa R, Galanello R, Ganz T, Giagu N, Maccioni L, Faa G, Nemeth E. 2007 Liver iron concentrations and urinary hepcidin in beta-thalassemia. Haematologica 92: 583–588

34. Papanikolaou G, Tzilianos M, Christakis JI, Bogdanos D, Tsimirika K, MacFarlane J, Goldberg YP, Sakellaropoulos N, Ganz T, Nemeth E. 2005 Hepcidin in iron overload disorders. Blood 105: 4103– 4105

35. Weizer-Stern O,Adamsky K,Amariglio N,Rachmilewitz E,Breda L,Rivella S,Rechavi G. 2006 mRNA expression of iron regulatory genes in beta-thalassemia intermedia and beta-thalassemia major mouse models. Am J Hematol 81: 479 – 483

36. Ganz T, Nemeth E. 2013 Hepcidin and iron homeostasis.Biochim Biophys Acta. 2012 Sep;1823(9):1434-43.

37. Benyamin B, Ferreira MA, Willemsen G, et al. 2009 Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet. 2009;41(11):1173-1175.

38. Chambers JC, Zhang W, Li Y et al. 2009 Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet 2009;41:1170e2.

39. Ganesh SK, Zakai NA, van Rooij FJ, Soranzo N et al. 2009 Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. Nat Genet. 2009 Nov;41(11):1191-8

40. Varilo T, Peltonen L 2004 Isolates and their potential use in complex gene mapping efforts. Curr Opin Genet Dev 14: 316–323.

41. Colonna V, Pistis G, Bomba L, Mona S, Matullo G, Boano R, Sala C, Viganò F, Torroni A, Achilli A, Hooshiar Kashani B, Malerba G, Gambaro G, Soranzo N, Toniolo D. 2013 Small effective population size and genetic homogeneity in the Val Borbera isolate. Eur J Hum Genet. 2013 Jan;21(1):89-94

42. Milani G, Masciullo C, Sala C, Bellazzi R, Buetti I, Pistis G, Traglia M, Toniolo D, Larizza C. 2011 Computer-based genealogy reconstruction in founder populations. J Biomed Inform. 2011 Dec;44(6):997-1003

43. Traglia M, Sala C, Masciullo C, Cverhova V, Lori F, Pistis G, Bione S, Gasparini P, Ulivi S, Ciullo M, Nutile T, Bosi E, Sirtori M, Mignogna G, Rubinacci A, Buetti I, Camaschella C, Petretto E, Toniolo D. 2009 Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One. 2009 Oct 22;4(10):e7554.

44. HapMap Project http://hapmap.ncbi.nlm.nih.gov/

45. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, McCarthy MI, Ramos EM, Cardon LR, Chakravarti A, Cho JH, Guttmacher AE, Kong A, Kruglyak L, Mardis E, Rotimi CN, Slatkin M, Valle D, Whittemore AS, Boehnke M, Clark AG, Eichler EE, Gibson G, Haines JL, Mackay TF, McCarroll SA, Visscher PM. 2009 Finding the missing heritability of complex diseases. Nature. 2009 Oct 8;461(7265):747-53.

46. The 1000 Genomes Project. 2012 Consortium An integrated map of genetic variation

47. 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, McVean GA. 2010 A map of human genome variation from population-scale sequencing. Nature. 2010 Oct 28;467(7319):1061-73

48. Esko T, Mezzavilla M, Nelis M, Borel C, Debniak T, Jakkula E, Julia A, Karachanak S, Khrunin A, Kisfali P, Krulisova V, Aušrelé Kučinskiené Z, Rehnström K, Traglia M, Nikitina-Zake L, Zimprich F, Antonarakis SE, Estivill X, Glavač D, Gut I, Klovins J, Krawczak M, Kučinskas V, Lathrop M, Macek M, Marsal S, Meitinger T, Melegh B, Limborska S, Lubinski J, Paolotie A, Schreiber S, Toncheva D, Toniolo D, Wichmann HE, Zimprich A, Metspalu M, Gasparini P, Metspalu A, D'Adamo P. 2013 Genetic characterization of northeastern Italian population isolates in the context of broader European genetic diversity. Eur J Hum Genet. 2013 Jun;21(6):659-65.

49. Girotto G, Pirastu N, Sorice R, Biino G, Campbell H, d'Adamo AP, Hastie ND, Nutile T, Polasek O, Portas L, Rudan I, Ulivi S, Zemunik T, Wright AF, Ciullo M, Hayward C, Pirastu M, Gasparini P. 2011 Hearing function and thresholds: a genome-wide association study in European isolated populations identifies new loci and pathways. J Med Genet. 2011 Jun;48(6):369-74.

50. Tepper BJ, Koelliker Y, Zhao L, Ullrich NV, Lanzara C, d'Adamo P, Ferrara A, Ulivi S, Esposito L, Gasparini P. 2008 Variation in the bitter-taste receptor gene TAS2R38, and adiposity in a genetically isolated population in Southern Italy. Obesity (Silver Spring). 2008 Oct;16(10):2289-95

51. Colonna V, Nutile T, Astore M, Guardiola O, Antoniol G, Ciullo M, Persico MG. 2007 Campora: a young genetic isolate in South Italy. Hum Hered. 2007;64(2):123-35

52. Pistis G, Okonkwo SU, Traglia M, Sala C, Shin SY, Masciullo C, Buetti I, Massacane R, Mangino M, Thein SL, Spector TD, Ganesh S; CHARGE Consortium Hematology Working, Pirastu N, Gasparini P, Soranzo N, Camaschella C, Hart D, Green MR, Toniolo D. 2013 Genome wide association analysis of a founder population identified TAF3 as a gene for MCHC in humans. PLoS One. 2013 Jul 31;8(7):e69206

53. Pirastu N, Kooyman M, Traglia M, Robino A, Willems SM, Pistis G, d'Adamo P, Amin N, d'Eustacchio A, Navarini L, Sala C, Karssen LC, van Duijn C, Toniolo D, Gasparini P. 2014 Association analysis of bitter receptor genes in five isolated populations identifies a significant correlation between TAS2R43 variants and coffee liking. PLoS One. 2014 Mar 19;9(3):e92065

54. Biino G, Santimone I, Minelli C, Sorice R, Frongia B, Traglia M, Ulivi S, Di Castelnuovo A, Gögele M, Nutile T, Francavilla M, Sala C, Pirastu N, Cerletti C, Iacoviello L, Gasparini P, Toniolo D, Ciullo M, Pramstaller P, Pirastu M, de Gaetano G, Balduini CL. 2013 Age- and sex-related variations in platelet count in Italy: a proposal of reference ranges based on 40987 subjects' data. PLoS One. 2013;8(1):e54289.

55. Barbieri C, Traglia M, Pagliardini L, Vanni V, Masciullo C, Sala CF, Cocca M, Portas L, Nutile T, Ulivi S, Ciullo M, Pirastu M, D'Adamo A, Gasarini P, La Marca A, Papaleo E, Panina P and Toniolo D. A large meta-analysis of anti-Mullerian hormone (AMH) in 1,200 European fertile women. [in preparation]

56. Benyamin B, Esko T, Ried JS, Radhakrishnan A, Vermeulen SH, Traglia M, Gögele M, Anderson D, Broer L, Podmore C, Luan J, Kutalik Z, Sanna S, van der Meer P, Tanaka T, Wang F, Westra HJ, Franke L, Mihailov E, Milani L, Häldin J, Winkelmann J, Meitinger T, Thiery J, Peters A, Waldenberger M, Rendon A, Jolley J, Sambrook J, Kiemeney LA, Sweep FC, Sala CF, Schwienbacher C, Pichler I, Hui J, Demirkan A, Isaacs A, Amin N, Steri M, Waeber G, Verweij N, Powell JE, Nyholt DR, Heath AC, Madden PA, Visscher PM, Wright MJ, Montgomery GW, Martin NG, Hernandez D, Bandinelli S, van der Harst P, Uda M, Vollenweider P, Scott RA, Langenberg C, Wareham NJ10; InterAct Consortium, van Duijn C, Beilby J, Pramstaller PP, Hicks AA, Ouwehand WH, Oexle K, Gieger C, Metspalu A, Camaschella C, Toniolo D, Swinkels DW, Whitfield JB. 2014 Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. Nat Commun. 2014 Oct 29;5:4926.

57. van der Harst P, Zhang W, Mateo Leach I, Rendon A, Verweij N, Sehmi J, Paul DS, Elling U, Allayee H, Li X, Radhakrishnan A, et al. 2012 Seventy-five genetic loci influencing the human red blood cell. Nature. 2012 Dec 20;492(7429):369-75.

58. Köttgen A, Albrecht E, Teumer A, Vitart V, Krumsiek J, Hundertmark C, Pistis G, Ruggiero D, et al.. 2013 Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. Nat Genet. 2013 Feb;45(2):145-54

59. Olden M, Corre T, Hayward C, Toniolo D, Ulivi S, Gasparini P, Pistis G, Hwang SJ, Bergmann S, Campbell H, Cocca M, Gandin I, Girotto G, Glaudemans B, Hastie ND, Loffing J, Polasek O, Rampoldi L, Rudan I, Sala C, Traglia M, Vollenweider P, Vuckovic D, Youhanna S, Weber J, Wright AF, Kutalik Z, Bochud M, Fox CS, Devuyst O. 2014 Common variants in UMOD associate with urinary uromodulin levels: a meta-analysis. J Am Soc Nephrol. 2014 Aug;25(8):1869-82.

60. Gieger C, Radhakrishnan A, Cvejic A, Tang W, Porcu E, Pistis G, Serbanovic-Canic J, Elling U, Goodall et al. 2011 New gene functions in megakaryopoiesis and platelet formation. Nature. 2011 Nov 30;480(7376):201-8

61. Perry JR, Corre T, Esko T, Chasman DI, Fischer K, Franceschini N, He C, Kutalik Z, Mangino M, Rose LM, Vernon Smith A, Stolk L, Sulem P, Weedon MN, Zhuang WV, Arnold A, Ashworth A, Bergmann S, Buring JE, Burri A, Chen C, Cornelis MC, Couper DJ, Goodarzi MO, Gudnason V, Harris T, Hofman A, Jones M, Kraft P, Launer L, Laven JS, Li G, McKnight B, Masciullo C, Milani L, Orr N, Psaty BM; ReproGen Consortium, Ridker PM, Rivadeneira F, Sala C, Salumets A, Schoemaker M, Traglia M, Waeber G, Chanock SJ, Demerath EW, Garcia M, Hankinson SE, Hu FB, Hunter DJ, Lunetta KL, Metspalu A, Montgomery GW, Murabito JM, Newman AB, Ong KK, Spector TD, Stefansson K, Swerdlow AJ, Thorsteinsdottir U, Van Dam RM, Uitterlinden AG, Visser JA, Vollenweider P, Toniolo D, Murray A. 2013 A genome-wide association study of early menopause and the combined impact of identified variants.Hum Mol Genet. 2013 Apr 1;22(7):1465-72.

62. Porcu E, Medici M, Pistis G, Volpato CB, Wilson SG, Cappola AR, Bos SD, Deelen J, den Heijer M, et al.. 2013 A meta-analysis of thyroid-related traits reveals novel loci and gender-specific differences in the regulation of thyroid function. PLoS Genet. 2013;9(2):e1003266.

63. Parsa A, Fuchsberger C, Köttgen A, O'Seaghdha CM, Pattaro Cet al. 2013 Common variants in Mendelian kidney disease genes and their association with renal function. J Am Soc Nephrol. 2013 Dec;24(12):2105-17.

64. Galesloot TE, Verweij N, Traglia M, van Dijk F, Geurts-Moespot AJ, Kiemeney LALM, Swertz MA, van der Meer P, CamaschellaC, Toniolo D, Vermeulen SH, van der Harst P, Swinkels DW. A meta-analysis of genome-wide association studies for serum hepcidin [in preparation]

65. UK10k Project - http://www.uk10k.org/

66. Timpson NJ, Walter K, Min JL, et al UK1OK Consortium Members; UK1OK Consortium Members. 2014 A rare variant in APOC3 is associated with plasma triglyceride and VLDL levels in Europeans. Nat Commun. 2014 Sep 16;5:4871.

67. Vrieze SI, Malone SM, Vaidyanathan U, Kwong A, Kang HM, Zhan X, Flickinger M, Irons D, Jun G, Locke AE, Pistis G, Porcu E, Levy S, Myers RM, Oetting W, McGue M, Abecasis G, Iacono WG. 2014 In search of rare variants: Preliminary results from whole genome sequencing of 1,325 individuals with psychophysiological endophenotypes. Psychophysiology. 2014 Dec;51(12):1309-20.

68. Deelen P, Menelaou A, van Leeuwen EM, et al Genome of the Netherlands Consortium 2014 Improved imputation quality of low-frequency and rare variants in European samples using the 'Genome of The Netherlands'. Eur J Hum Genet. 2014 Nov;22(11):1321-6.

69. Lim ET, Würtz P, Havulinna AS, Palta P, Tukiainen T, Rehnström K, Esko T, Mägi R, Inouye M, Lappalainen T, Chan Y, Salem RM, Lek M, Flannick J, Sim X, Manning A, Ladenvall C, Bumpstead S, Hämäläinen E, Aalto K, Maksimow M, Salmi M, Blankenberg S, Ardissino D, Shah S, Horne B, McPherson R, Hovingh GK, Reilly MP, Watkins H, Goel A, Farrall M, Girelli D, Reiner AP, Stitziel NO, Kathiresan S, Gabriel S, Barrett JC, Lehtimäki T, Laakso M, Groop L, Kaprio J, Perola M, McCarthy MI, Boehnke M, Altshuler DM, Lindgren CM, Hirschhorn JN, Metspalu A, Freimer NB, Zeller T, Jalkanen S, Koskinen S, Raitakari O, Durbin R, MacArthur DG, Salomaa V, Ripatti S, Daly MJ, Palotie A; Sequencing Initiative Suomi (SISu) Project. 2014 Distribution and medical impact of loss-of-function variants in the Finnish

founder population. PLoS Genet. 2014 Jul 31;10(7):e1004494.

70. Colonna V, Cocca M. Genetic drift and purgin in Italian isolates. In preparation.

71. Panoutsopoulou K, Hatzikotoulas K, Xifara DK, Colonna V, Farmaki AE, Ritchie GR, Southam L, Gilly A, Tachmazidou I, Fatumo S, Matchan A, Rayner NW, Ntalla I, Mezzavilla M, Chen Y, Kiagiadaki C, Zengini E, Mamakou V, Athanasiadis A, Giannakopoulou M, Kariakli VE, Nsubuga RN, Karabarinde A, Sandhu M, McVean G, Tyler-Smith C, Tsafantakis E, Karaleftheri M, Xue Y, Dedoussis G, Zeggini E. 2014 Genetic characterization of Greek population isolates reveals strong genetic drift at missense and trait-associated variants. Nat Commun. 2014 Nov 6;5:5345.

72. Polasek O, Marusić A, Rotim K, Hayward C, Vitart V, Huffman J, Campbell S, Janković S, Boban M, Biloglav Z, Kolcić I, Krzelj V, Terzić J, Matec L, Tometić G, Nonković D, Nincević J, Pehlić M, Zedelj J, Velagić V, Juricić D, Kirac I, Belak Kovacević S, Wright AF, Campbell H, Rudan I. 2009 Genome-wide association study of anthropometric traits in Korcula Island, Croatia.Croat Med J. 2009 Feb;50(1):7-16.

Part I

A population-based study of hepcidin

The first part of the study includes the characterization of interactions of hepcidin, the main regulatory molecule of iron homeostasis, with iron and other cellular and environmental factors in the large population of Val Borbera, an Italian genetic isolate.

The two isoforms hepcidin-20 and hepcidin-25 present in serum and their ratio were measured and analyzed in relation with age, sex and iron parameters.

Patients may present concurrent liver iron overload and metabolic syndrome (MetS), a dosorder known as dysmetabolic hyperferritinemia (DHF). The possible role of hepcidin in the disease has been evaluated in a subset of Val Borbera individuals affected by metabolyc syndrome to try to dissect the correlation between MetS and hepcidin level.

Chapter 2

Serum levels of the hepcidin-20 isoform in a large general population: The Val Borbera study

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Journal of Proteomics. 2012 Dec 5;76 Spec No.:28-35

Abstract

Hepcidin, a 25 aminoacid liver hormone, has recently emerged as the key regulator of iron homeostasis. Proteomic studies in limited number of subjects have shown that biological fluids can also contain truncated isoforms, whose role remains to be elucidated. We report, for the first time, data about serum levels of the hepcidin-20 isoform (hep-20) in a general population, taking advantage of the Val Borbera (VB) study where hepcidin-25 (hep-25) was measured by SELDI-TOF-MS. Detectable amount of hep-20 were found in sera from 854 out of 1577 subjects (54.2%), and its levels were about 14% of hep-25 levels. A small fraction of subjects (n=30, 1.9%) had detectable hep-20 but undetectable hep-25. In multivariate regression models, significant predictors of hep-20 were hep-25 and age in males, and hep-25, age, serum ferritin and body mass index in females. Of note, the hep-25:hep-20 ratio was not constant in the VB population, but increased progressively with increasing ferritin levels. This is not consistent with the simplistic view of hep-20 as a mere catabolic byproduct of hep-25. Although a possible active regulation of hep-20 production needs further confirmation, our results may also have implications for immunoassays for serum hepcidin based on antibodies lacking specificity for hep-25.

Introduction

In the last decade hepcidin, a small peptide hormone, has emerged as the key regulator of systemic iron homeostasis[1,2]. Hepcidin inhibits the intestinal absorption of dietary iron
and the release of iron from macrophages through the interaction with the transmembrane iron exporter ferroportin, causing its internalization and degradation in lysosomes[3]. The iron bioactive form of hepcidin is a 25-amino acid peptide (hep-25) that shares high homology with defensins, a family of antimicrobial peptides of the innate immunity[4]. It is produced mainly by the liver as an 84-amino acid precursor that subsequently undergoes proteolytic cleavages to generate the mature form[5]. Further hep-25 processing can result in the generation of two amino-terminal truncated isoforms, hepcidin-22 (hep-22) and hepcidin-20 (hep-20), whose physiological role is still unclear[6]. The development of a reliable hepcidin assay has been proven difficult, particularly with classical immunological methods, yielding to a number of different approaches[7]. Among these, Mass Spectrometry (MS) based studies in limited number of subjects have identified and measured small amounts of hep-20 in both serum and urine, while hep-22 has been found only in urine[8]. Of note, functional studies have demonstrated that the two truncated isoforms almost completely loss the ability to interact with ferroportin[9]. Being inactive in iron regulation, they have been postulated to be degradation byproducts of hep-25. On the other hand, recent studies have suggested that hep-20 may retain greater antimicrobial and fungicidal activity than hep-25, particularly at acidic pH (pH 5.0)[10,11]. In the small series published until now, relatively high levels of hep-20 have been detected in heterogeneous pathological conditions like acute myocardial infarction (AMI)[12], anemia of chronic disease (ACD)[13], and, particularly, in chronic kidney disease (CKD)[14-16]. In our previous study using an improved Surface Enhanced Laser Desorption/Ionization Time of Flight-MS (SELDI-TOF-MS) approach where 54 patients in chronic hemodialysis were compared with 57 controls, hep-20 was detectable in 100% and 39%, respectively[15]. Kroot et al.[17] evaluated 186 patients with various diseases and 23 healthy controls by a weak cation exchange (WCX)-TOF-MS assay. No isoforms were found in sera from healthy subjects and patients with low hepcidin concentrations, while relevant amount of hep-20 were found in some conditions, again particularly in CKD. These studies highlighted that the contribution of hep-20 to "total" serum hepcidin measured by a non-selective ELISA assay is not constant but may vary substantially between healthy subjects and patients with different diseases. However, large-scale information about serum hep-20 concentration is lacking. Thus, the present study was aimed at quantifying serum hep-20 within the framework of the recently completed Iron Section of the Val Borbera (VB) study[18], where serum hepcidin was measured by SELDI-TOF-MS. To the best of our knowledge, we report here for the first time in a large general population the levels of hep-20 according to age and sex, their determinants, and the variations of hep-25:hep-20 ratio according to the iron status.

Material and methods

Subjects and biochemical analyses

This study included 1577 subjects aged 18–98 years enrolled in the Iron Section of the VB study. Details about the general design of this study, as well as the enrollment criteria have been extensively reported elsewhere[18,19]. The study was approved by the San Raffaele Hospital and Regione Piemonte ethical committees, and all subjects gave written informed consent. For each participant, anthropometric, complete blood cell count, and biochemical parameters including serum iron, transferrin, transferrin saturation, ferritin, creatinine, and markers of inflammation were available. Serum hepcidin isoforms were determined using a SELDI-TOF-MS assay previously used for their identification and characterization[8], and subsequently validated for quantification by several investigators including our group[12-18,20].

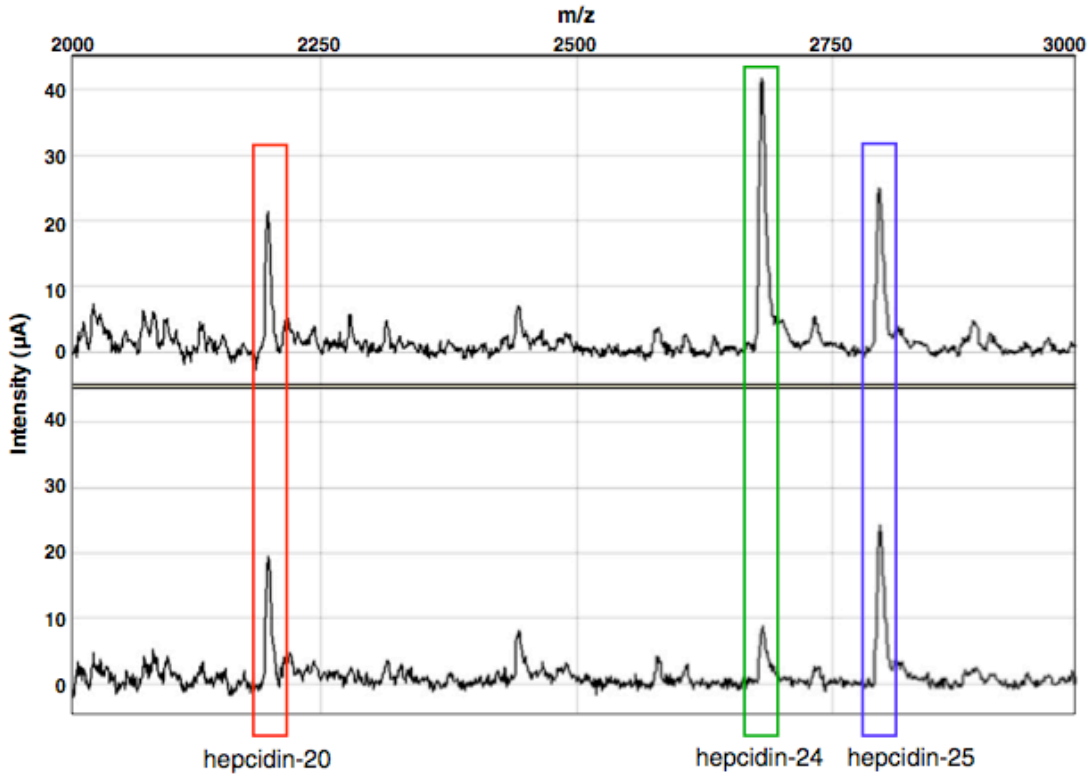


Figure 1. Representative SELDI-TOF-MS profile of serum samples from Val Borbera cohort with (A) and without (B) hep-24 internal standard. The hepcidin isoforms hep-20, hep-24 (synthetic analogue), and hep-25 are indicated by rectangles.

Briefly, copper loaded immobilized metal affinity capture ProteinChip arrays (IMAC-30 Cu2+) were selected as chromatographic surface. A synthetic hepcidin analogue, hepcidin-24 (hep-24, purchased from Peptide International, Louisville, KY), was used as internal standard for quantification. Spectra were collected in duplicate for each serum samples, with or without spiking of the internal standard at a concentration of 10 nM (Fig. 1). With respect to hep-20 quantification, we used as reference peak hep-20 kindly provided by Dr. Elizabeta Nemeth (University of

California Los Angeles, CA). Hep-20 concentration was expressed in nM resulting from the following equation: (sample 2192 m/z peak intensity) × 10 nM/(hep-24 spiked sample 2673 m/z peak intensity–nonspiked sample 2673 m/z peak intensity). Based on the measured background noise in MS spectra, the lower limit of detection (LLOD) for both hepcidin isoforms was 0.55 nM. It was calculated on 20 randomly selected VB sera, and was in agreement with the LLOD previously published by our group and others[20]. Adequate linear standard curve (y = 1.566 × − 0.254, R2 = 0.998) was obtained by serially diluting synthetic hepcidin-20 in blank serum (diluted 1:100 in water). Intra-day precision was determined by using three different concentrations of hep-20 (5, 10, and 25 nM), each with seven replicates. This was repeated on four separate days. Precision was assessed by coefficient of variation (CV%). The within and between run precisions at the low concentrations similar to what found in VB subjects were 6.9% and 7.0%, respectively. The mean peak intensity ratio hepcidin-24/hepcidin-20 in blank serum spiked with both peptides at 7 different concentration combinations (25, 20, 15, 12.5, 10, 7.5, and 5 nM) was 0.85, i.e. similar to what obtained for the main isoform hepcidin-25[15].

Statistical analyses

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as means ± standard deviations, while those with a skewed distribution, including hep-25, hep-20, hep-25:hep-20 ratio, ferritin, CRP and creatinine, were log-transformed and expressed as geometric means with 95% confidence intervals (CIs). Considering that in many subjects the levels of hepcidin (in particular those of hep-20) were not detectable, not allowing a correct log-transformation, the relative statistical analyses in the whole study population were performed adding the value of 0.1 to each hepcidin value and considering not detectable hepcidin levels as 0 (thus, the log transformable 0.1 [0 + 0.1] for statistical analysis). For sake of completeness, the analyses on hep-25 and hep-20 were performed also in the subgroups with detectable levels. On the other hand, the analyses on hep-25: hep-20 ratio were performed only in subjects with detectable levels of both isoforms. Quantitative data were analyzed using the Student's t test or by analysis of variance (ANOVA) with polynomial contrasts for linear trend when indicated. Sex specific correlations between quantitative variables were assessed using Pearson's test. Qualitative data were analyzed using the chi-square test, with analysis for linear trend when indicated. Independent determinants of serum hep-20 and hep-25:hep-20 ratio were estimated by means of linear regression models, including all the variables significantly associated at univariate analysis. Two-sides P values < 0.05 were considered statistically significant.

Results

The main characteristics of the total population are listed in Table 1. This table also lists data stratified by gender, since we previously observed significant gender differences in serum levels of

the bioactive hep-25 isoform, as reported in detail elsewhere[18].

Table 1.

Main characteristics of the total population.

	Total population N = 1577	Males N = 706	Females N = 871	P
Age (years)	55.7 ± 17.8	55.3 ± 17.6	56.0 ± 18.1	0.436
BMI	25.9 ± 4.4	26.5 ± 3.8	25.5 ± 4.8	< 0.001
s-iron (µg/dl)	97.58 ± 34.11	105.83 ± 36.15	90.86 ± 30.80	< 0.001
Transferrin (mg/dl)	242.04 ± 41.52	235.97 ± 36.89	246.98 ± 44.34	< 0.001
Transferrin Saturation %	29.30 ± 11.62	32.30 ± 12.11	26.85 ± 10.60	< 0.001
Ferritin[a] (ng/ml)	69 (65–72)	120 (114–128)	44 (41–47)	< 0.001
Hb (g/dl)	14.4 ± 1.4	15.4 ± 1.2	13.7 ± 1.1	< 0.001
CRP[a] (mg/l)	0.17 (0.16–0.18)	0.16 (0.15–0.17)	0.17 (0.16–0.18)	0.220
Creatinine[a] (µmol/l)	0.85 (0.84–0.86)	0.96 (0.95–0.98)	0.77 (0.76–0.78)	< 0.001
Hep-20[a] (nmol/l)[b]	0.69 (0.63–0.75)	0.82 (0.72–0.94)	0.60 (0.53–0.67)	< 0.001
Hep-20[a] (nmol/l)[c]	3.31 (3.17–3.46)	3.26 (3.06–3.47)	3.35 (3.16–3.56)	0.530
Hep-20 detectable %	54.2	59.1	50.2	–
Hep-25[a] (nmol/l)[b]	4.96 (4.59–5.35)	7.09 (6.46–7.78)	3.71 (3.31–4.16)	< 0.001
Hep-25[a] (nmol/l)[c]	7.86 (7.54–8.19)	8.89 (8.38–9.44)	7.02 (6.63–7.44)	< 0.001
Hep-25 detectable %	89.1	94.6	84.6	–

[a]Variables not normally distributed are expressed as geometric means with 95% CIs.

[b]Geometric mean of hep-20 and hep-25 with 95% CIs calculated on whole population (1577 subjects).

[c]Geometric mean of hep-20 and hep-25 with 95% CIs calculated on subjects with detectable hepcidin levels i.e. n = 854 and n = 1405 respectively.

Briefly recapitulating, hep-25 was significantly lower in women aged <50 years as compared to men of same age, while after this time-point (nearly corresponding to women's menopause) hep-25 levels tended to be similar in both sexes and relatively stable over the following decades. This reflected mainly the lower iron status (i.e. lower ferritin levels) of women during the fertile period, where iron absorption from the gut needs to be allowed by low hep-25 to counterbalance iron losses with menses. Hep-20 was detectable in 854 out of 1577 (54.2%) subjects, at variance with hep-25, which was detectable in 89.1% of subjects. The main characteristics of the subjects stratified for detectable serum hepcidin isoform are reported in Supplementary Table 1. Of note, there was a group, albeit small (n=30, 1.9%), of subjects in whom only the “minor” hep-20 isoform was detectable. In general, as compared with subjects with undetectable hep-20, subjects with detectable hep-20 were older and had higher body iron status (as reflected by transferrin saturation

and ferritin levels) and hep-25 levels (Supplementary Table 2).

The two hepcidin isoforms were significantly and positively correlated in both sexes (males: r = 0.48, P < 0.001; females: r = 0.45, P < 0.001; Fig. 2). At univariate analyses, hep-20 also significantly correlated with age, hemoglobin, and C-Reactive Protein (CRP) in men, and with age, body mass index (BMI), ferritin, CRP and creatinine in women (Table 2). Multivariate linear regression models showed hep-25 and age as independent significant predictors of hep-20 in men, while hep-25, age, BMI, and ferritin were significant predictors of hep-20 in women (Table 3A and B). Of note, the beta coefficient of ferritin in women was negative (Table 3B). Fig. 3A and B shows the variations of hep-20 in the VB population according to different ranges of age and iron status (reflected by serum ferritin levels), respectively. In addition, Supplementary Fig. 1 shows the corresponding hep-25 levels after stratification for ferritin levels.

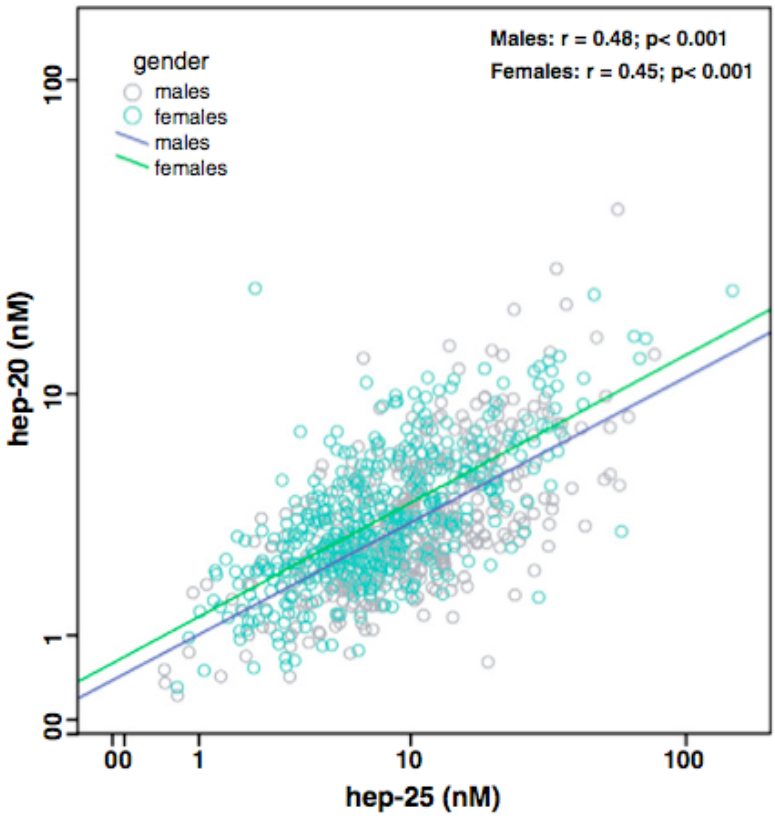


Figure 2. Correlation plot between hep-20 and hep-25 (logarithmic scale)

Table 2.

Sex specific correlation analysis of hepcidin-20.

	Males		Females	
	Correlation	***P***	**Correlation**	***P***
Hep-25 (nmol/l)	0.480	**< 0.001**	0.449	**< 0.001**
Age (years)	0.096	**0.049**	0.178	**< 0.001**
BMI	0.049	0.315	0.186	**< 0.001**
s-iron (µg/dl)	− 0.028	0.571	− 0.023	0.627
Transferrin (mg/dl)	− 0.039	0.430	− 0.049	0.308
Transferrin saturation %	− 0.005	0.912	− 0.013	0.789
Ferritin[a] (ng/ml)	0.062	0.206	0.149	**0.002**
Hb (g/dl)	− 0.110	**0.024**	− 0.048	0.320
CRP[a] (mg/l)	0.115	**0.052**	0.173	**0.002**
Creatinine[a] (µmol/l)	− 0.028	0.583	0.109	**0.029**

Values in bold are those considered statistically significant ($P < 0.05$).

[a]Variables not normally distributed are expressed as geometric means with 95% CIs.

We then focused on the ratio between the two isoforms, and particularly on its behavior in relation with age and iron parameters of VB subject (Table 4 and 5). This analysis could be properly done only in subjects with both the hepcidin isoforms detectable (n = 824). The hep-25:hep-20 ratio was clearly lower in women aged <50 years (i.e. premenopausal) as compared to men of corresponding age, while differences attenuated in the subsequent decades (Fig. 4A). Of note, the hep-25:hep-20 ratio was not constant in the VB population, but increased progressively according to increasing ferritin levels (Fig. 4B). Fig. 5A and B summarizes the relative percentages of hep-25 and hep-20 according to increasing ferritin levels. In both sexes, the relative percentage of hep-20 progressively and significantly decreased with increased ferritin levels.

Discussion

Although much is known on the mechanism of action of hepcidin through the binding with its receptor/cellular exporter ferroportin[3], the mechanism(s) of hepcidin processing, secretion, and catabolism are still poorly elucidated.

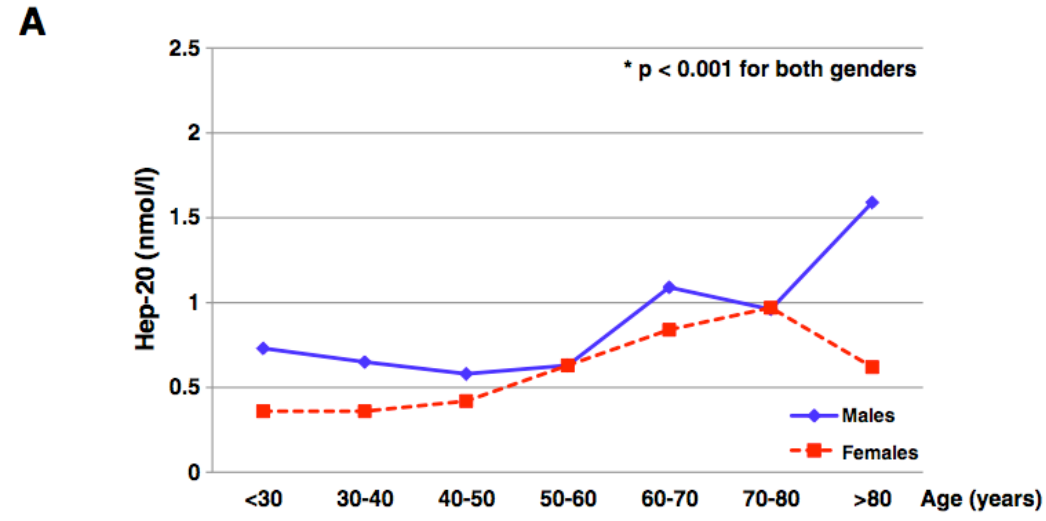
Table 3.

Predictors of Hep-20 levels in males (A) and females (B).

A		
	Males	
	β-coefficient	***P***
Hepcidin25 (nmol/l)	**0.627**	**< 0.001**
Age (years)	**0.126**	**0.007**

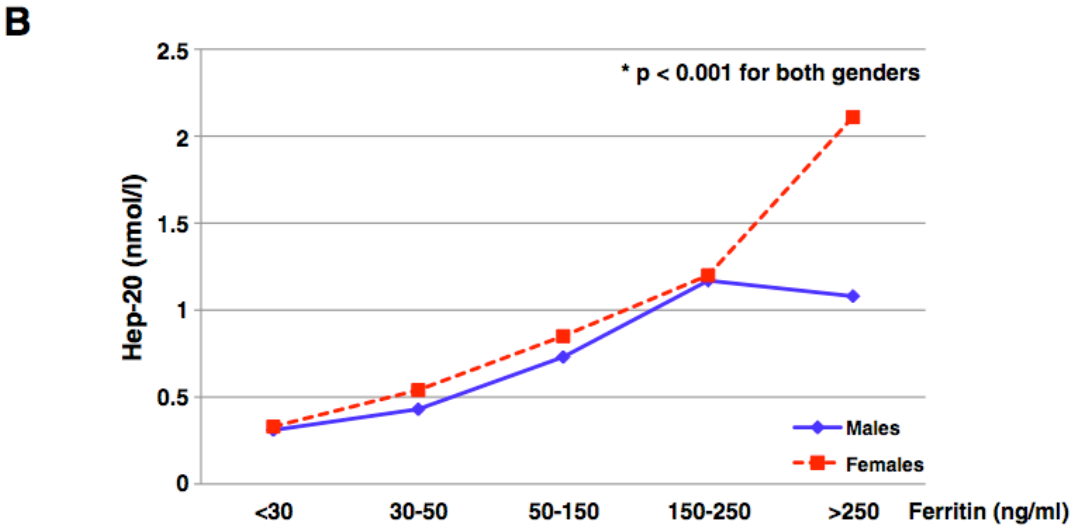
B		
	Females	
	β-coefficient	*P*
Hepcidin25 (nmol/l)	**0.553**	**< 0.001**
Age (years)	**0.177**	**0.004**
Ferritin (μg/l)	**− 0.255**	**0.001**
BMI	**0.163**	**0.003**

Initial efforts to establish reliable assays for this hormone have indicated that the entire pre-pro-hormone (84 amino acid) is also present in the circulation[21], while, at variance with hep-25, its concentration correlates poorly with iron status[22]. Moreover, small studies by MS-based techniques[7,8] have found that two further N-terminal truncated isoforms, namely hep-22 and hep-20, are present in biological fluids, particularly hep-20 in certain disease conditions like CKD [15,17]. Even though hep-20 may be either quantitatively or qualitatively the most relevant isoform of "mature" hepcidin, no large-scale population study has been conducted so far on its serum levels relative to hep-25, as well as on its possible determinants. This study establishes for the first time that more than half individuals at population level have detectable amount of hep-20 in the circulation.



Hep-20 (nmol/l) Males	0.73 (0.45-0.85)	0.65 (0.44-0.94)	0.58 (0.41-0.82)	0.63 (0.47-0.84)	1.09 (0.81-1.48)	0.96 (0.67-1.36)	1.59 (1.03-2.44)
Hep-20 (nmol/l)Females	0.36 (0.25-0.52)	0.36 (0.26-0.48)	0.42 (0.30-0.57)	0.63 (0.47-0.85)	0.84 (0.64-1.09)	0.97 (0.70-1.34)	0.62 (0.41-0.92)

*by ANOVA with polynomial contrasts for linear trend



Hep-20 (nmol/l) Males	0.31 (0.18-0.52)	0.43 (0.24-0.78)	0.73 (0.61-0.89)	1.17 (0.90-1.53)	1.08 (0.77-1.51)
Hep-20 (nmol/l) Females	0.33 (0.27-0.40)	0.54 (0.41-0.71)	0.85 (0.70-1.03)	1.20 (0.77-1.87)	2.11 (0.68-6.53)

*by ANOVA with polynomial contrasts for linear trend

Figure 3. Behavior of hep-20 in VB population according to different ranges of age (A) and ferritin (B), respectively. Males are indicated by continuous blue line, females by a red dotted line.

These individuals were generally included among those with discrete amount of serum hep-25, who in turn represented approximately 89% of the total VB population. As described in detail elsewhere[18], the remaining 11% of individuals were mostly represented by pre-menopausal women with highly prevalent low iron status[23], implying the need to suppress hepcidin for up-regulating the absorption of dietary iron[2]. The large VB database also allowed us to investigate the relevant variables associated with hep-20 levels. To put our results into perspective, we will discuss in more

detail two main possible practical implications.

Hepcidin-20 as a possible caveat in hepcidin assay for clinical purpose

To the best of our knowledge, the VB is the first large-scale population study on serum hepcidin using a MS-based assay. The high correlation of hep-25 with iron status was reported elsewhere[18], and paralleled what observed in a Dutch population by another group using an ELISA method[24]. Notwithstanding recent considerable progress in the complex field of hepcidin assay[7], we still lack a gold reference method[25], and each approach has relative advantages and caveats.

Table 4.

Sex specific correlation analysis of hep-25:hep-20 ratio.

	Males		Females	
	Correlation	***P***	**Correlation**	***P***
Age (years)	− 0.119	**0.015**	0.136	**0.006**
BMI	− 0.027	0.588	0.073	0.137
s-iron (µg/dl)	0.098	**0.046**	0.118	**0.016**
Transferrin (mg/dl)	− 0.074	0.135	− 0.193	**< 0.001**
Transferrin saturation %	0.106	**0.032**	0.172	**< 0.001**
Ferritin[a] (ng/ml)	0.497	**< 0.001**	0.564	**< 0.001**
Hb (g/dl)	− 0.009	0.850	0.134	**0.006**
CRP[a] (mg/l)	0.043	0.472	0.063	0.284
Creatinine[a] (µmol/l)	− 0.061	0.234	0.037	0.470

[a]Variables not normally distributed are expressed as geometric means with 95% CIs.

In particular, ELISA methods are cheaper, easy, and have the potential for wide diffusion in clinical settings, but lack absolute specificity for hepcidin isoforms because of various degree of antibody cross-reactivity that is hard to eliminate. On the other hand, MS-based methods are costly and require dedicated personnel, but can properly distinguish the isoforms. Indeed, in the only study published so far that directly compared two second-generation hepcidin assays, Kroot et al. showed that the observed differences in absolute concentrations were explained, at least partially, by the isoforms detected by MS, as opposed to "total" hepcidin detected by ELISA[17]. Until now, data on hep-20 in healthy subjects were limited and could only be inferred from small case–control studies. We previously reported by SELDI- TOF-MS that hep-20 was detectable in sera from 35 out of 57 (62%) healthy controls, at variance with CKD patients in whom hep-20 was always detectable[15]. Kroot et al., using WCX-TOF-MS assay, compared data from 23 healthy controls with several groups of patients with heterogeneous disorders of iron metabolism[17]. Hep-20 was not

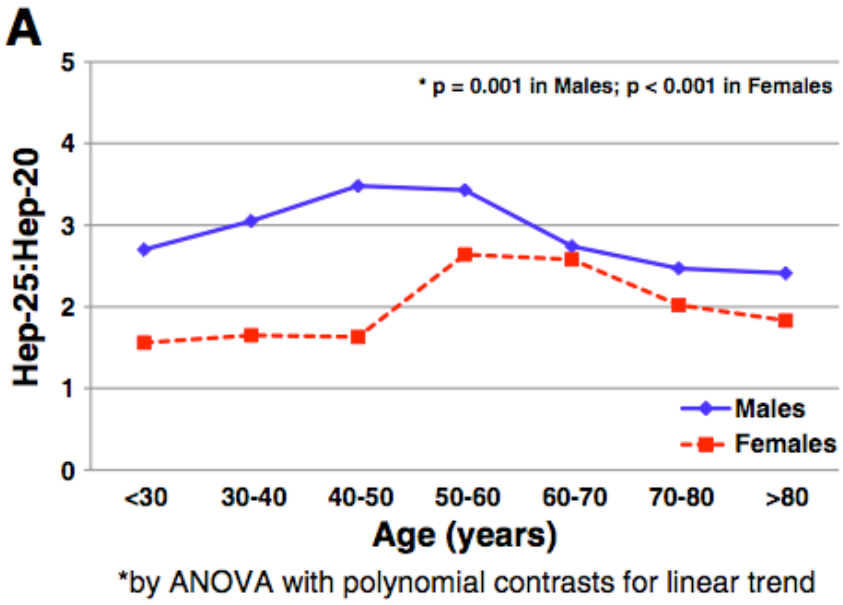
detectable in the few of controls, but was frequently high in several diseases, particularly in CKD.

Table 5.

Predictors of hep-25:hep-20 ratio in males and females.

	Males		Females	
	β-coefficient	*P*	β-coefficient	*P*
Ferritin (μg/l)	0.504	< 0.001	0.662	< 0.001
Age (years)	− 0.147	0.001	− 0.198	< 0.001

Different percentage in controls with detectable hep-20 between the two studies may be explained by the fact that we used more stringent criteria for "control" definition, excluding subjects with even subclinical iron deficiency and hence virtually eliminating those with low/ absent hepcidin production. Anyway, the present study suggests that the relative contribute of hep-20 to "total" serum hepcidin is not negligible at population level. In the total population, mean hep-20 levels were about 14% of corresponding hep-25 levels. On the other hand, when considering only subjects with detectable hep-25 levels (n= 1405), this percentage rose up to 42.1%.



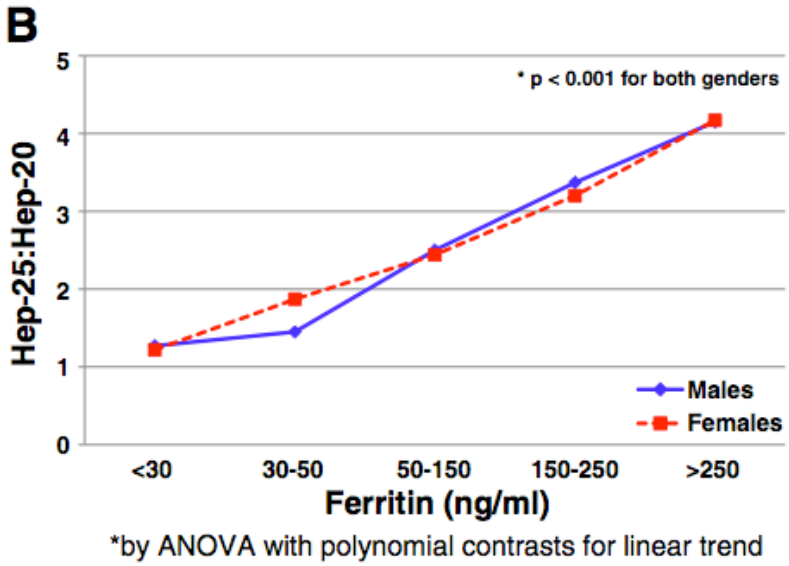


Figure 4. Hep-25:hep-20 ratio in groups of individuals classified according to age (A) and ferritin levels (B), respectively. Males are indicated by blue continuous line, females by a red dotted line.

Hepcidin-20: more than simply a "fixed" degradation products?

The presence of circulating truncated hepcidin isoforms raises questions about their origin and biological meaning. Until now, little is known about the processing of the 22-mer, and 20-mer peptides. Biochemical studies from Schranz and co-workers[26] proposed that the two truncated isoforms may result from the sequential action of unknown aminopeptidases on the mature hep-25 peptide. The Authors suggested Dipeptidylpeptidase IV as a strong candidate for the generation of hep-20 from hep-22, based on the presence of a proline at the cleavage site[27]. Whatever the molecular mechanism, whether or not the processing is actively regulated remains elusive. Theoretically, hep-20 may represent either the final inactive product of constitutive (i.e. not regulated) degradation of hep-25, or a possibly functional peptide whose production may be modulated by body's need relative to the need of hep-25.

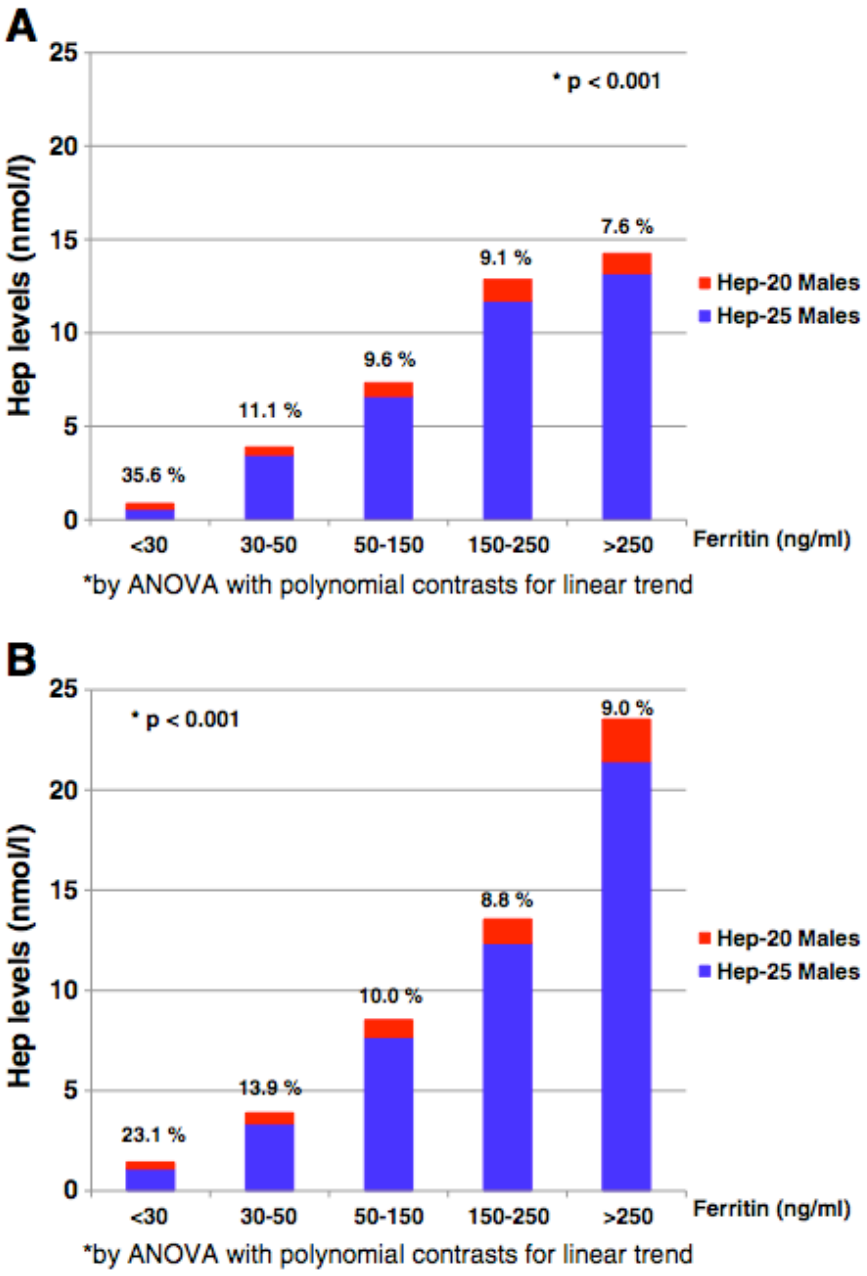


Figure 5. Relative percentages of hep-25 and hep-20 according to increasing ferritin levels in males (A) and females (B), respectively. The percentage value over the column represents the proportion of hep-20 on total hep (calculated as hep-20+hep-25).

Clues to the latter hypothesis are the recent studies on the potentially relevant antimicrobial properties of hep-20[10,11], as well as the pilot studies on its increased concentration in several diseases[12,13,15,16]. Although our study cannot inform on the putative hep-20 function, it may point toward a putative active regulation of hep-20 production and/or hep-25 degradation. This is illustrated particularly by analyzing the hep-25:hep-20 ratio. A simple degradation process should result in a relatively stable ratio between the bioactive peptide (hep-25) and its catabolic product (hep-20). On the other hand, as depicted in Fig. 4A and B, the ratio was consistently lower in women during the fertile period than in men of corresponding age, and increased progressively in

both sexes with increasing ferritin levels. Accordingly, a multivariate model showed that ferritin was an independent predictor of hep-20 in women with a negative coefficient, and not positive as it would be expected if hep-20 would merely represent a constitutive degradation product of hep-25 (Table 5). Taken together, these observations suggest that in subjects with iron deficiency the few hep-25 produced may be, in addition, efficiently degraded to keep the iron bioactive peptide as low as possible to maximize intestinal iron absorption. On the other hand, in subjects with adequate or high iron status hep-25 degradation may proceed less efficiently to keep a normal iron balance and/or prevent dangerous iron load.

Study limitations

Although fascinating, the hypothesis of an active regulation of hep-20 needs further specific studies, being only indirectly supported by our data. Other limitations of our study are represented by the lack of mechanistic explanation on hep-20 formation/function, as well as the relatively high lower limit of detection of our assay that did not allow proper evaluation of the hep-25:hep-20 ratio in subjects with undetectable levels of both isoforms. Moreover, our data suffer from the lack of standardization of current hepcidin assays[25], and need confirmation once a "gold reference" assay will be established.

Conclusions

Considering the recent discovery of hepcidin, we are likely only at the beginning of a story in which much has to be yet discovered. While in the last decade we have learned much on hepcidin regulation at transcriptional level[1,2], times may be mature for in depth investigations on the post-translational regulation of this small peptide hormone. If confirmed, our data may suggest an active regulation of hep-25 degradation according to body iron need. The protease(s) responsible of hep-25 processing, once identified, might be therapeutic target(s) for the control of iron metabolism.

Acknowledgments

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REFERENCES

1. Ganz T. 2011 Hepcidin and iron regulation, 10 years later. Blood 2011;117:4425-33.

2. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. 2010 Two to tango: regulation of mammalian iron metabolism. Cell 2010;142:24-38.

3. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. 2004 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-3

4. Ganz T. 2003 Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3:710-20.

5. Valore EV, Ganz T. 2008 Posttranslational processing of hepcidin in human hepatocytes is mediated by the prohormone convertase furin. Blood Cells Mol Dis 2008;40: 132-8.

6. Park CH, Valore EV, Waring AJ, Ganz T. 2001 Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806-10.

7. Castagna A, Campostrini N, Zaninotto F, Girelli D. 2010 Hepcidin assay in serum by SELDI-TOF-MS and other approaches. J Proteomics 2010;73:527-36.

8. Kemna EH, Tjalsma H, Podust VN, Swinkels DW. 2007 Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin Chem 2007;53:620-8.

9. Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. 2006 The N-terminus of hepcidin is essential for its interaction with ferroportin: structure–function study. Blood 2006;107:328-33.

10. Maisetta G, Petruzzelli R, Brancatisano FL, Esin S, Vitali A, Campa M, et al. 2010 Antimicrobial activity of human hepcidin 20 and 25 against clinically relevant bacterial strains: effect of copper and acidic pH. Peptides 2010;31:1995-2002.

11. Tavanti A, Maisetta G, Del Gaudio G, Petruzzelli R, Sanguinetti M, Batoni G, et al. 2011 Fungicidal activity of the human peptide hepcidin 20 alone or in combination with other antifungals against Candida glabrata isolates. Peptides 2011;32:2484-7.

12. Suzuki H, Toba K, Kato K, Ozawa T, Tomosugi N, Higuchi M, et al. 2009 Serum hepcidin-20 is elevated during the acute phase of myocardial infarction. Tohoku J Exp Med 2009;218:93-8.

13. Tomosugi N, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H, et al. 2006 Detection of serum hepcidin in renal failure and inflammation by using ProteinChip system. Blood 2006;108:1381-7.

14. Tessitore N, Girelli D, Campostrini N, Bedogna V, Solero PG, Castagna A, et al. 2010 Hepcidin is not useful as a biomarker for iron needs in haemodialysis patients on maintenance erythropoiesis-stimulating agents. Nephrol Dial Transplant 2010;25:3996-4002.

15. Campostrini N, Castagna A, Zaninotto F, Bedogna V, Tessitore N, Poli A, et al. 2010 Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. J Biomed Biotechnol 2010;2010:329646.

16. Peters HP, Laarakkers CM, Swinkels DW, Wetzels JF. 2010 Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010;25:848-53.

17. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede AE, et al. 2010 Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570-9.

18. Traglia M, Girelli D, Biino G, Campostrini N, Corbella M, Sala C, et al. 2011 Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 2011;48:629-34.

19. Traglia M, Sala C, Masciullo C, Cverhova V, Lori F, Pistis G, et al. 2009 Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One 2009;4:e7554.

20. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, et al. 2008 Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.

21. Kulaksiz H, Gehrke SG, Janetzko A, Rost D, Bruckner T, Kallinowski B, et al. 2004 Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004;53:735-43.

22. Hadley KB, Johnson LK, Hunt JR. 2006 Iron absorption by healthy women is not associated with either serum or urinary prohepcidin. Am J Clin Nutr 2006;84:150-5.

23. Looker A, Dallman P, Carroll M, Gunter E, Johnson C. 1997 Prevalence of iron deficiency in the United States. JAMA 1997;277:973-6.

24. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, et al. 2011 Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.

25. Kroot JJ, Kemna EH, Bansal SS, Busbridge M, Campostrini N, Girelli D, et al. 2009 Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. Haematologica 2009;94:1748-52.

26. Schranz M, Bakry R, Creus M, Bonn G, Vogel W, Zoller H. 2009 Activation and inactivation of the iron hormone hepcidin: biochemical characterization of prohepcidin cleavage and sequential degradation to N-terminally truncated hepcidin isoforms. Blood Cells Mol Dis 2009;43:169-79.

27. Barrett AJ. 1992 Cellular proteolysis. An overview. Ann N Y Acad Sci 1992;674:1–15.

Chapter 3

Increased Serum Hepcidin Levels in Subjects with the Metabolic Syndrome: A Population Study

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Abstract

The recent discovery of hepcidin, the key iron regulatory hormone, has changed our view of iron metabolism, which in turn is long known to be linked with insulin resistant states, including type 2 diabetes mellitus and the Metabolic Syndrome (MetS). Serum ferritin levels are often elevated in MetS (Dysmetabolic hyperferritinemia - DHF), and are sometimes associated with a true mild-to-moderate hepatic iron overload (dysmetabolic iron overload syndrome - DIOS). However, the pathophysiological link between iron and MetS remains unclear. This study was aimed to investigate, for the first time, the relationship between MetS and hepcidin at population level. We measured serum hepcidin levels by Mass Spectrometry in 1,391 subjects from the Val Borbera population, and evaluated their relationship with classical MetS features. Hepcidin levels increased significantly and linearly with increasing number of MetS features, paralleling the trend of serum ferritin. In multivariate models adjusted for relevant variables including age, C-Reactive Protein, and the HFE C282Y mutation, ferritin was the only significant independent predictor of hepcidin in males, while in females MetS was also independently associated with hepcidin. Overall, these data indicate that the fundamental iron regulatory feedback is preserved in MetS, i.e. that hepcidin tends to progressively increase in response to the increase of iron stores. Due to recently discovered pleiotropic effects of hepcidin, this may worsen insulin resistance and contribute to the cardiovascular complications of MetS.

Introduction

The "metabolic syndrome" (MetS) is a condition highly prevalent in western countries, involving near one fourth of the adult population[1]. Although definitions vary, the essential features of MetS are represented by the deadly quartet of hyperglycemia, dyslipidemia, hypertension, and obesity[2], leading to a substantial cardiovascular risk, but also to risk of hepatic diseases, namely nonalcoholic fatty liver disease (NAFLD). In 1997, Moirand et al. first reported the presence of histologically proven liver iron overload in overweight subjects with abnormal glucose metabolism and dyslipidemia[3]. This condition, later designated as dysmetabolic iron overload syndrome (DIOS)[4], is now known to occur in about one third of subjects with NAFLD and represents the most severe counterpart of the so-called dysmetabolic hyperferritinemia (DHF) (for a recent extensive review, see Dongiovanni et al[5]). The latter in turn is by far the commonest cause of consultation for increased serum ferritin levels in clinical practice[6]. Nevertheless, the complex pathophysiological links between iron and metabolic derangements remain poorly understood[5]. In the last ten years, hepcidin has emerged as the key iron-regulatory hormone[7]. This defensin-like 25 amino acid peptide is mainly produced by the liver in response to increased plasma or tissue iron to homeostatically down-regulate absorption and recycling of the metal[8]. At the molecular level, hepcidin acts by binding and inactivating its cell membrane receptor ferroportin, the only known cellular iron exporter[9]. Ferroportin is particularly expressed by cells critical for iron homeostasis, like absorbing duodenal enterocytes, reticuloendothelial macrophages (involved in iron storage and recycling), and hepatocytes (involved in iron storage and endocrine regulation)[9]. Hepcidin is also upregulated by inflammatory cytokines, a response believed to contribute to host defense by subtracting iron from invading pathogens[10]. Given its central role in iron homeostasis, hepcidin represents an appealing candidate to be investigated in subjects with MetS features, but until now methodological difficulties[11] have hampered large epidemiological studies. Taking advantage from the recently completed iron section of the Val Borbera Study (VBS)[12], this study was aimed to investigate the relationships between hepcidin and the main features of MetS at population level.

Materials and Methods

Details on the VBS population have been previously reported elsewhere[12]. Individuals aged 18 years or older were eligible to participate in the study. In this analysis we included subjects with available complete data allowing their classification according to established criteria for MetS[2]. In detail, the following features were considered: 1) abdominal obesity, defined as the presence of waist circumference ≥94 cm in men or ≥80 cm in women; 2) fasting plasma glucose ≥100 mg/dL or drug treatment for elevated blood glucose; 3) serum triglycerides ≥150 mg/dL or drug treatment for elevated triglycerides; 4) serum HDL cholesterol (HDL-C) <40 mg/dL in men and <50 mg/dL in women or drug treatment for low HDL-C; 5) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure. Subjects were considered to have MetS when they had at least three of

the above-mentioned five traits. Homozygotes for the hemochromatosis mutation (C282Y on the HFE gene) were excluded (n = 7). A total of 1,391 subjects, 616 men and 775 women were finally included in the present study. Fasting blood samples obtained early in the morning were analyzed the same day or stored at −80°C for further analysis. Routine blood parameters and serum hepcidin were determined by standard methods and by mass spectrometry, respectively, as previously described[12]. The study was approved by the ethical committees of San Raffaele Hospital Milano, Regione Piemonte, and Azienda Integrata Ospedaliera Universitaria of Verona, Italy. All subjects gave written informed consent.

Statistical Analyses

All calculations were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). As many of the continuous variables of interest, including serum hepcidin and ferritin, showed a non-Gaussian distribution, their values were log-transformed and expressed as geometric means with 95% confidence intervals (CIs).

Since some subjects had serum hepcidin levels below the lower limit of detection (LLOD) for our method (0.55 nM), to allow a correct analysis these subjects were considered as having hepcidin "0", and hepcidin was log-transformed after the addition of 0.1 to each value in the dataset. Quantitative data were analyzed using the Student's t test or by analysis of variance (ANOVA) with polynomial contrasts for linear trend, when appropriate. Qualitative data were analyzed with the $\chi 2$ test and with $\chi 2$ analysis for linear trend, when appropriate. Correlations between quantitative variables were assessed using Pearson's coefficient. Most of the hepcidin-related analyses were done separately in males and females, since we[12] and others[13] recently reported substantial gender differences in hepcidin serum levels. Particularly, women during the fertile period showed hepcidin levels significantly lower (i.e. less than half) than men of the same age range. Similarly, since MetS subjects were older than those without MetS, analyses were always adjusted for age. Independent determinants of serum hepcidin levels were assessed through a series of linear regression models, using either MetS by itself or individual MetS features as covariates, and adjusting for age, ferritin, C-Reactive Protein (CRP) and C282Y HFE mutation. Two-sided p values <0.05 were

Results

Table 1 summarizes the main clinical, anthropometrical and biochemical features of the population studied, including stratification by gender. Using these data, we calculated the population prevalence of MetS features (shown in Table S1). Overall, 304 individuals (21.9%) could be classified as having MetS using the criteria defined above. Table 2 shows the biochemical iron parameters of the VB subjects stratified for having or not the MetS. Of note, MetS subjects had significantly higher serum levels of both ferritin and hepcidin as compared to subjects without

MetS (geometric means for ferritin: 102 versus 61 µg/L, P<0.001; for hepcidin: 7.95 versus 4.29 nM, P<0.001). Such results remained statistically significant also after adjusting for age and sex (Table 2, last column). Beyond mean values, to evaluate the proportion of MetS individuals with high hepcidin values, we stratified hepcidin levels into quartiles considering subjects with no or just one MetS feature as the reference group. Of note, subjects with undetectable hepcidin levels were significantly underrepresented in the MetS group as compared to the non-MetS group (Table 2). As shown in Figure S1, near 50% of subjects with ≥4 MetS features had hepcidin values in the top quartile of the reference group. We then evaluated the behavior of these two parameters according to the number of MetS features (0 to 5, where the last two categories were merged because of the small number of subjects with all five the features). According to our previous data in a different population[14], serum ferritin levels increased linearly according to increasing number of MetS features, both in males and in females (Figures S2 A–C). The same behavior was observed for serum hepcidin levels, again both in males and in females (Figures 1 A–C). At univariate analyses (Table S2), the variable showing the strongest association with hepcidin was ferritin (beta coefficients = 0.559, and 0.585 in males and females, respectively; P<0.001 for both). Of note, beta coefficients and slopes were quite similar when correlations were made separately in subjects with or without the MetS (Figure S3), suggesting that the homeostatic loop of hepcidin in response to iron stores is well preserved in MetS.

We then performed a series of multiple logistic models to assess the influence of MetS or its individual components on hepcidin levels in both sexes after adjustment for age and all the other relevant covariates, i.e. ferritin, CRP, and C282Y HFE mutation (whose allelic frequency in the VB population was 0.065)[12]. When considering MetS as a comprehensive covariate (Table 3) in a model adjusted for age and serum ferritin, it was independently associated with hepcidin in females but not in males, although the standardized beta coefficient (0.093) for MetS was quite lower than that for ferritin (standardized beta coefficient = 0.580). This association remained statistically significant also after adjustment for CRP and C282Y HFE mutation (standardized beta coefficient = 0.080; P = 0.012), as well as after including in the model hemoglobin, uric acid, and creatinine (standardized beta coefficient = 0.073; P = 0.028). Considering the individual MetS features as covariates (Table S3), the only independent association was observed for abnormal glucose metabolism in females, again with a beta coefficient (0.080) much lower than that of ferritin (0.638). Since the interaction term between ferritin and MetS was significant in females (P<0.001), hepcidin levels were stratified in this group according to both ferritin levels and presence/absence of MetS. As shown in Figure S4, the MetS-associated increase of hepcidin was particularly evident (and statistically significant) in females with the lower ferritin values. A similar trend was not observed in males (data not shown). Finally, when females were stratified on the basis of hepcidin levels, the prevalence of MetS increased progressively from the lowest to the highest strata (Figure S5A). This association remained statistically significant after adjustment for age and ferritin

(Figure S5B).

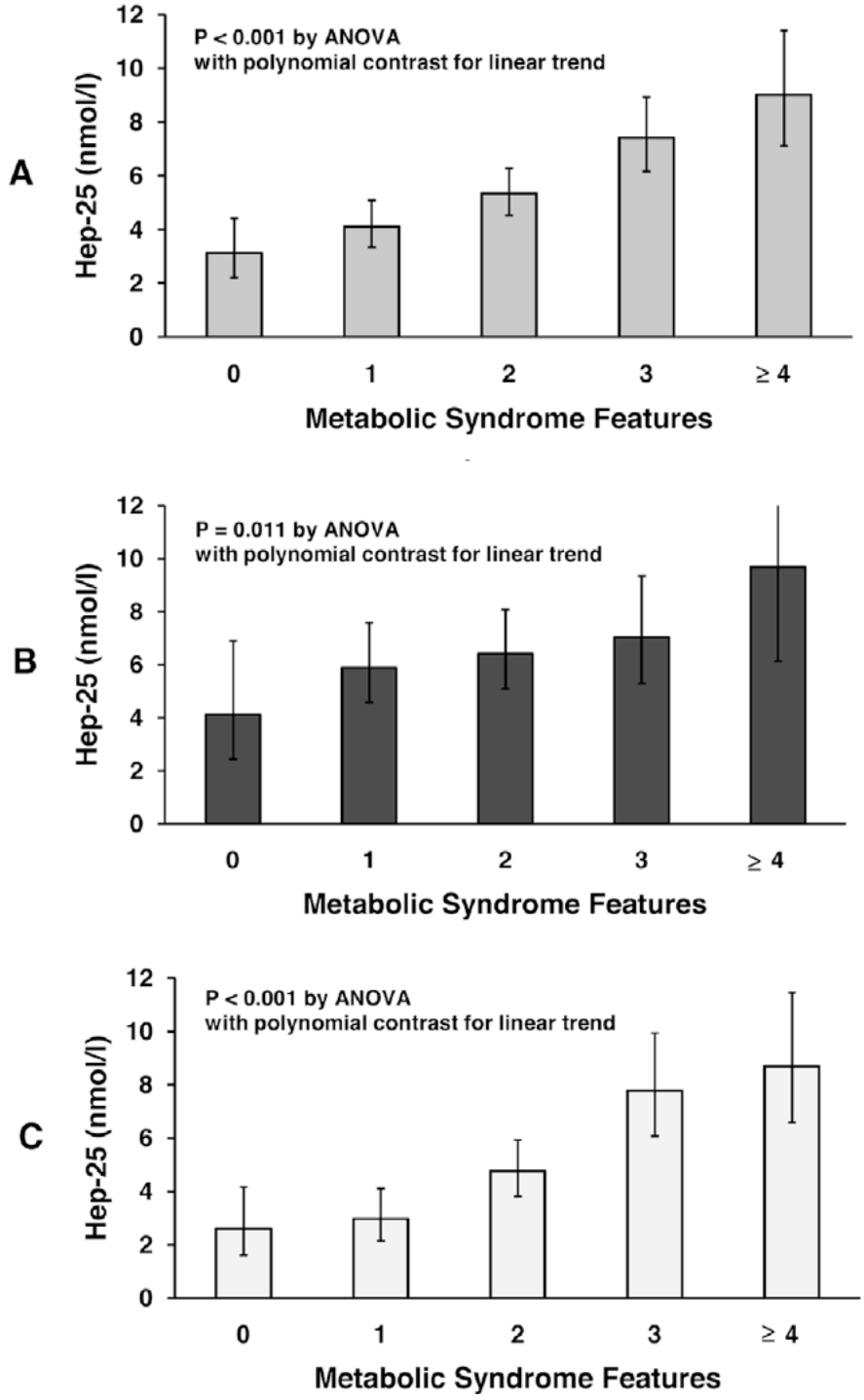

Figure 1. Serum hepcidin levels in the Val Borbera population according to increasing number of MetS features. (A) whole population, (B) males and (C) females.

Discussion

In the recent years, a bulk of evidence, particularly from epidemiological studies[14-17] have established a link between iron metabolism and insulin resistant states, including type 2 diabetes

mellitus and the MetS (for recent reviews, see Dongiovanni et al[5] and Rajpathak et al[18]). Accordingly, experimental studies[19], recently confirmed by a sophisticated approach in C. Elegans[20], have revealed a complex interplay between insulin/IGF-1 signaling and ferritin expression. On the other hand, some prospective studies[16,17] have shown a positive association between baseline levels of ferritin, i.e. the best available serum marker of body iron stores[21], and development of type 2 diabetes. On this basis, it has been postulated that iron may promote insulin resistance through its well-known pro-oxidant properties[22]. Although this causal link remains debated, it is undisputed that dysmetabolic subjects often have high serum ferritin levels, being the so-called dysmetabolic hyperferritinemia (DHF) the commonest cause of mild to moderate hyperferritinemia in clinical practice[6]. The histopathological entity known as dysmetabolic iron overload syndrome (DIOS, formerly designated as "insulin resistance-associated hepatic iron overload – IRHIO)[4] is now believed to represent the most severe clinical expression of DHF[5], where variable degrees of stainable iron coexist with classical features of NAFLD, and serum ferritin levels predicts advanced hepatic fibrosis[23].

Our view of iron overload disorders has radically changed by the discovery of hepcidin[7], which has been demonstrated to be inappropriately low in genetic hemochromatosis[8]. On the other hand, pilot studies have found high hepcidin levels in either serum[24] or urine[25] of few DIOS subjects (n = 16 to 24), suggesting a distinct pathogenesis. Supporting and extending these observations, our results establish for the first time at population level that subjects with MetS have increased serum levels of hepcidin. In subjects of both sexes hepcidin increased linearly with increasing number of the five classical MetS features, paralleling the previously described behavior of serum ferritin[14].

Table 1

Clinical, anthropometrical, and biochemical data of the VB population.

	All (n = 1,391)	Male (n = 616)	Female (n = 775)	*P*
Age (years)	55.8±18.2	55.6±17.8	56.0±18.4	0.715
Weight (Kg)	70.0±14.1	77.8±12.3	63.7±12.3	<0.001
Waist (cm)	90.0±11.8	92.5±10.3	88.0±12.5	<0.001
Height (cm)	164.1±11.4	171.7±7.3	158.0±10.5	<0.001
BMI	25.9±4.5	26.4±3.8	25.4±4.9	<0.001
Total Cholesterol (mg/dl)	203±42	199±41	207±42	<0.001
HDL Cholesterol (mg/dl)	59±15	54±13	63±15	<0.001
LDL Cholesterol (mg/dl)	124±35	122±34	125±36	0.122
Triglycerides* (mg/dl)	89 (87–92)	98 (94–102)	83 (80–86)	<0.001
Glucose* (mg/dl)	90 (89–91)	92 (91–94)	88 (87–89)	<0.001
Diabetes %	3.0	2.6	3.3	0.481
Uric acid (mg/dl)	5.0±1.3	5.8±1.2	4.5±1.1	<0.001
Creatinine* (mg/dl)	0.85 (0.84–0.86)	0.97 (0.95–0.98)	0.77 (0.76–0.78)	<0.001
CRP* (mg/l)	0.17 (0.16–0.18)	0.16 (0.15–0.17)	0.17 (0.16–0.18)	0.207
s-Iron (µg/l)	97.3±34.3	105.5±36.5	90.7±30.9	<0.001
Transferrin (mg/dl)	241.2±43.5	235.3±38.3	245.9±46.8	<0.001
Transferrin saturation (%)	29±11	32±12	27±10	<0.001
Ferritin* (µg/l)	68 (65–72)	116 (109–124)	45 (42–48)	<0.001
Hb (g/dl)	14.4±1.5	15.4±1.2	13.6±1.3	<0.001
Hematocrit (%)	43.6±4.1	45.9±3.3	41.8±3.8	<0.001
RBC (10^6/µl)	4.8±0.5	5.1±0.4	4.6±0.4	<0.001
MCV (fl)	90.7±6.1	91.1±4.9	90.4±6.9	0.027
Hepcidin* (nmol/l)	4.9 (4.5–5.3)	6.8 (6.1–7.6)	3.8 (3.4–4.3)	<0.001
Hepcidin undetectable (n/%)	152/10.9%	37/6.0%	115/14.8%	<0.001
Hepcidin/ferritin* Ratio (nmol/µg × 1000)	71.9 (67.3–76.9)	58.8 (53.9–64.0)	84.5 (76.7–93.0)	<0.001

*variables not normally distributed were log-transformed and expressed as geometric means with 95% CIs.

Of note, serum ferritin was the strongest predictor of hepcidin, while in our analyses CRP, the classical systemic marker of inflammation, was not a significant determinant of both parameters. Taken together, these data indicate that the fundamental iron regulatory feedback is preserved in MetS, i.e. that hepcidin tends to progressively increase in response to a moderate increase of iron stores, likely in the attempt to counterbalance it by limiting intestinal iron absorption. As a corollary, once simple and cheap hepcidin assays will be available in the future, the hepcidin:ferritin ratio may be proven helpful in practice for rapid distinction of DHF/DIOS from other iron overload disorders where hepcidin is inappropriately low, as mentioned above.

Table 2

Biochemical iron parameters in the VB population stratified for presence or absence of MetS.

	Metabolic Syndrome NO(n = 1,087)	Metabolic Syndrome YES(n = 304)	*Unadjusted P*	*Sex- and age-Adjusted P*
Age (years)	53.1±18.6	65.6±12.2	<0.001	
Male Sex (%)	44.6	43.1	0.636	
s-Iron (µg/l)	97.7±34.9	95.6±31.9	0.342	0.721
Transferrin (mg/dl)	243.4±44.1	233.5±40.6	<0.001	0.046
Transferrin saturation (%)	29±11	30±12	0.510	0.575
Ferritin* (µg/l)	61 (58–65)	102 (92–112)	<0.001	<0.001
Hepcidin* (nmol/l)	4.3 (3.9–4.7)	8.0 (7.0–9.1)	<0.001	<0.001
Hepcidin undetectable (n/%)	140/12.9%	12/3.9%	<0.001	<0.001
Hepcidin/Ferritin* Ratio (nmol/µg × 1000)	70.4 (65.2–76.0)	77.8 (68.5–88.3)	0.222	0.093
Hb (g/dl)	14.3±1.5	14.6±1.5	0.003	<0.001

*variables not normally distributed were log-transformed and expressed as geometric means with 95% CIs.

While our data definitively exclude hepcidin deficiency as the underlying mechanism, the key-point that remains to be addressed is the primum movens leading to an increase of iron stores in some dysmetabolic subjects. Aigner et al. proposed that some cytokines produced by the expanding adipose tissue (i.e. TNF-α and other "adipokines") may down-regulate hepatic ferroportin leading to intracellular iron accumulation and compensatory stimulation of hepcidin[26]. Things are further complicated by the fact that the adipose tissue by itself may be a source of hepcidin[27]. On the other hand, some findings in women may be in agreement with these hypotheses. Indeed, we found that in women MetS was independently associated to hepcidin in multivariate models. Of note, when women with or without MetS were stratified by ferritin levels, MetS women with ferritin in the lower range had hepcidin levels significantly higher than non-MetS counterpart. Since this was particularly evident in women with ferritin levels indicating true iron deficiency (i.e. <30 µg/l) where hepcidin is generally almost completely suppressed[12], this suggests that some MetS-related factors may affect hepcidin in this subgroup. On the other hand, the influence of MetS per se on hepcidin levels appears limited when iron stores are abundant. Recent experimental studies have found that leptin, one of the main adipokines, is able to stimulate hepatic hepcidin production[28], and a positive correlation has been found between serum levels of leptin and hepcidin in obese children[29]. Our results may warrant further studies on adults in this direction, particularly focusing on differences by gender. Indeed, the reason(s) why we observed an independent influence on hepcidin only in women remain to be elucidated. Nonetheless, some clues in literature also suggest that the link between iron and dysmetabolic features may be particularly relevant in women. Sheu et al. found a relationship between ferritin and insulin resistance only in women but not in men[15]. Similarly, the largest prospective study showing ferritin as an independent predictor of future development of type 2 diabetes mellitus included only women[16].

Table 3

Predictors of hepcidin-25 in males and females, considering MetS as a comprehensive binary (present versus absent) covariate.

	Male		Female	
	β-coefficient	*P*	β-coefficient	*P*
Age (years)	−0.056	0.098	−0.050	0.135
Metabolic Syndrome	−0.040	0.244	0.093	0.003*
S-Ferritin	0.569	<0.001	0.580	<0.001

*this association remained statistically significant also after adjusting for CRP, C282Y HFE mutation, hemoglobin, uric acid, and creatinine.

Chronic Hyperhepcidinemia in Metabolic Syndrome: more than Simply a Bystander?

Whatever the mechanism(s) behind, this study establishes for the first time at population level that hepcidin levels tend to be high in MetS. In view of the rapidly growing evidence for pleiotropic effects of hepcidin, this may have relevant implications for the MetS pathophysiology. First, studies in cellular models have recently demonstrated that hepcidin binding to ferroportin is able to activate Janus kinase 2/Signal Transducer and Activator 3 (Jak2/STAT3) signaling, leading in turn to an increased production of Suppressor of cytokine signaling 3 (SOCS3)[30], a central player in inducing hepatic steatosis, and MetS in mouse models[31]. Thus, hyperhepcidinemia might prime a vicious circle worsening MetS through SOCS3 induction over time. Second, high hepcidin levels may theoretically contribute to the well-known cardiovascular morbidity in MetS subjects. Indeed, three very recent experimental studies[32-34] have concordantly indicated that hepcidin may promote atherosclerosis, particularly by destabilizing the plaques through macrophage overactivation after erythrophagocytosis[34].

Study Limitations

Due to its observational design, our study cannot provide any mechanistic explanation, particularly with regards to whether increased hepcidin levels are cause or consequence of insulin resistance in subjects with MetS. Similarly, the lack of data on insulin levels precluded a direct analysis of the relationship between hepcidin and estimates of insulin resistance.

Conclusions

Our population study provides the first evidence for chronic hyperhepcidinemia as a new additional feature of MetS. The strong association between hepcidin and ferritin, as well as their parallel behavior as a function of increasing number of MetS features, suggest that hyperhepcidinemia may occur mainly in response to mild-to-moderate increase of body iron stores. Due to the recently discovered pleiotropic effects of hepcidin, our study suggests future investigations on the possible role of this hormone in worsening insulin resistance and in promoting the cardiovascular complications of MetS.

REFERENCES

1. Ford ES, Giles WH, Dietz WH 2002 Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 287: 356–359

2. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, et al. 2009 Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120: 1640–1645

3. Moirand R, Mortaji AM, Loréal O, Paillard F, Brissot P, et al. 1997 A new syndrome of liver iron overload with normal transferrin saturation. Lancet 349: 95–97

4. Riva A, Trombini P, Mariani R, Salvioni A, Coletti S, et al. 2008 Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome. World J Gastroenterol 14: 4745–4752

5. Dongiovanni P, Fracanzani AL, Fargion S, Valenti L 2011 Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. J Hepatol 55: 920–932

6. Adams PC, Barton JC 2011 A diagnostic approach to hyperferritinemia with a non-elevated transferrin saturation. J Hepatol 55: 453–458

7. Ganz T 2011 Hepcidin and iron regulation, 10 years later. Blood 117: 4425–4433

8. Hentze MW, Muckenthaler MU, Galy B, Camaschella C 2010 Two to tango: regulation of Mammalian iron metabolism. Cell 142: 24–38

9. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, et al. 2004 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306: 2090–2093

10. Wrighting DM, Andrews NC 2006 Interleukin-6 induces hepcidin expression through STAT3. Blood 108: 3204–3209

11. Castagna A, Campostrini N, Zaninotto F, Girelli D 2010 Hepcidin assay in serum by SELDI-TOF-MS and other approaches. J Proteomics 73: 527–536

12. Traglia M, Girelli D, Biino G, Campostrini N, Corbella M, et al. 2011 Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 48: 629–634

13. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, et al. 2011 Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 117: e218–225

14. Bozzini C, Girelli D, Olivieri O, Martinelli N, Bassi A, et al. 2005 Prevalence of body iron excess in the metabolic syndrome. Diabetes Care 28: 2061–2063

15. Sheu WH, Chen YT, Lee WJ, Wang CW, Lin LY 2003 A relationship between serum ferritin and the insulin resistance syndrome is present in non-diabetic women but not in non-diabetic men. Clin Endocrinol Oxf 58: 380–385

16. Jiang R, Manson JE, Meigs JB, Ma J, Rifai N, et al. 2004 Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 291: 711–717

17. Jehn ML, Guallar E, Clark JM, Couper D, Duncan BB, et al. 2007 A prospective study of plasma ferritin level and incident diabetes: the Atherosclerosis Risk in Communities ARIC Study. Am J Epidemiol 165: 1047–1054

18. Rajpathak SN, Crandall JP, Wylie-Rosett J, Kabat GC, Rohan TE, et al. 2009 The role of iron in type 2

diabetes in humans. Biochim Biophys Acta 1790: 671–681

19. Yokomori N, Iwasa Y, Aida K, Inoue M, Tawata M, et al. 1991 Transcriptional regulation of ferritin messenger ribonucleic acid levels by insulin in cultured rat glioma cells. Endocrinology 128: 1474–1480

20. Ackerman D, Gems D 2012 Insulin/IGF-1 and Hypoxia Signaling Act in Concert to Regulate Iron Homeostasis in Caenorhabditis elegans. PLoS Genet 8: e1002498.

21. Cook JD, Flowers CH, Skikne BS 2003 The quantitative assessment of body iron stores. Blood 101: 3359–3364

22. Fernandez-Real JM, Lopez-Bermejo A, Ricart W 2002 Cross-talk between iron metabolism and diabetes. Diabetes 51: 2348–2354

23. Kowdley KV, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, et al. 2012 Serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 55: 77–85

24. Ruivard M, Lainé F, Ganz T, Olbina G, Westerman M, et al. 2009 Iron absorption in dysmetabolic iron overload syndrome is decreased and correlates with increased plasma hepcidin. J Hepatol 50: 1219–1225

25. Trombini P, Paolini V, Pelucchi S, Mariani R, Nemeth E, et al. 2011 Hepcidin response to acute iron intake and chronic iron loading in dysmetabolic iron overload syndrome. Liver Int 31: 994–1000

26. Aigner E, Theurl I, Theurl M, Lederer D, Haufe H, et al. 2008 Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. Am J Clin Nutr 87: 1374–1383

27. Bekri S, Gual P, Anty R, Luciani N, Dahman M, et al. 2006 Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. Gastroenterology 131: 788–796

28. Chung B, Matak P, McKie AT, Sharp P 2007 Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells. Journal of Nutrition 137: 2366–2370

29. del Giudice EM, Santoro N, Amato A, Brienza C, Calabrò P, et al. 2009 Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. J Clin Endocrinol Metab 94: 5102–5107

30. De Domenico I, Zhang TY, Koening CL, Branch RW, London N, et al. 2010 Hepcidin mediates transcriptional changes that modulate acute cytokine-induced inflammatory responses in mice. J Clin Invest 120: 2395–2405

31. Ueki K, Kondo T, Tseng YH, Kahn CR 2004 Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. Proc Natl Acad Sci U S A 101: 10422–10427

32. Valenti L, Dongiovanni P, Motta BM, Swinkels DW, Bonara P, et al. 2011 Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations. Arterioscler Thromb Vasc Biol 31: 683–690

33. Saeed O, Otsuka F, Polavarapu R, Karmali V, Weiss D, et al. 2012 Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler Thromb Vasc Biol 32: 299–307

34. Li JJ, Meng X, Si HP, Zhang C, Lv HX, et al. 2012 Hepcidin Destabilizes Atherosclerotic Plaque via Overactivating Macrophages After Erythrophagocytosis. Arterioscler Thromb Vasc Biol 32: 1158–1166

Part II

Effect of A736V TMPRSS6 on hepcidin and iron metabolism in healthy individuals and in case controls studies

The next two studies focused on the serine protease matriptase-2 TMPRSS6, the negative regulator of hepcidin and of the BMP/SMAD pathway. TMPRSS6 inactivation causes iron-deficiency-anemia refractory to iron administration both in humans and mice. Genome wide association studies have shown that the SNP rs855791, which causes the matriptase-2 V736A amino acid substitution, is associated with variations of serum iron, transferrin saturation, hemoglobin and erythrocyte traits.

In the first study the activity of hepcidin inhibition of rs855791, has been verified in vitro and then tested in healthy Val Borbera population.

In the second study the effect of TMPRSS6 polymorphism rs855791 on hepcidin and erythropoiesis has been studied in patients with chronic hemodialysis (CHD) to clarify whether rs855791 influences iron metabolism and anemia during chronic inflammation and renal failure.

Chapter 4

TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals

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Abstract

The iron hormone hepcidin is inhibited by matriptase-2, a liver serine-protease encoded by TMPRSS6 gene. Cleaving the BMP-coreceptor hemojuvelin, matriptase-2 impairs the BMP/SMAD signaling pathway, downregulates hepcidin and facilitates iron absorption. TMPRSS6 inactivation causes iron-deficiency-anemia refractory to iron administration both in humans and mice. Genome wide association studies have shown that the SNP rs855791, which causes the matriptase-2 V736A amino acid substitution, is associated with variations of serum iron, transferrin saturation, hemoglobin and erythrocyte traits. Here we show that in vitro matriptase-2 736^{A} inhibits hepcidin more efficiently than 736^{V}. Moreover, in a genotyped population, after exclusion of samples with iron deficiency and inflammation, hepcidin, hepcidin/transferrin saturation and hepcidin/ferritin ratios were significantly lower and iron parameters were consistently higher in homozygotes 736^{A} than in 736^{V}. Our results indicate that rs855791 is a TMPRSS6 functional variant and strengthen that even a partial inability to modulate hepcidin influences iron parameters and indirectly erythropoiesis.

Introduction

Hepcidin is the key regulator of iron homeostasis, controlling surface expression of the iron exporter ferroportin on enterocytes and macrophages[1]. Inactivation of hepcidin causes severe iron overload in mice and humans, whereas hepcidin overexpression causes iron deficiency anemia[2]. Hepcidin expression is up-regulated in response to increased body iron, through the Bone Morphogenetic Protein (BMP)-hemojuvelin (HJV)-Son of Mothers Against Decapentaplegic (SMAD) pathway[3] and inhibited by matriptase-2 (MT2), a type II transmembrane serine protease, encoded by the TMPRSS6 gene[4,5] that in vitro cleaves the BMP-coreceptor HJV[6]. In vivo "Mask" mice, which have a deleted serine protease domain[4], and Tmprss6 null mice[7] show microcytic anemia due to high hepcidin levels. TMPRSS6 deleterious mutations in humans cause iron-refractory iron-deficiency anemia (IRIDA), unresponsive to oral iron administration[5]. The same mutations show partial inhibition of the hepcidin promoter activity when overexpressed with HJV in vitro in hepatoma cells[6,8].

Recently genome-wide association studies (GWAS) reported the association of common genetic variants of TMPRSS6 (rs855791 and rs4820268) with serum iron and transferrin saturation[9-11], hemoglobin (Hb), MCV and MCH[12,13], highlighting a role for MT2 in the control of iron and erythrocyte parameters. The SNP rs855791 (2321G->A) causes a non-synonymous alanine to valine change (A736V) in the catalytic domain, whereas the SNP rs4820268 leads to a synonymous change at 521 and is in linkage disequilibrium with rs855791. Since rs855791 affects the MT2 catalytic domain, a common speculation was that its effects were hepcidin-mediated[9,14].

We tested this hypothesis using an in vitro assay, based on luciferase reporter gene driven by the hepcidin promoter and showed that the $MT2^{736A}$ inhibits hepcidin more efficiently than $MT2^{736V}$. We also demonstrated that this variant affects hepcidin levels of normal individuals.

Material and methods

In vitro studies

The in vitro analyses (western blot, hepcidin promoter activity assay and binding assay) were previously reported[6] and are detailed in supplemental material. The TMPRSS6 variant, encoding 736A ($MT2^{736A}$) was obtained by mutagenesis of $MT2^{736V}$ plasmid by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA)

Human studies

The population of the genetic isolate "Val Borbera" (VB) was previously described15. The study was approved by San Raffaele Ethical Committee. Serum hepcidin levels were measured by SELDI-TOF-Mass Spectrometry16 and detailed results are reported elsewhere[11].

Statistical analysis

Association of TMPRSS6 rs855791 was first analyzed in 655 unrelated (pairwise kinship coefficient <0.0625) individuals selected using the Greffa software[17]. We included in the final analysis only individuals with hepcidin levels above the lower limit of detection (0.55 nM) and subjects with ferritin 30ng/ml and CRP 1mg/dL (Subset 1), Mean values were adjusted for sex, age, squared age and by interaction between them (sex*age, sex*squared age) by ANOVA (95% C.I.) using SPSS 17.0 software (SPSS Chicago, IL, US) and in house R-2.8.1 scripts (The R Project for Statistical Computing at http://www.r-project.org).

Results and discussion

In vitro function of MT2 A736V variants

We first demonstrated that the proportion of $MT2^{736A}$ and $MT2^{736V}$ expressed on the surface of transfected cells was similar (Fig. 1A). Then we observed that MT2736A inhibited the luciferase-hepcidin promoter more efficiently than $MT2^{736V}$ with a dose-dependent effect at low concentration (Fig. 1B). In agreement with the luciferase assay the release of the serine protease domain, that in our hands correlates with the protease activity[6,8], was slightly increased in cells transfected with the more active $MT2^{736A}$ variant compared with $MT2^{736V}$ (Fig. S1 upper panel). These results suggest that rs855791 is a functional variant. Western blot on cell lysates and phospholipase C were not enough sensitive to detect a significant difference in the cleavage of HJV between the two variants (Fig. S1 lower panel).

Based on gene expression arrays analysis, it was proposed that rs4820268, the other TMPRSS6 variant significantly associated with iron and erythrocyte traits[10,18] might cause a differential allelic expression (60:40 ratio) of TMPRSS6 mRNA[19]. However, it is unlikely that the modest difference observed results in detectable changes of the protease activity. rs4820268 is in linkage disequilibrium (r2 = 0.811 in VB cohort) with rs855791: thus its association with iron and erythrocyte traits might simply be secondary to that of rs855791.

Human database indicates V at 736 position as the “wild type” matriptase-2. However, comparative analysis indicates A as the ancestral amino acid, since A is evolutionary conserved in all the species where an annotated matriptase-2 sequence is available (Fig. 1C). This observation suggests that the $MT2^{736V}$ variant, that leads to increased hepcidin production and inhibition of iron absorption, is evolutionary a recent change.

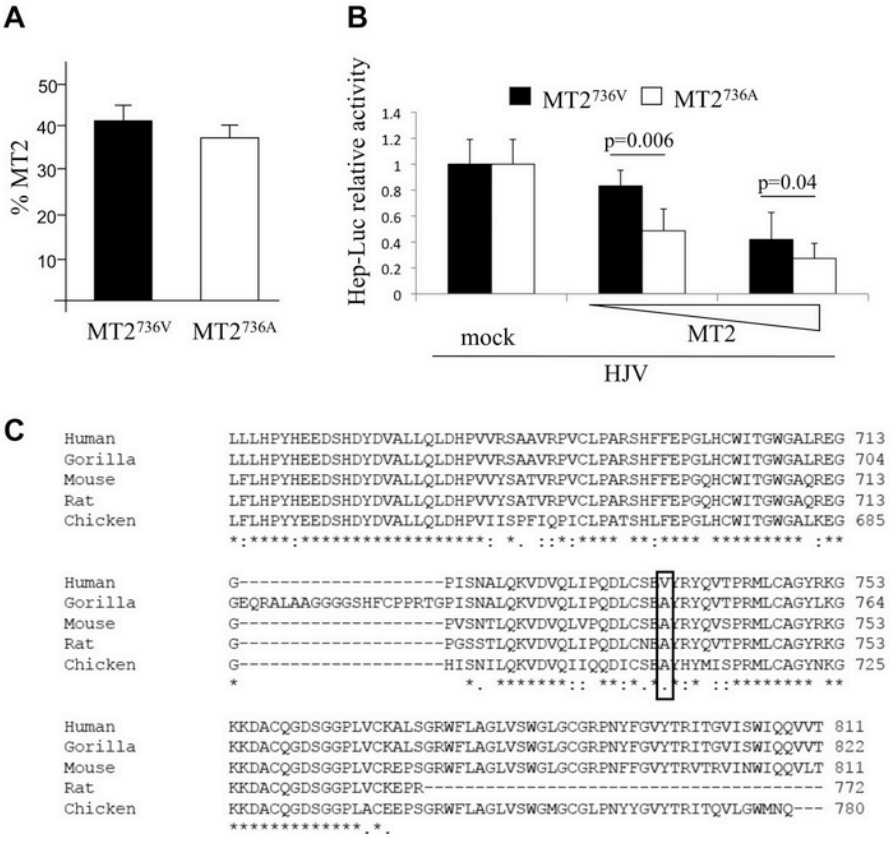

Figure 1. In vitro characterization of the function of MT2 variants and evolutionary conservation of the catalytic domain. (A) Quantification of membrane-bound MT2 (MT2) by binding assay. HeLa cells were transiently transfected with the TMPRSS6 cDNA encoding MT2^{736V}, MT2^{736A}, or the empty vector (mock) and analyzed for the percentage of MT2 on the cell surface[6]. The amount of surface MT2 was calculated as the ratio between the absorbance of unpermeabilized and permeabilized cells. Error bars indicate SD. (B) Hepcidin promoter activity assay. Hep3B cells were transiently transfected with 0.25 μg of pGL2-basic reporter vector (Promega) containing the 2.9-kb fragment of the human hepcidin promoter[23] in combination with pRL-TK Renilla luciferase vector (Promega) and HJV, as described previously[6]. Increasing doses (from 0.002 to 0.01 μg/mL) of MT2^{736V} or MT2^{736A}-expressing vectors were used. Relative luciferase activity was calculated as the ratio of firefly (reporter) to Renilla luciferase activity and is expressed as a multiple of the activity of cells transfected with the reporter alone. Experiments were performed in triplicate. The statistical significance is indicated above the bars. (C) Alignment of part of the serine protease domain of MT2 of different species by multiple sequence alignment ClustalW (EMBL-EBI) program. The sequence is highly conserved. The human 736 and the orthologous position in the other species are boxed.

Hepcidin levels of normal carriers of MT2 736 variants

We validated our in vitro results in the VB cohort, which had serum hepcidin levels measured. Since in a genetic isolate many individuals are related only a group of 655 unrelated individuals was studied. Furthermore we selected 545 normal subjects, after excluding iron deficient individuals (serum ferritin <30 ng/ml) and individuals with clinically relevant inflammatory conditions (CRP >1 mg/dL)[11]. Serum hepcidin levels were lower in AA compared to VV homozygous individuals. The difference was not significant in the whole series but only in the selected subset (p = 0.038) (Fig. 2 and Table S1). Since hepcidin expression is strongly dependent

on both iron stores and plasma iron we normalized hepcidin on ferritin and on transferrin saturation. In both cases we confirmed that the normalized values were significantly lower in AA compared to VV homozygotes (p=0.038 for hepcidin/ferritin and p=0.056 for hepcidin/transferrin saturation respectively) in subset 1 (Fig. 2 and Table S1). Consistently iron and transferrin saturation were higher in AA than in VV homozygotes (Fig. 2 and Table S1), as observed[9]. MCV and MCH showed a similar trend, although the difference did not reach statistical significance (Fig. S2). No difference was found for ferritin, transferrin and Hb levels (Fig. S1 and not shown).

Our results suggest that MT2 influences normal hepcidin response to both plasma and total body iron. Hepcidin regulation is complex[2]. In mice the hepcidin response to isolated increase of transferrin saturation20 or to an acute iron increase[21] differs from the response to increased total body iron or to chronic iron treatment. Both responses are based on the same BMP signaling pathway and on SMAD activation, but only the second entails a BMP6 increase[20,21]. The difference in the hepcidin/transferrin saturation and hepcidin/ferritin ratios observed between the two TMPRSS6 genotypes strengthens a role for MT2 in counterbalancing both BMP6-dependent and BMP6- independent hepcidin upregulation. The reduced activity of $MT2^{736V}$ demonstrated by the in vitro assay is in agreement with the effect observed in vivo.

$MT2^{736V}$ is the less frequent allele with a frequency of 0.45 in Val Borbera, as in other Caucasian populations. From the available studies the distribution among different populations is not homogeneous (Table S2). Although the samples analyzed are limited, $MT2^{736A}$ seems largely prevalent among Africans (0.80-0.90) compared to Caucasians (0.50)[9,11,14] and Japanese (0.40)[22]. Whether the variant might provide an advantage enhancing iron absorption in conditions of limited dietary availability or alternatively might have conferred protection against some infections remains to be clarified in future studies.

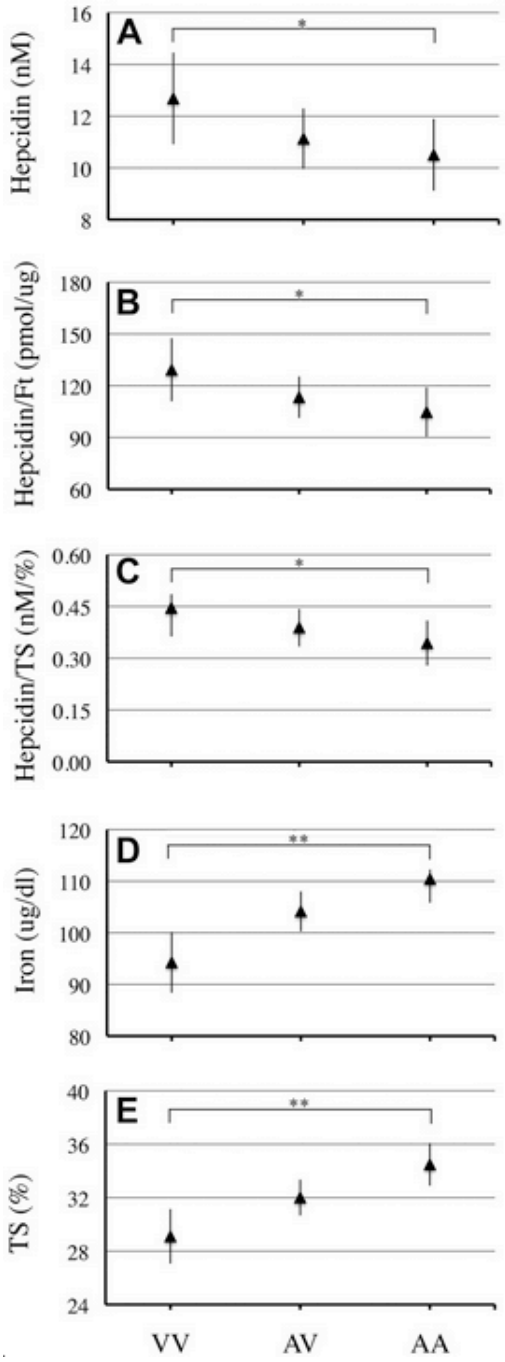

Figure 2. Hepcidin traits and iron parameter mean levels in individuals from subset 1 classified according to MT2 genotypes (AA, AV, and VV). Hepcidin (A), hepcidin/ferritin ratio (B), hepcidin/transferrin saturation ratio (C), serum iron (D), and transferrin saturation (E) are shown. Data are expressed as mean values and are corrected by sex, age, squared age, and interaction by ANOVA (95% confidence intervals are shown). VV indicates homozygotes for the TMPRSS6 alleles encoding valine; AA, homozygotes for the TMPRSS6 alleles encoding alanine; and AV, compound heterozygotes for the 2 alleles. P values refer to comparison between AA and VV homozygotes; *P < .05; **P < .0005.

In conclusion our data indicate that TMPRSS6 rs855791 has a functional role in determining the protease activity and regulating hepcidin expression both in vitro and in normal subjects, suggesting that it influences hepcidin response to the increase of both circulating and total body iron.

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REFERENCE

1. Nemeth E, Tuttle MS, Powelson J, et al. 2204 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004;306(5704):2090-2093.

2. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. 2010 Two to tango: regulation of Mammalian iron metabolism. Cell. 2010;142(1):24-38.

3. Babitt JL, Huang FW, Wrighting DM, et al. 2006 Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet. 2006;38(5):531-539.

4. Du X, She E, Gelbart T, et al. 2008 The serine protease TMPRSS6 is required to sense iron deficiency. Science. 2008;320(5879):1088-1092.

5. Finberg KE, Heeney MM, Campagna DR, et al. 2008 Mutations in TMPRSS6 cause iron- refractory iron deficiency anemia (IRIDA). Nat Genet. 2008;40(5):569-571.

6. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. 2008 The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab. 2008;8(6):502-511.

7. Folgueras AR, de Lara FM, Pendas AM, et al. 2008 Membrane-bound serine protease matriptase- 2 (Tmprss6) is an essential regulator of iron homeostasis. Blood. 2008;112(6):2539-2545.

8. Silvestri L, Guillem F, Pagani A, et al. 2009 Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. Blood. 2009;113(22):5605-5608.

9. Benyamin B, Ferreira MA, Willemsen G, et al. 2009 Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet. 2009;41(11):1173-1175.

10. Tanaka T, Roy CN, Yao W, et al. 2010 A genome-wide association analysis of serum iron concentrations. Blood. 2010;115(1):94-96.

11. Traglia M, Girelli D, Campostrini N, et al. 20011 The association of HFE and TMPRSS6 genetic variants to iron and erythrocyte parameters is only in part dependent on serum hepcidin levels. Journal of Medical Genetics. 2011

12. Soranzo N, Sanna S, Wheeler E, et al. 2010 Common variants at 10 genomic loci influence hemoglobin A(C) levels via glycemic and nonglycemic pathways. Diabetes. 2010;59(12):3229- 3239.

13. Oexle K, Ried JS, Hicks AA, et al. 2011 Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferrin receptor (sTfR) levels. Hum Mol Genet. 2011;20(5):1042-1047.

14. Chambers JC, Zhang W, Li Y, et al. 2009 Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet. 2009;41(11):1170-1172.

15. Traglia M, Sala C, Masciullo C, et al. 2009 Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One. 2009;4(10):e7554.

16. Girelli D, Trombini P, Busti F, et al. 2011 A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis. Haematologica. 2011;96(4):500-506.

17. Falchi M, Forabosco P, Mocci E, et al. 2004 A genomewide search using an original pairwise sampling approach for large genealogies identifies a new locus for total and low-density lipoprotein cholesterol in two genetically differentiated isolates of Sardinia. Am J Hum Genet. 2004;75(6):1015-1031.

18. Pichler I, Minelli C, Sanna S, et al. 2011 Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum Mol Genet. 2011;20(6):1232- 1240.

19. Serre D, Gurd S, Ge B, 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. 2008;4(2):e1000006.

20. Corradini E, Meynard D, Wu Q, et al. 2011 Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. Hepatology. 2011:54(1):273- 284.

21. Ramos E, Kautz L, Rodriguez R, et al. 2011 Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. Hepatology. 2011;53(4):1333-1341.

22. Kamatani Y, Matsuda K, Okada Y, et al. 2010 Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet. 2010;42(3):210-215.

23. Pagani A, Silvestri L, Nai A, Camaschella C. 2008 Hemojuvelin N-terminal mutants reach the plasma membrane but do not activate the hepcidin response. Haematologica. 2008;93(10):1466- 1472.

Chapter 5

The A736V TMPRSS6 polymorphism influences hepcidin and iron metabolism in chronic hemodialysis patients: TMPRSS6 and hepcidin in hemodialysis

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Abstract

Background: Aim of this study was to evaluate whether the A736V TMPRSS6 polymorphism, a major genetic determinant of iron metabolism in healthy subjects, influences serum levels of hepcidin, the hormone regulating iron metabolism, and erythropoiesis in chronic hemodialysis (CHD).

Methods: To this end, we considered 199 CHD patients from Northern Italy (157 with hepcidin evaluation), and 188 healthy controls without iron deficiency, matched for age and gender. Genetic polymorphisms were evaluated by allele specific polymerase chain reaction assays, and hepcidin quantified by mass spectrometry.

Results: Serum hepcidin levels were not different between the whole CHD population and controls (median 7.1, interquartile range (IQR) 0.55-17.1 vs. 7.4, 4.5-17.9 nM, respectively), but were higher in the CHD subgroup after exclusion of subjects with relative iron deficiency ($p = 0.04$). In CHD patients, the A736V TMPRSS6 polymorphism influenced serum hepcidin levels in individuals positive for mutations in the HFE gene of hereditary hemochromatosis ($p < 0.0001$). In particular, the TMPRSS6 736 V variant was associated with higher hepcidin levels ($p = 0.017$). At multivariate analysis, HFE and A736V TMPRSS6 genotypes predicted serum hepcidin independently of ferritin and C reactive protein ($p = 0.048$). In patients without acute inflammation and overt iron deficiency (C reactive protein <1 mg/dl and ferritin >30 ng/ml; $n = 86$), hepcidin was associated with lower mean corpuscular volume ($p = 0.002$), suggesting that it contributed to iron-restricted erythropoiesis. In line with previous results, in patients without acute inflammation and severe iron deficiency the “high hepcidin” 736 V TMPRSS6 variant was associated with higher erythropoietin maintenance dose ($p = 0.016$), independently of subclinical inflammation ($p = 0.02$).

Conclusions: The A736V TMPRSS6 genotype influences hepcidin levels, erythropoiesis, and anemia management in CHD patients. Evaluation of the effect of TMPRSS6 genotype on clinical outcomes in prospective studies in CHD may be useful to predict the outcomes of hepcidin manipulation, and to guide treatment personalization by optimizing anemia management.

Background

Patients with end stage renal disease (ESRD) undergoing chronic hemodialysis (CHD) are commonly affected by anemia, which is related to erythropoietin (Epo) deficiency, blood losses, and chronic inflammation[1]. Treatment is based on erythropoiesis stimulating agents in association with intravenous (i.v.) iron formulations, but is of often difficult to achieve and maintain the desired hemoglobin (Hb) levels without incurring in side effects[2,3].

ESRD is characterized by major alterations in iron metabolism including low transferrin saturation (TS), resulting in reduced iron availability for the erythroblasts, and hyperferritinemia[2,4]. Upregulation of serum levels of hepcidin, the hepatic hormone regulating systemic iron metabolism, has been proposed to explain the alterations of iron metabolism of CHD patients and the resistance to anemia treatment[5,6]. Increased serum levels of hepcidin have indeed been reported in ESRD and CHD[2,5,7-11]. In response to increased iron stores, hepcidin inhibits intestinal iron absorption and iron recycling from monocytes by binding and inactivating the iron exporter Ferroportin-1. The consequent inhibition of iron export from duodenocytes and macrophages results in decreased TS, and increases serum ferritin as a result of iron entrapment into macrophages. Increased hepcidin in ESRD may result from reduced glomerular filtration, subclinical inflammation, as hepcidin is an acute phase reactant, and increased iron stores due to chronic supplementation. On the other hand, hepcidin is downregulated by anemia, hypoxia, and erythropoietin[12].

The upregulation of hepcidin transcription in response to iron is mediated by a mechanism depending on the interaction of various proteins including the hereditary hemochromatosis protein HFE, and matriptase-2 (TMPRSS6). We previously reported that in CHD patients common HFE mutations that alter hepatic iron sensing[13] were associated with lower hepcidin levels relatively to iron stores[6,14], achievement of target Hb levels for lower doses of iron, and with reduced mortality due to sepsis and cardiovascular disease, previously linked to more intense iron supplementation[15-18]. These initial results are in line with the hypothesis that inhibition of hepcidin in CHD may improve anemia control, and even survival in CHD patients[2,3,19,20].

The TMPRSS6 gene encodes for matriptase-2, a membrane-bound protease that decreases hepcidin transcription by cleaving hemojuvelin. Rare loss-of-function germline mutations of TMPRSS6 cause iron-refractory iron-deficiency anemia related to extremely high hepcidin levels, whereas the common rs855791 polymorphisms resulting in the p.A736V substitution is a major determinant of iron status in healthy subjects. Indeed, in the general population the p.736 V allele (henceforth 736 V) has been associated with lower serum iron, higher hepcidin[20,21], and decreased Hb[22-24], due to a less efficient inhibition of hepcidin transcription[21]. Furthermore, the p.A736V polymorphism has been shown to influence iron overload in hereditary hemochromatosis and nonalcoholic fatty liver disease[25,26]. However, it is not known whether the A736V variant influences iron metabolism during chronic inflammation and renal failure.

In the hypothesis that increased hepcidin is involved in the deregulation of iron metabolism and the anemia of CHD, the aim of this study were to evaluate whether the TMPRSS6 A736V polymorphism influences hepcidin levels and erythropoiesis parameters in CHD patients.

Methods

Subjects

We considered 199 CHD patients treated at the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico from June 2006 to June 2011[14]. Patients were dialyzed with synthetic biocompatible membranes and bicarbonate dialysate thrice in week (t.i.w.), and given i.v. recombinant human Epo (EprexW) t.i.w., at a dose aimed to maintain hemoglobin (Hb) between 10.5 and 12 g/dl. Iron was administered i.v. as Fe^{3+}-gluconate (FerlixitW) when TS was less than 30% or ferritin <200 ng/ml, and suspended when ferritin was above 500 ng/ml [27]. Iron infusion was started once weekly and titrated according to requirements.

Baseline venous blood samples for complete blood count, iron parameters, and markers of inflammation (tested by standard methods) were collected in the morning before hemodialysis (the first weekly session) at standardized times after the last administration of therapies potentially altering iron status and hepcidin release: one week after the last dose of i.v. iron, and 3 days after the last dose of Epo (all these conditions were contemporarily satisfied for all patients). Aliquots of serum samples for hepcidin-25 and hepcidin-20 measurement, which were stored at −80°C until the analysis, were available in a subset of 157 and 99 patients, respectively [6]. Transferrin (TF) levels were only available from 2007, so that this variable could not be included in multivariate analyses.

We further selected a subgroup of 86 patients without inflammation or severe iron deficiency at baseline evaluation, arbitrarily defined on the basis of C reactive protein (CRP) levels <1 mg/dl and serum ferritin concentration >30 ng/ml, to avoid the confounding effect of acute inflammation and severe iron deficiency.

As reference population for hepcidin levels and for the prevalence of the genetic variants under study, we randomly selected 188 unrelated controls from the database of Val Borbera study [20]. Controls were unrelated subjects, with normal Hb (12–16 g/dl in females, 14–18 g/dl in males), ferritin (30–200 ng/ml in females, 40–300 ng/ml in males), and TS (16-45%), absence of homozygosity for the C282Y HFE mutation, and normal kidney function (estimated glomerular filtration rate according to simplified MDRD >60 ml/min), matched for age (± 5 years) and sex with CHD patients (for 11 patients, no match could be found).

Table 1.

Demographic, and clinical features of 199 consecutive CHD patients from Northern Italy with available evaluation of C282Y and H63D HFE genotype (183 with A736V TMPRSS6 evaluation) and 188 healthy controls

	All patients	Ferritin >30 ng/ml and CRP <1 mg/dl	Controls
N=	199	86	188
Gender F	79 (40)	40 (47)	82 (44)
Age years	64.3±14 [c]	63.8±14	60.0±17
BMI Kg/m^2	22.5±5 [c]	21.9±4 [c]	26.2±4.2
Dialysis duration months	34 {13-82}	37 {16-87}	-
Kt/V	1.3±0.2	1.3±0.2	-
Creatinine mg/dl	9.9±2.5 [c]	10.0±3 [c]	0.8±0.2
Active smoke	47 (24)	17 (20)	23 (16)
Albumin g/100 ml	3.7±0.5	3.8±0.4	-
CRP mg/dl	0.83 {0.4-2.5} [c]	0.40 {0.3-0.6} [c]	0.1 {0-0.2}
Hb g/dl	10.8±1.2 [c]	10.9±1.1 [c]	14.7±1.1
MCV fl	96±8 [c]	96±8 [c]	92±4
Serum iron µg/dl	55±23 [c]	58±19 [c]	98±25
Transferrin mg/dl	190±37 [c]	182±32 [c]	240±34
TS%	24.7±10 [c]	26.5±10 [c]	29.0±7
Ferritin ng/ml	265 {155-411} [c]	280 {204-445} [c]	84 {55-128}
Epo IU/Kg/week	100 {59-180}	106 {69-179}	0
Fe i.v. mg/month	94 {0-185}	93 {0-142}	0
Hepcidin-20 [a] nM	0.55 {0.55-5.3} [c]	1.39 {0.55-5.8}	0.87 {0.55-3.35}
Hepcidin-20 detectable	44 (45)	23 (51)	104 (55)
Hepcidin-25 [b] nM	7.1 {0.55-17.1}	6.8 {0.55-17.6}	7.4 {4.5-11.9}
Hepcidin-25 / ferritin [b]	0.024 [c]	0.021 [c]	0.080
	{0.007-0.067}	{0.005-0.051}	{0.046-0.14}

():% values, {}: interquartile range, CRP: C reactive protein, Hb: hemoglobin, MCV: mean corpuscular volume, TS: transferrin saturation, Epo: erythropoietin, IU: international units, i.v.: intravenous, wt: wild-type, available in 99 CHD patients (45 with ferritin >30 ng/ml and CRP <1 mg/dl) [b] available in 157 CHD patients (57 with ferritin >30 ng/ml and CRP <1 mg/dl); [c] $p < 0.05$ vs. healthy controls.

Clinical, genetic and demographic features of subjects included in the study are shown in Table 1. Each patient gave written informed consent. The study was conducted according to the principles contained in the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda hospital of Milan.

Genetic analysis and serum hepcidin assay

DNA was extracted from peripheral blood by the phenolchloroform method. HFE genotype (C282Y and H63D variants) and the TMPRSS6 rs855791 C > T polymorphism, (p.A736V variant) were assessed by sequence allele specific PCR as previously described [25,28]. Random samples were confirmed by direct sequencing. Quality controls were performed to verify the reproducibility of the results.

Table 2.

C282Y and H63D HFE and A736V TMPRSS6 genotypes of 183 CHD patients and 188 healthy controls from the Val Borbera study (p = ns)

	All patients	Controls
HFE C282Y and H63D*	N = 199	N = 188
wt/wt	139 (69.0)	112 (59.6)
H63D/wt	46 (23.0)	54 (28.7)
H63D/H63D	5 (2.5)	8 (4.3)
C282Y/wt	8 (4.0)	12 (6.4)
C282Y/H63D	3 (1.5)	2 (1.0)
TMPRSS6 A736V	N = 183	N = 188
A/A	61 (33.3)	60 (31.9)
A/V	75 (41.0)	93 (49.5)
V/V	47 (25.7)	35 (18.6)

Valid genotypic data were obtained for 100% of subjects analyzed. For 13 patients (6.5%) only HFE genotype was available from a previous study, due to the lack of possibility to get the consent for new genetic studies.

Table 3.

Clinical and genetic variables associated with hepcidin-25 levels at Spearman's rho test (univariate analysis) and multivariate analysis in CHD patients from Northern Italy

	Univariate		GLM model 1		GLM model 2	
	R	P	Estimate	p	Estimate	p
Ferritin ng/ml	+0.30	0.0001	0.27	0.004	0.27	0.005
CRP mg/dl	+0.29	0.0004	0.29	0.01	0.28	0.010
Transferrin mg/dl	−0.20	0.03	-	-	-	-
Epo IU/Kg/week	+0.16	0.06	0.18	0.94	-	-
HDL mg/dl	−0.20	0.06	-	-	-	-
Albumin g/l	−0.15	0.06	-	-	-	-
TMPRSS6 A/A HFE muts + vs. other genotypes	−0.19	0.02	-	-	−0.17	0.048

Generalized linear model (GLM) 1 included clinical variables correlated with hepcidin-25 at univariate analysis (reported for $p < 0.1$), which were available in the majority of patients, i.e. ferritin, CRP levels, and Epo dose (157 subjects included). The second model considered the major clinical independent determinants of hepcidin, plus the genetic factors analyzed (143 subjects included).

CRP: C reactive protein; Epo: erythropoietin, IU: international units, HDL: high density lipoprotein cholesterol; HFE muts +: positive for HFE mutations; GLM: generalized linear model.

For hepcidin measurement, we used a protocol based on SELDI-TOF mass spectrometry and copper-loaded immobilized metal-affinity capture ProteinChip arrays (IMAC30-Cu2+)[29], extensively validated in previous studies[13,20,21]. Concentrations of serum hepcidin-25 and hepcidin-20 were expressed as nM.

Statistical analysis

Results are expressed as means ± SD for normally distributed variables and as median {interquartile range} for non-normally distributed variables. Variables were correlated by Spearman's rho test, and data compared between groups by t-test or Wilcoxon test, according to data distribution. Frequencies were compared by Chi-square test. We also evaluated the hepcidin-25/ferritin ratio (H/F), an established marker of adequacy of hepcidin response to iron stores. Independent predictors of serum hepcidin-25 and Epo requirements were evaluated by multivariate analysis (generalized linear model, GLM) including the variables identified as significantly associated with hepcidin at univariate analysis and available for all subjects, as specified in the result section. Log transformations were applied to normalize skewed variables before multivariate analysis. Results were considered significant when p was lower than 0.05 (two-tailed).

Results

Frequency distribution of HFE and TMPRSS6 variants and hepcidin levels in patients and controls

The frequency distribution of the C282Y and H63D HFE variants and A736V of the TMPRSS6 variant did not violate Hardy-Weinberg equilibrium in both patients and controls ($p > 0.1$; Table 2), and was not significantly different between the two groups (p=ns). Serum hepcidin-25 levels were not significantly different between the whole group of CHD patients and controls (Table 1), whereas H/F ratio was lower in patients (Table 1). One hundred-four (52.3%) of CHD patients were classified as "iron-deficient" on the basis of guidelines for iron treatment in CHD, which takes into account the effect of inflammation on ferritin (ferritin <200 ng/ml and TS <30%) [27]. After the exclusion of these subjects, hepcidin-25 was higher in patients (9.31 {3.11-22.4} nM) than in controls ($p = 0.04$).

Table 4.

Clinical variables associated with hepcidin-25 levels at Spearman's rho test (univariate analysis) in 86 CHD patients from Northern Italy with ferritin levels > 30 ng/ml and CRP < 1 mg/dl (the characteristics of this subset of patients are reported in Table 1 and related results; associated variables are shown for p < 0.1)

	R	p
Ferritin ng/ml	+0.38	<0.0001
MCV fl	−0.47	0.002
Transferrin mg/dl	−0.23	0.012
TS%	+0.23	0.069
CRP mg/dl	+0.21	0.095

MCV: mean corpuscular volume; TS: transferrin saturation; CRP: C reactive protein.

Clinical determinants of hepcidin in CHD patients

Clinical variables associated with hepcidin-25 levels in CHD patients are shown in Table 3. Hepcidin-25 was correlated with ferritin and CRP levels, and negatively associated with TF. In patients without severe iron deficiency and with normal CRP levels (Table 4), hepcidin-25 was correlated with ferritin, and inversely correlated with TF and mean corpuscular volume (MCV) values. The method used for the assessment of hepcidin-25 allows also the quantification of hepcidin-20, an amino-terminal truncated isoform, which is postulated to represent a degradation product of hepcidin-25 with no activity on iron metabolism, but possibly involved in antimicrobial response. Regarding the truncated hepcidin isoform (hepcidin-20)[30], the absolute levels were

slightly lower in the whole CHD group than in controls, but these data were available in a limited subgroup of patients, and the prevalence of detectable hepcidin-20 levels was the same as in controls. On the other hand, absolute hepcidin-20 levels were slightly higher in the CHD subgroup of patients without functional iron deficiency (Table 1), while the difference did not reach statistical significance. Because of the small subgroups that could be analyzed, it is possible that these nominal differences represent false positive results, and no definite conclusion can be drawn. Variables associated with hepcidin-20 levels are shown in Table 5.

Table 5.

Clinical variables associated with hepcidin-20 levels at at Spearman's rho test (univariate analysis) and multivariate generalized linear model (GLM) in 99 CHD patients from Northern Italy (reported for $p < 0.1$ for clinical variable)

	Univariate		GLM	
	R	P	Estimate	p
Hepcidin-25 nM	+0.60	<0.0001	+0.63	<0.0001
Active smoke	−0.25	<0.0001	−0.27	0.0016
Transferrin mg/dl	−0.23	0.032	-	-
TS%	+0.21	0.039	+0.13	0.22
TMPRSS6 A/A HFE muts + vs. other genotypes	−0.18	0.12	+0.13	0.99

TS: transferrin saturation; HFE muts +: positive for HFE mutations; GLM: generalized linear model.

The major determinant of hepcidin-20 was hepcidin-25, but active smoking was also independently associated with lower hepcidin-20. In control subjects, serum hepcidin-20 levels were not significantly lower in active smokers vs. non-smokers and previous smokers (median 0, IQR {2.99-6.38}, vs. 1.67 {3.68-6.59} nM, respectively; p = 0.17), even after correction for hepcidin-25 (p = 0.08).

Effect of TMPRSS6 and HFE variants on hepcidin, iron, and erythropoiesis

The effect of HFE genotype (wild-type, heterozygosity for C282Y and H63D mutations, and other genotypes) on hepcidin-25 in CHD patients is shown in Figure 1A. Hepcidin-25 was lower in patients with HFE mutations than in those without (p=0.01), in particular in those carrying the C282Y mutation or homozygous for the H63D mutation (p = 0.0004). The effect of HFE genotype on the H/F ratio is shown in Figure 1B. The H/F ratio was lower in patients with HFE mutations

than in those without (p = 0.04). Therefore, we grouped together patients with any HFE mutation in further analyses, in order to better characterize the effect of TMPRSS6 genotype.

The combined HFE (presence or absence of HFE mutations) and TMPRSS6 A736V genotypes influenced serum hepcidin-25 levels (p < 0.0001; Figure 2A). In line with the hypothesized negative effect of HFE mutations and of the 736A allele on hepcidin transcription, patients negative for HFE mutations had higher hepcidin-25 levels than patients with 736A/A and positive for HFE mutations (p < 0.05). Furthermore, in patients with HFE mutations, those with the 736 V/V genotype had higher hepcidin-25 than those with the 736A/A genotype (p = 0.017). Similar results were obtained for the H/F ratio in CHD patients, i.e. the 736 V/V genotype was associated with significantly higher H/F than the 736A/A genotype in patients with, but not in those without HFE mutations (not shown). At multivariate analysis (Table 3), the HFE positive 736A/ A genetic status was associated with lower hepcidin-25 levels independently of ferritin and CRP levels (p = 0.048). Patients negative for HFE mutations had higher hepcidin-20 levels than patients with 736A/A plus HFE mutations (Figure 2B). At multivariate analysis (Table 5), the effect of genetic factors on hepcidin-20 levels was not independent of hepcidin-25 levels.

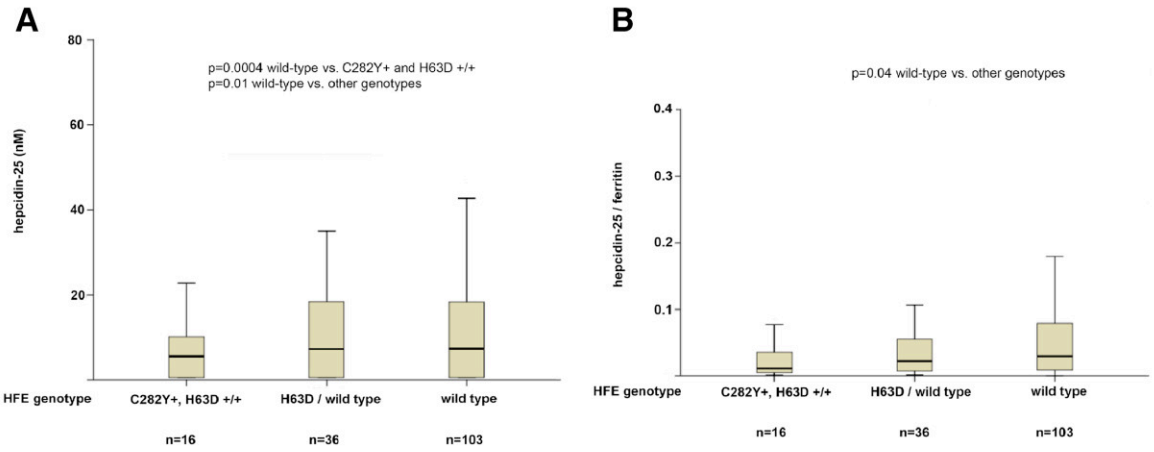

Figure 1. Effect of HFE C282Y and H63D genotype status (wild-type, heterozygosity for the H63D mutation, other genotypes) on hepcidin-25 levels (panel A), and hepcidin-25 / ferritin ratio (panel B) in 155 CHD patients from Northern Italy. doi:10.1186/1471-2369-14-48

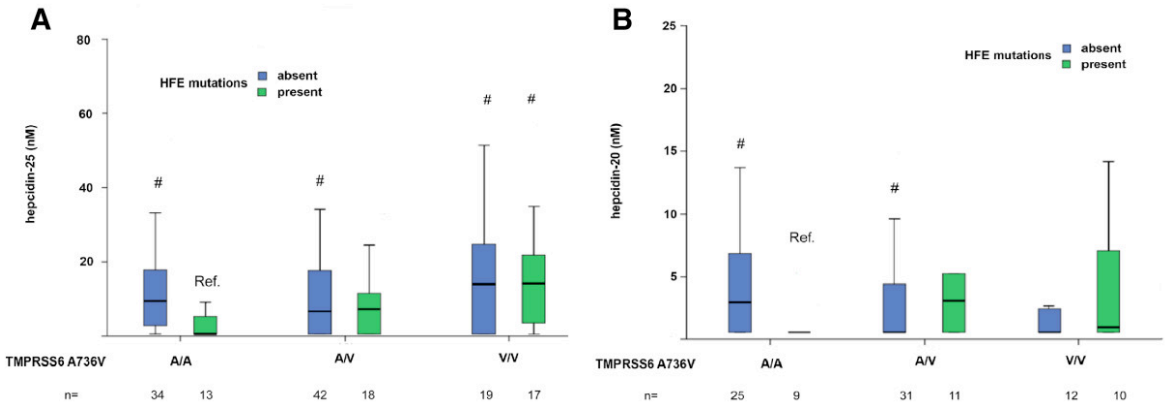

Figure 2. Combined effect of HFE C282Y and H63D and TMPRSS6 rs855791 (A736V) polymorphisms on hepcidin-25 levels in 143 (panel A), and on hepcidin-20 in 99 (panel B) CHD patients from Northern Italy. # $p < 0.05$ vs. TMPRSS6 A/A and HFE mutations present. doi:10.1186/1471-2369-14-48

Besides with hepcidin-25 values, as in the general population[20] the presence of HFE mutations was associated with lower TF levels ($p = 0.03$), and with a lower dose of iron supplementation to achieve the Hb target ($p = 0.03$).

TMPRSS6 A736V polymorphism was not associated with Hb levels and iron parameters in the overall CHD cohort. In order to avoid the confounding effect of acute inflammation and severe iron deficiency, we analyzed whether the TMPRSS6 A736V polymorphism influenced iron parameters and erythropoiesis in patients with CRP < 1 mg/dl and ferritin > 30 ng/ml. Results are presented in Table 6. In this subset of patients, the number of "high hepcidin" 736 V alleles was correlated with higher Epo requirement to control anemia ($p = 0.027$). In line with the negative effect of increased hepcidin on iron availability and erythropoiesis, there was also a trend for an association between the 736 V allele and lower iron and MCV values. At multivariate analysis adjusted for CRP levels, the number of 736 V TMPRSS6 alleles carried by CHD patients was associated with the weight-adjusted Epo dosage required to achieve the target Hb levels ($p = 0.02$, estimate coefficient 45, 95% c.i. 7–82).

Discussion

In CHD patients, it is usually difficult to control anemia because of a complex derangement of iron metabolism, which is due to chronic inflammation, blood losses, and concomitant Epo administration[2]. Increased serum levels of hepcidin, the hepatic hormone regulating iron metabolism, have been suggested to contribute to the functional iron deficiency that limit erythropoiesis in CHD [2,3,31]. Differently from what reported in smaller series of patients with unmatched controls, including previous studies from our group[5-8,32,33], we found that hepcidin-25 levels were not significantly increased in the whole CHD population. Nevertheless, accordingly to the previous reports[5-8,32,33], the major determinants of hepcidin were serum ferritin and CRP levels. The failure to confirm a relative hyper-hepcidinemia in the overall CHD cohort could be explained

by a number of reasons: i) the inclusion of a large and less selected CHD population compared to previous studies, more closely reflecting patients observed in clinical practice, including those requiring high doses of Epo and with relative iron deficiency (factors both known to reduce hepcidin), as suggested by higher hepcidin levels in patients without functional iron deficiency; ii) the relatively low average iron stores in this cohort (as reflected by median serum ferritin levels of only 265 ng/ml, Table 1) because of a local policy aimed at minimizing iron supplementation, due to long standing interest in iron metabolism and the side effects of iron overload; and iii) at variance with previous studies, e.g. Zaritsky et al. [32], the meticulous matching of the controls for age and gender, recently established as major determinants of hepcidin-25 at population level[20,34], as well as the systematic exclusion of even subclinical iron deficiency in controls, which both contributed to a more realistic comparison of hepcidin-25 levels than those made until now. Accordingly with these considerations, however, when CHD patients with relative iron deficiency were excluded from these analyses, hepcidin-25 was actually higher in CHD than in controls. Anyway, the comparison of hepcidin levels between CHD patients and controls was not the main aim of the present study, which was not therefore specifically designed to achieve this goal. Our results in this sense need to be confirmed in similarly large patient populations and matched controls.

Table 6.

Association of TMPRSS6 A736V polymorphism (736 V allele, additive model) with iron and erythropoietic parameters at Spearman's rho test (univariate analysis) in 86 CHD patients from Northern Italy with ferritin > 30 ng/ml and CRP < 1 mg/dl (the characteristics of this subset of patients are reported in Table 1 and related results; associated variables are shown for p < 0.1)

	R	p
Serum iron µg/ml	−0.26	0.077
CRP mg/dl	+0.22	0.061
MCV fl	−0.28	0.061
Epo IU/Kg/week	+0.28	0.027

CRP: C reactive protein; MCV: mean corpuscular volume; Epo: erythropoietin; IU: international units.

Clearance of hepcidin by hemodialysis[29] may possibly compensate for increased production, and explain the reduced H/F ratio in patients compared to controls. Notwithstanding, in patients without severe iron deficiency and active inflammation at the time of evaluation, hepcidin was associated with lower MCV, i.e. with iron-restricted erythropoiesis, suggesting that it negatively influences

iron availability to the erythron, and that it represents a potential therapeutic target to improve anemia management.

The specific aim of this study was to evaluate whether the A736V TMPRSS6 polymorphism regulating hepcidin transcription, a determinant of iron-restricted erythropoiesis in the general population[21,35], influences hepcidin levels and erythropoiesis in CHD. To increase the power of this analysis, patients were stratified for the presence of loss-of-function HFE mutations, that we preliminarily confirmed to influence hepcidin in this series[6]. The major finding was that the 736 V TMPRSS6 loss-of -function variant appears to modulate the effect of HFE mutations on hepcidin. Indeed, the A736V polymorphism influenced serum hepcidin in patients positive for HFE mutations. This suggests that the 736 V variant with defective proteolytic activity determining increased hepcidin transcription[21] may abrogate the inhibitory effect of HFE mutations on hepcidin. Thus, the A736V TMPRSS6 variant appears as a modifier of the phenotypic expression of HFE mutations in patients with CHD, who are characterized by chronic subclinical inflammation.

Furthermore, in patients without overt iron deficiency and acute inflammation, the 736 V variant was associated with higher hepcidin levels and with higher requirement of Epo for anemia management, thus suggesting that the effect of TMPRSS6 genotype translates into clinically detectable differences in erythropoiesis. Importantly, at multivariate analysis the association between TMPRSS6 genotype and Epo maintenance dose was independent of subclinical inflammation, as indicated by CRP levels. These data are in line with the association between TMPRSS6 736 V with hepcidin levels, and in turn with the positive association of hepcidin with the Epo maintenance dose in the same subgroup. Therefore, inhibition of hepcidin might be helpful for a better control of anemia in patients predisposed to high hepcidin release[19]. Evaluation of the impact of HFE and TMPRSS6 genotype on the survival of CHD patients after adequate follow-up would be instrumental to fully define their clinical impact.

Conclusions

In conclusion, in CHD patients the A736V TMPRSS6 genotype influences hepcidin levels, and in the absence of acute inflammation and severe iron deficiency, also erythropoiesis and anemia management. Evaluation of the effect of TMRPSS6 genotype on clinical outcomes in prospective studies in CHD patients may be useful to predict the outcomes of hepcidin manipulation, to develop new approaches to optimize anemia management, and to guide treatment personalization.

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REFERENCES

1. Paganini EP 1989 Overview of anemia associated with chronic renal disease: primary and secondary mechanisms. Semin Nephrol 1989, 9:3–8.

2. Canavesi E, Valenti L Modulation of Iron Metabolism and Hepcidin Release by HFE Mutations in Chronic Hemodialysis Patients: Pathophysiological and Therapeutic Implications. In Hemodialysis. Different Aspects. Edited by Maria Goretti P. doi:10.5772/22735. Available from: www.intechopen.com/books/hemodialysis-different-aspects/ modulation-of-iron-metabolism-and-hepcidin-release-by-hfe-mutations-in- chronic-hemodialysis-patients. ISBN 978-953-307-315-6.

3. Babitt JL, Lin HY 2010 Molecular mechanisms of hepcidin regulation: implications for the anemia of CKD. Am J Kidney Dis 2010, 55:726–741.

4. Kalantar-Zadeh K, Rodriguez RA, Humphreys MH 2004 Association between serum ferritin and measures of inflammation, nutrition and iron in haemodialysis patients. Nephrol Dial Transplant 2004, 19:141–149.

5. Kato A, Tsuji T, Luo J, Sakao Y, Yasuda H, Hishida A 2008 Association of prohepcidin and hepcidin-25 with erythropoietin response and ferritin in hemodialysis patients. Am J Nephrol 2008, 28:115–121.

6. Valenti L, Girelli D, Valenti GF, Castagna A, Como G, Campostrini N, Rametta R, Dongiovanni P, Messa P, Fargion S 2009 HFE mutations modulate the effect of iron on serum hepcidin-25 in chronic hemodialysis patients. Clin J Am Soc Nephrol 2009, 4:1331–1337.

7. Tomosugi N, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H, Ishikawa I 2006 Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. Blood 2006, 108:1381–1387.

8. Kuragano T, Itoh K, Shimonaka Y, Kida A, Furuta M, Kitamura R, Yahiro M, Nanami M, Otaki Y, Hasuike Y, et al 2011 Hepcidin as well as TNF-{alpha} are significant predictors of arterial stiffness in patients on maintenance hemodialysis. Nephrol Dial Transplant 2011, 26:2663–2667.

9. Malyszko J, Mysliwiec M 2007 Hepcidin in anemia and inflammation in chronic kidney disease. Kidney Blood Press Res 2007, 30:15–30.

10. Morelle J, Labriola L, Jadoul M 2009 Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009, 76:1116. author reply 1116.

11. Costa E, Swinkels DW, Laarakkers CM, Rocha-Pereira P, Rocha S, Reis F, Teixeira F, Miranda V, do Sameiro Faria V, Loureiro A, et al: 2009 Hepcidin serum levels and resistance to recombinant human erythropoietin therapy in haemodialysis patients. Acta Haematol 2009, 122:226–229.

12. Pinto JP, Ribeiro S, Pontes H, Thowfeequ S, Tosh D, Carvalho F, Porto G 2008 Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. Blood 2008, 111:5727–5733.

13. Girelli D, Trombini P, Busti F, Campostrini N, Sandri M, Pelucchi S, Westerman M, Ganz T, Nemeth E, Piperno A, Camaschella C 2011A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis. Haematologica 2011, 96:500–506.

14. Valenti L, Valenti G, Como G, Santorelli G, Dongiovanni P, Rametta R, Fracanzani AL, Tavazzi D, Messa PG, Fargion S 2008 HFE Genotype Influences Erythropoiesis Support Requirement in Hemodialysis Patients: A Prospective Study. Am J Nephrol 2008, 28:311–316.

15. Valenti L, Valenti G, Como G, Burdick L, Santorelli G, Dongiovanni P, Rametta R, Bamonti F, Novembrino C, Fracanzani AL, et al 2007 HFE gene mutations and oxidative stress influence serum ferritin, associated with vascular damage, in hemodialysis patients. Am J Nephrol 2007, 27:101–107.

16. Kalantar-Zadeh K, Regidor DL, McAllister CJ, Michael B, Warnock DG 2005 Time-dependent associations between iron and mortality in hemodialysis patients. J Am Soc Nephrol 2005, 16:3070–3080.

17. Valenti L, Dongiovanni P, Motta BM, Swinkels DW, Bonara P, Rametta R,
Burdick L, Frugoni C, Fracanzani AL, Fargion S 2011 Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations. Arterioscler Thromb Vasc Biol 2011, 31:683–690.

18. Valenti L, Swinkels DW, Burdick L, Dongiovanni P, Tjalsma H, Motta BM, Bertelli C, Fatta E, Bignamini D, Rametta R, et al 2010 Serum ferritin levels are associated with vascular damage in patients with nonalcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2010, 21:568–575.

19. Sasu BJ, Cooke KS, Arvedson TL, Plewa C, Ellison AR, Sheng J, Winters A, Juan T, Li H, Begley CG, Molineux G 2010 Antihepcidin antibody treatment modulates iron metabolism and is effective in a mouse model of inflammation-induced anemia. Blood 2010, 115:3616–3624.

20. Traglia M, Girelli D, Biino G, Campostrini N, Corbella M, Sala C, Masciullo C, Vigano F, Buetti I, Pistis G, et al 2011 Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 2011, 48:629–634.

21. Nai A, Pagani A, Silvestri L, Campostrini N, Corbella M, Girelli D, Traglia M, Toniolo D, Camaschella C 2011 TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. Blood 2011, 118:4459–4462.

22. Finberg KE 2009 Iron-refractory iron deficiency anemia. Semin Hematol 2009, 46:378–386.

23. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP, Hottenga JJ, Henders AK, Campbell MJ, Wallace L, et al 2009 Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009, 41:1173–1175.

24. Chambers JC, Zhang W, Li Y, Sehmi J, Wass MN, Zabaneh D, Hoggart C, Bayele H, McCarthy MI, Peltonen L, et al 2009 Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet 2009, 41:1170–1172.

25. Valenti L, Fracanzani A, Rametta R, Fraquelli M, Soverini G, Pelusi S, Dongiovanni P, Conte D, Fargion S 2012 Effect of the A736V TMPRSS6 polymorphism on the penetrance and clinical expression of hereditary hemochromatosis. J Hepatol 2012, 57:1319–1325.

26. Valenti L, Rametta R, Dongiovanni P, Motta BM, Canavesi E, Pelusi S, Pulixi EA, Fracanzani AL, Fargion S 2012 The A736V TMPRSS6 Polymorphism Influences Hepatic Iron Overload in Nonalcoholic Fatty Liver Disease. PLoS One 2012, 7:e48804.

27. Locatelli F, Aljama P, Barany P, Canaud B, Carrera F, Eckardt KU, Horl WH, Macdougal IC, Macleod A, Wiecek A, Cameron S 2004 Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. Nephrol Dial Transplant 2004, 19(Suppl 2):ii1–ii47.

28. Valenti L, Canavesi E, Galmozzi E, Dongiovanni P, Rametta R, Maggioni P, Maggioni M, Fracanzani AL, Fargion S 2010 Beta-globin mutations are associated with parenchymal siderosis and fibrosis in patients with non- alcoholic fatty liver disease. J Hepatol 2010, 53:927–933.

29. Campostrini N, Castagna A, Zaninotto F, Bedogna V, Tessitore N, Poli A, Martinelli N, Lupo A, Olivieri O, Girelli D 2010 Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. J Biomed Biotechnol 2010, 2010:329646.

30. Campostrini N, Traglia M, Martinelli N, Corbella M, Cocca M, Manna D, Castagna A, Masciullo C, Silvestri L, Olivieri O, et al 2012 Serum levels of the hepcidin-20 isoform in a large general population: The Val Borbera study. J Proteomics 2012, 76:28–35.

31. Babitt JL, Lin HY 2012 Mechanisms of anemia in CKD. J Am Soc Nephrol 2012, 23:1631–1634.

32. Zaritsky J, Young B, Gales B, Wang HJ, Rastogi A, Westerman M, Nemeth E, Ganz T, Salusky IB 2010 Reduction of serum hepcidin by hemodialysis in pediatric and adult patients. Clin J Am Soc Nephrol 2010, 5:1010–1014.

33. Bratescu LO, Barsan L, Munteanu D, Stancu S, Mircescu G 2010 Is hepcidin-25 a clinically relevant parameter for the iron status in hemodialysis patients? J Ren Nutr 2010, 20:S77–S83.

34. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, van Tienoven D, Wetzels JF, Kiemeney LA, Sweep FC, den Heijer M, Swinkels DW 2011 Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011, 117:e218–e225.

35. Delbini P, Vaja V, Graziadei G, Duca L, Nava I, Refaldi C, Cappellini MD 2010 Genetic variability of TMPRSS6 and its association with iron deficiency anaemia. Br J Haematol 2010, 151:281–Äì284.

Part III

Identification of novel loci affecting the storage and distribution of body iron: a genetic approach

The following chapters report GWAS performed on hepcidin and iron traits, risk factor for common genetic disorders of iron homeostasis, to highlight the role of known loci and to find novel loci involved in iron metabolism.

The first study dissects the association of HFE and TMPRSS6 to erythroid traits and iron parameters and the role of hepcidin. For the first time hepcidin was measured in a large population and variantion in its level could be associated to HFE and TMPRSS6 variants and studied in correlation with the other iron parameters.

To highlight novel loci associated to hepcidin levels in healthy individuals, a first meta-analysis has been organized on 6,000 European individuals from the VB cohort and two Dutch cohorts and the results are shown in the second study.

The third analysis reports genetic association studies of serum iron, ferritin, transferrin and transferrin saturation in a large international study. Meta analysis of GWAS of eleven cohorts of european origin, including 48,000 individuals, have been carried out and several novel loci affecting iron homeostasis and lipid metabolism in humans were highlighted, confirming the correlation between iron and lipid metabolism.

In the last study a subset of cohorts from the meta-analysis has been used to quantify the level of serum iron in Parkinson patients and the possible effect of the known HFE and TMPRSS6 variants as predictor of Parkinson disease (PD) risk.

Chapter 6

Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations

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Abstract

Background Hepcidin is the main regulator of iron homeostasis: inappropriate production of hepcidin results in iron overload or iron deficiency and anaemia.

Aims To study variation of serum hepcidin concentration in a normal population.

Results Hepcidin showed age and sex dependent variations that correlated with ferritin but not with serum iron and transferrin saturation. The size of the study population was underpowered to find genome wide significant associations with hepcidin concentrations but it allowed to show that association with serum iron, transferrin saturation and erythrocyte traits of common DNA variants in HFE (rs1800562) and TMPRSS6 (rs855791) genes is not exclusively dependent on hepcidin values. When multiple interactions between environmental factors, the iron parameters and hepcidin were taken into account, the HFE variant, and to lesser extent the TMPRSS6 variant, were associated with ferritin and with hepcidin normalised to ferritin (the hepcidin/ferritin ratio).

Conclusions The results suggest a mutual control of serum hepcidin and ferritin concentrations, a mechanism relevant to the pathophysiology of HFE haemochromatosis, and demonstrate that the HFE rs1800562 C282Y variant exerts a direct pleiotropic effect on the iron parameters, in part independent of hepcidin.

Introduction

Iron is essential for multiple biological functions in all tissues, but especially for haemoglobin synthesis, as shown by anaemia that results from iron deficiency. Excess iron is toxic, because it favours oxidative stress and cell damage[1]. For this reason, the amount of plasma iron is maintained within narrow limits and is tightly regulated by the liver peptide hepcidin according to the body's needs[2]. Hepcidin controls the surface expression of the iron exporter ferroportin on enterocytes and iron recycling macrophages[3]. Genetic disorders of the hepcidineferroportin pathway lead to opposite conditions. Haemochromatosis is caused by mutations in genes which encode upstream hepcidin activating proteins (HFE, TFR2, hemojuvelin) or mutations in hepcidin itself. All these forms of haemochromatosis are characterised by inappropriately high iron absorption, elevated transferrin saturation and serum ferritin, and inappropriately low/undetectable hepcidin expression[4]. More rarely mutations affect ferroportin, the downstream target of hepcidin; as a consequence, either iron is not recycled and remains sequestered in macrophages or the mutant is not internalised because is hepcidin resistant[5]. Iron refractory, iron deficiency anaemia (IRIDA) is caused by mutations of TMPRSS6, which encodes the liver expressed hepcidin inhibitor serine protease matriptase-2. Mask mice homozygous for a truncated matriptase-2 lacking the serine protease domain[6], Tmprss6 null mice[7], and patients with IRIDA[8] do not efficiently absorb oral iron because they are unable to fully suppress hepcidin activation[9,10]. They display very low transferrin saturations but moderately decreased serum ferritin because of iron retention in macrophage stores[11]. Genetic variants of two of the hepcidin regulatory genes, TMPRSS6 and HFE, affect serum iron concentration[12] and transferrin saturation[13,14] in normal populations. Furthermore, single nucleotide polymorphisms (SNPs) at TMPRSS6 and HFE loci were found to be associated with quantitative variations of haemoglobin (Hb) concentrations and erythrocyte traits.[15-18] However, it remains uncertain whether the association is iron mediated or dependent on a direct effect of the variants on erythropoiesis. In addition, the effect of the 'iron gene' variants was ascribed to variations in hepcidin concentrations[15,16,18], but serum hepcidin was not measured.

We report here the analysis of serum hepcidin concentrations, measured by a mass spectrometry based method[19], in 1657 normal individuals from the Val Borbera (VB) genetic isolate in Northern Italy. We explored relationships between hepcidin and a set of anthropometric, haematologic, and iron parameters and tested the association of two common variants rs1800562 and rs855791 in the HFE and TMPRSS6 genes, respectively, with iron, erythrocyte parameters and hepcidin values in 1545 genotyped individuals. We demonstrate a reciprocal control of serum hepcidin and ferritin concentrations that may be relevant to the pathophysiology of HFE haemochromatosis, and demonstrate that the HFE C282Y variant exerts a direct pleiotropic effect on several of the iron parameters, partly independent of hepcidin.

Methods

Study subjects

The VB population has been previously described[20]. Only individuals aged 18 years or older were eligible to participate in the study. The study was approved by the San Raffaele Hospital and Regione Piemonte ethical committees. The health status of the population was assessed as reported[20]. Blood tests Fasting blood samples were obtained in the early morning. Blood was analysed the same day or aliquoted and stored at -80°C for further analysis. Blood cell counts and erythrocyte indexes were determined with an automated cell counter[21]. Other blood tests, serum iron, transferrin, and ferritin were determined by standard methods. Transferrin saturation was calculated dividing serum iron by transferrin (mg) X 1.42[22]. Serum hepcidin assay Serum hepcidin was measured in all samples with a validated mass spectrometry based method, that is surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) using a PCS4000 (Bio-Rad, Hercules, California, USA) mass spectrometer, copper loaded immobilised metal affinity capture ProteinChip arrays (IMAC30-Cu2+), and a synthetic hepcidin analogue (hepcidin-24, Peptides International, Louisville, Kentucky, USA) as an internal standard[19], with recent technical improvements[23]. The lower limit of detection was 0.55 nM. The intra- and inter-assay coefficient of variations of this method ranged from 6.1-7.3% and from 5.7-11.7% (mean 7.7%), respectively. In order to produce comparable results and to override the circadian rhythm of hepcidin[24,25], measurements were performed on samples obtained in all cases after an overnight fast.

Statistical analysis

Statistical analyses were performed by using STATA V.9 (StataCorp). Comparisons of all measured parameters in men and women were performed using the t test. Sex specific correlation analysis was used to assess the linear relationship between s-hepcidin and all other parameters. Subsequently, simple and multiple linear regression analyses were employed to find best predictors of serum hepcidin.

Heritability analysis.

The heritability analysis was performed using SOLAR (Sequential Oligogenic Linkage Analysis Routines ver. 4.1.2) (http://solar.sfbrgenetics.org/), as described[20]. As the distributions were not normal, a log10 transformation was performed for hepcidin, hepcidin/ferritin, and ferritin. For all phenotypes, individuals presenting values more than four SDs from the mean were removed.

Genotyping and association analysis.

One thousand six hundred and sixty-four DNAs from the VB population were genotyped using the Illumina 370 Quad-CNV array, v3. DNAs with more than 10% missing genotypes, SNPs that failed

the Hardy-Weinberg Equilibrium test ($p<10^{-6}$) and with minor allele frequency <0.01 were removed. 343 866 SNPs, which included common SNPs in HFE, TMPRSS6 and transferrin (TF) genes, were used in the analyses. Genome-wide association analysis (GWAS) was done on the directly genotyped SNPs using the GenABEL package[26] that takes into account the relatedness among the VB population, using genomic kinship. To account for multiple testing, the p value cut-off for GW significance was 1.5E-7. An additive model was used on the standardised residuals of each quantitative trait adjusted for the effects of sex, age, sex*age, squared age and sex*square age. Each trait was checked for normality with non-parametric tests. A log10 transformation was performed for hepcidin, hepcidin/ferritin ratio, and ferritin. All the other traits (serum iron, transferrin, transferrin saturation) did not require normalisation. For all phenotypes, individuals with values more than four SDs were removed. All the analyses were done on the whole sample and on a selected subset (subset 1) where individuals affected by acquired conditions known to alter iron metabolism and hepcidin concentrations were omitted. This includes subjects with C reactive protein (CRP) >1 mg/dl (as marker of clinical inflammatory conditions) and serum ferritin <30 ng/ml (as marker of iron deficiency).

Results

Serum hepcidin concentrations show age and sex related changes and strongly correlate with serum ferritin Serum hepcidin was determined in 1657 subjects, 929 females and 728 males, age range 18e98, mean age 55.4617.8 years (supplemental figure S1). Anthropometric data, red cell parameters, serum iron, transferrin, transferrin saturation, and ferritin concentrations were available for all samples (supplemental table S1).

Hepcidin and most iron parameters showed striking age and sex dependent variations (figure 1 and supplemental table S2), particularly evident for ferritin (figure 1B), hepcidin (figure 1C) and, to a lesser extent, for transferrin saturation (figure 1A). Ferritin concentrations were lower in females aged <50 years and significantly higher in older females, while they remained stable across the different age groups in males (figure 1B). Serum hepcidin concentrations showed variations analogous to those of ferritin in individuals <50 years old, were similar in males and females aged 50e70, whereas among the elderly they were lower in females. The hepcidin/ferritin ratio, used to correct for hepcidin changes according to iron stores[27,28], clearly indicated the large difference between young males and females that sharply decreased with ageing (figure 1D).

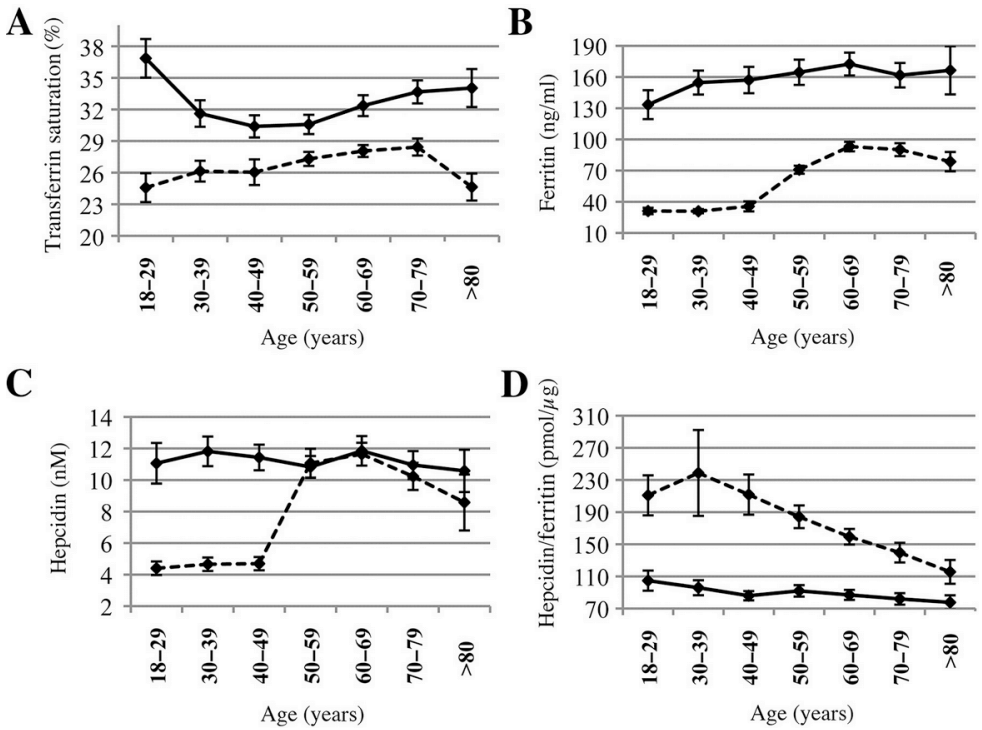

Figure 1. Age and sex dependent variations of transferrin saturation (A), serum ferritin (B), serum hepcidin (C), and hepcidin/ferritin ratio (D) in the whole population. Males are indicated by a continuous line, females by a dotted line. Bars indicate SEs.

Table 1 Multiple regression analysis of serum hepcidin by sex

	Males		Females	
Trait	**β**	**p**	**β**	**p**
Age	−0.0048	**	0.0430	****
Squared age	—	NS	−0.0004	****
Ferritin	0.0018	****	0.0036	****
Hb	−0.0669	**	—	NS
MCHC	−0.0923	****	—	NS
Total cholesterol	—	NS	0.0018	**

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0005.
Hb, haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Serum hepcidin correlation analysis by sex (supplemental table S3) showed that Pearson's correlations were negligible for all measured parameters except for ferritin (r=0.32 and r=0.53 in men and women, respectively) and CRP (r=0.25 in women) and were always greater in females. Hepcidin concentrations are known to be decreased or even suppressed by iron deficiency and increased by iron overload and inflammatory cytokines. To reduce the effect of these confounding environmental variables, 41 males and 296 females with iron deficiency defined by ferritin <30 ng/ml and 75 subjects with CRP values >1 mg/dl (eight with concomitant iron deficiency) or individuals missing the information were excluded from the analyses. We also excluded 50 individuals with undetectable hepcidin who had multiple causes that might account for hepcidin suppression, such as heavy alcohol intake, ß-thalassaemia trait, blood donations, and advanced age.

The remaining 1203 individuals (642 males and 561 females, mean age 56.86±17) are indicated as subset 1. To study correlations between hepcidin and iron and red cell parameters, separate linear regression analysis were performed in males and females from subset 1, using log transformed hepcidin and age as covariate (supplemental table S4). Significant variables were tested in multiple regression models (table 1). Age, ferritin, Hb and mean corpuscular haemoglobin concentration (MCHC) were independent predictors of hepcidin concentrations in males, accounting for 13.5% of the total hepcidin variability; in females age, ferritin and total cholesterol accounted for 17.7% of the total phenotypic variation. Only age and ferritin were common to both sexes. Serum iron, transferrin, and transferrin saturation lost their correlations with hepcidin, when adjusted for the other parameters. Consistent with serum ferritin being a predictor of hepcidin concentrations, when we clusterised the subjects according to ferritin values in three classes corresponding to iron deficiency (Ft<30 ng/ml), normal iron balance (30≤Ft≤200 ng/ml in females and 30≤Ft≤300 ng/ml in males) and iron overload (Ft>200 ng/ml in females and >300 ng/ml in males), mean hepcidin concentrations increased progressively and differed significantly among the three groups ($p<0.001$) (figure 2A). Considering classes of transferrin saturation that define iron deficiency (<16%), normal iron status (16-45%), and iron overload (>45%), no significant differences in hepcidin concentrations were observed (figure2B).

Hepcidin heritability is low and increases when hepcidin is corrected for ferritin concentrations.

The genetic component of the variability (heritability=H^2) of all iron parameters is quite relevant and was estimated also for the VB population, thanks to the availability of a complete genealogy[20]. The heritability of hepcidin, even calculated in subset 1, devoid of acquired confounding factors, was instead very low (H^2=0.098) and non-significant (table 2). In this analysis ferritin was the most significant covariate (p=1.5E-28). Accordingly, if the serum hepcidin concentrations were normalised to ferritin and the hepcidin/ ferritin ratio was considered, the heritability increased (H^2=0.219). Age and sex explained about 12% of the variability. None of the other iron parameters contributed to the model. We calculated the heritability of ferritin in the same dataset. Ferritin H^2 was significantly higher in subset 1 (H^2=0.455) than in the whole population (H^2=0.27) (table 2). Consistent with the correlation between hepcidin and ferritin, the heritability of ferritin decreased (H^2=0.381) in subset 1 if hepcidin was included as a covariate. In this case the covariates sex and hepcidin explained 29.9% of the variability. None of the other iron parameters was a significant covariate for ferritin heritability.

Association of TMPRSS6 and HFE variants with iron and erythrocyte parameters is replicated in the Val Borbera population.

The size of the study population was underpowered to find GW significant associations with hepcidin or hepcidin/ferritin ratio values (supplemental figure S2). However the availability of the hepcidin concentrations allowed us to evaluate better the effect of the common variants in two genes, TMPRSS6 and HFE, involved in monogenic disorders of iron metabolism[1]. These variants were previously found associated with iron parameters and red blood cells traits[12-14] and their effect was hypothesised to be dependent on hepcidin variation. Most findings of previous GWAS for iron parameters and erythrocyte traits were replicated in the VB population (supplemental table S5). In our series HFE rs1800562, corresponding to the C282Y variant, which at the homozygous state is responsible of hereditary haemochromatosis, was associated with serum iron (p¼3.95E-9), transferrin (p=4.95E-11), and transferrin saturation (p=2.64E-15) at GW significance and to lesser extent to mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and MCHC. rs855791, corresponding to the A736V of the serine protease TMPRSS6,13 was associated at GW significance with serum iron (p=9.41E-11) and transferrin saturation (p=3.89E-9). rs3811647 in the TF gene was associated only with transferrin concentrations (p=2.1E-16)[14].

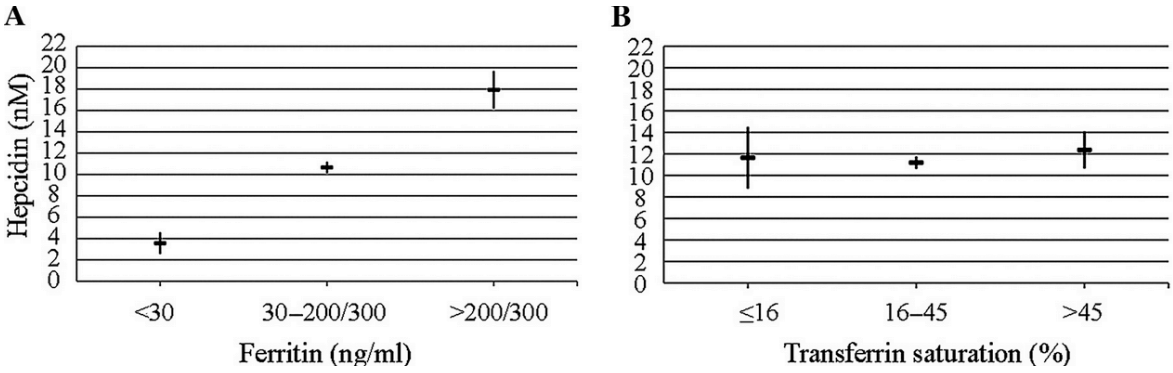

Figure 2. Serum hepcidin in groups of individuals classified according to serum ferritin (A) or transferrin saturation (TfSat) (B) concentrations. Three classes are shown: iron deficiency (ferritin <30 ng/ml and TfSat <=16%), normal iron status (intermediate values), and iron overload (ferritin >200 ng/ml in females/ >300 ng/ml in males and TfSat >45%). Mean values are age and sex adjusted by ANOVA (95% CI).

Table 2 Hepcidin and ferritin heritability

	Basic model		p Value of covariates								
Trait	H^2	p	Sex	Age	Age^2	Sex*age	$Sex*age^2$	Ft	Hep	Tf Sat	Effect of covariates
Log_{10}(hepcidin)	0.098	NS	**	NS	NS	****	**	ND	ND	ND	0.045
Log_{10}(hepcidin)†	0.080	NS	NS	NS	NS	****	****	****	ND	NS	0.139
Log_{10}(hepcidin/ferritin)‡	0.219	***	****	NS	NS	***	****	ND	ND	NS	0.117
Log_{10}(ferritin)	0.455	****	****	****	*	NS	NS	ND	ND	ND	0.225
Log_{10}(ferritin)§	0.381	****	****	NS	NS	NS	NS	ND	****	ND	0.299
Log_{10}(ferritin)¶	0.362	****	****	****	****	NS	*	ND	****	NS	0.399

*p<0.05; **p<0.01; ***p<0.001;****p<0.0005.
All models included sex, age, age^2, sex*age, $sex*age^2$ as covariates.
H2: Heritability corresponding to the genetic component of the variability of each trait; age^2: squared age; sex*age: interaction between sex and age; $sex*age^2$: interaction between sex and squared age.
†Ferritin, Tf Sat,Tf, Iron, MCH, MCHC, MCV, Hb, Hct were also included in the model. The only covariates significant (p<0.01) were iron and ferritin (p<0.0005).
‡This model included also all the variables but ferritin.
§This model included also hepcidin as covariate.
¶This model included also hepcidin, Tf Sat,Tf, Iron, MCH, MCHC, MCV, Hb and Hct.
Ft, ferritin; Hb, haemoglobin; Hct, haematocrit; Hep, hepcidin; MCH, mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; ND, not done; Tf Sat, transferrin saturation.

Association analysis of subset 1 showed an increased genetic effect of HFE and TMPRSS6 SNPs on iron and transferrin saturation and a smaller increase of TMPRSS6 effect on MCV and MCH (supplemental table S5). A large increase of the genetic effect was also found for the Tf SNP on iron and particularly on Tf. The association of TMPRSS6 rs855791 variant to red cell traits was reported as mostly dependent on the amount of iron available for erythropoiesis[13]. By using iron parameters as covariates in the regression analysis for MCV, MCH, and MCHC we found that iron, transferrin saturation and ferritin reduced the effect of both the HFE and TMPRSS6 in subset 1 while transferrin did not. Considering together iron, transferrin saturation and ferritin, HFE association was abolished and that of TMPRSS6 greatly reduced (supplemental table S6).

Association of common TMPRSS6 and HFE variants with iron parameters is not dependent on hepcidin concentrations.

Based on the results described above we were able to assess whether the effect of the two common genetic variants in TMPRSS6 and HFE considered was mediated by hepcidin. We used hepcidin as covariate in the association analysis of 1545 genotyped individuals that had serum hepcidin measured. For all iron and red blood cells parameters, the association of the HFE and TMPRSS6 SNPs did not change significantly (supplemental table S7), suggesting that the two variants may exert a direct effect on these parameters.

A novel association of hepcidin/ferritin ratio to TMPRSS6 and HFE variants.

The association of HFE rs1800562 variant with ferritin, which was GW borderline significant in the whole cohort (p=3.06E-7), became highly significant (p=7.49E-10) in subset 1, and the significance further increased if hepcidin was used as covariate (p=1.64E-10). TMPRSS6 rs855791 association with ferritin was nominally significant and did not greatly change after adjusting for covariates (table 3). We also tested whether the HFE and TMPRSS6 variants were associated with hepcidin. As expected from the heritability results, hepcidin was not associated unless it was normalised to ferritin (table 3). The hepcidin/ferritin ratio was associated with both variants in subset 1: the HFE rs1800562 variant was significantly associated (p=6.36E-04) but the effect was smaller than that observed for the other iron parameters. It explained only 1% of the variance compared to around 4% for ferritin and 3% for the other iron parameters (supplemental table S5). The VB cohort does not have enough statistical power to definitively demonstrate association of the TMPRSS6 rs855791 that was only nominally associated (p=1.49E-02).

Discussion

We report here the first large scale epidemiological and genetic study of serum hepcidin, the main regulator of plasma iron concentration, in the general adult population.

Table 3 Association of rs1800562 and rs855791 to hepcidin and ferritin

	Whole cohort					Subset 1				
Trait†	N	β	SE	p	R^2* (%)	N	β	SE	p	R^2* (%)
rs1800562 HFE§										
Log_{10}(hepcidin)	1545	0.064	0.075	3.97E-01	–	1130	0.150	0.087	8.27E-02	–
Log_{10}(hepcidin/ferritin)	1537	−0.207	0.076	6.80E-03	0.4	1128	−0.300	0.088	6.36E-04	1.0
Log_{10}(ferritin)	1656	0.389	0.076	3.06E-07	1.6	1130	0.556	0.090	7.49E-10	3.9
Log_{10}(ferritin)‡	1538	0.377	0.079	1.61E-06	1.6	1128	0.576	0.090	1.64E-10	4.2
rs855791 TMPRSS6¶										
Log_{10}(hepcidin)	1545	−0.015	0.037	6.86E-01	–	1130	0.021	0.043	6.20E-01	–
Log_{10}(hepcidin/ferritin)	1537	0.039	0.037	2.99E-01	–	1128	0.105	0.043	1.49E-02	0.5
Log_{10}(ferritin)	1656	−0.070	0.037	5.68E-02	–	1130	−0.111	0.044	1.18E-02	0.6
Log_{10}(ferritin)‡	1538	−0.092	0.038	1.51E-02	0.3	1128	−0.129	0.044	3.22E-03	0.9

*R^2: Adjusted R square of the linear regression corresponding to the variance of the trait explained by each locus.
†All the traits were corrected for covariates: sex, age, squared age, interaction between sex and age and interaction between sex and squared age.
‡This model included hepcidin as covariate.
§Minor allele is A for rs1800562 *HFE* encoding C282Y (frequency=0 0.065 in the whole cohort; frequency=0.068 in subset 1).
¶Minor allele is A for rs855791 *TMPRSS6* encoding A736V (frequency=0.45 in both datasets).

Hepcidin concentrations under normal iron homeostasis showed striking gender differences and variation across ages. While in males concentrations were rather stable, in females more dynamic changes were observed, paralleling the well known age related ferritin changes. Young females had significantly lower concentrations than males. Females aged 50-60 years showed hepcidin concentrations comparable to those of age matched males, but had significantly lower serum ferritin. This underlines that the threshold for hepcidin increase in response to body iron is lower in females. Among the elderly, hepcidin concentrations decreased in both genders paralleling ferritin reduction, even if the hepcidin decrease was more evident in females (figure 1). Hepcidin regulation was studied in human disorders of iron metabolism and in animal models: it is known to respond rapidly to increased circulating iron loaded transferrin[3,29] (measured by transferrin saturation) and tissue iron, whose surrogate index is serum ferritin. In our series serum hepcidin strongly correlated with serum ferritin in both sexes, confirming results previously observed in a small number of individuals[25,30] , but did not correlate with serum iron, transferrin, and transferrin saturation. Thus, although an acute increase in transferrin saturation triggers an hepcidin response[27,29,31], in steady conditions hepcidin appears mainly influenced by stored iron. Accordingly, hepcidin concentrations differed in groups of individuals whose iron status was assessed according to ferritin values, but not in groups classified according to transferrin saturation. The strong correlation between hepcidin and ferritin underscores the relevance of normalising the hepcidin concentrations using the hepcidin/ ferritin ratio, as proposed in hereditary haemochromatosis[27,28]. The correlation between hepcidin and ferritin concentrations was positive, reflecting the response of hepcidin to iron stores concentrations, likely through increased BMP6 and activation of the BMP signalling pathway[32,33]. However, the reverse might also be true as hepcidin might modulate the concentration of serum ferritin by degradation of the iron exporter ferroportin[3], thus favouring iron retention in macrophages and an increase in cytosolic and serum ferritin[34]. The previously reported associations of two common TMPRSS6 and HFE variants to iron were replicated in the VB cohort where they reached GW significance. We confirmed that association of the TMPRSS6 rs855791variant with all red cell traits was mostly dependent on the amount of iron available for erythropoiesis[13] and we showed that HFE C282Y association with all

red cell traits was abolished if iron, transferrin saturation and ferritin were considered together as covariates in association analysis. The availability of serum hepcidin concentrations allowed us to directly test whether hepcidin could be the molecule mediating the association of the two TMPRSS6 and HFE variants. Our results showed that this was not the case. Since including hepcidin as a covariate in association analysis did not change the results, we suggest that the association of TMPRSS6 and HFE variants to iron parameters could result from a direct pleiotropic effect on the iron parameters. Alternatively, as hepcidin concentrations are homeostatically regulated by iron and erythropoietic activity, genetic effects may be masked by the hormone nature of hepcidin, which controls iron by a negative feedback and is strongly influenced by environmental factors. Accordingly, the heritability of hepcidin was negligible. The heritability was higher for the hepcidin/ferritin ratio (H^2=0.219), confirming that genetic factors modifying hepcidin values may be masked by the rapid changes of hepcidin concentration in response to increased body iron stores or to other factors.

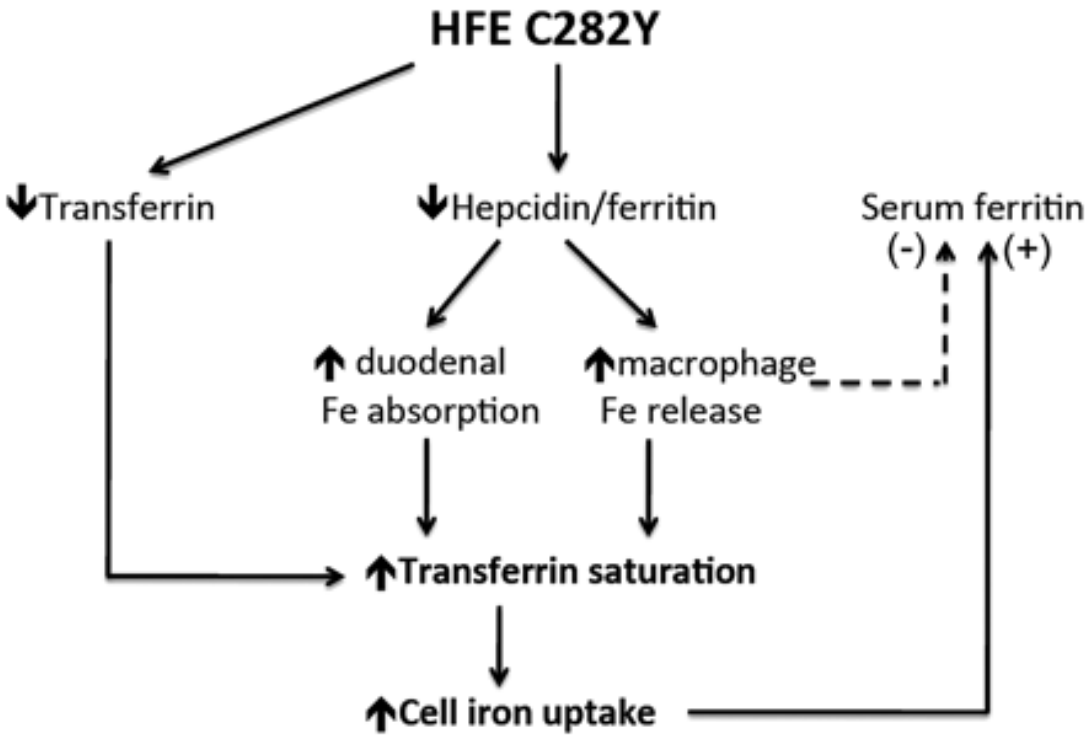

Figure 3. Model of the effect of HFE C282Y on transferrin saturation and hepcidin (see text for details). The dotted line indicates the hypothetical effect of reduced hepcidin on serum ferritin.

We therefore tested association of the hepcidin/ ferritin ratio to the HFE and TMPRSS6 variants considered. This resulted in a significant association of the hepcidin/ferritin ratio with HFE (and borderline with TMPRSS6) (table 3), demonstrating that the HFE C282Y mutation can indeed affect hepcidin, but with a modest effect that could not account for the strong effect of the same mutation on the other iron parameters.

Altogether, the two common variants considered appear to affect iron in part through hepcidin, likely through modulation of the BMP signalling pathway that integrates signals from erythropoiesis and iron stores to activate or repress hepcidin transcription.1 However, it is quite difficult to account for the direct and strong effect of the HFE C282Y mutation on transferrin.

We cannot exclude the possibility that the HFE C282Y mutation affects additional and novel pathway(s) (figure 3) able to regulate iron homeostasis in normal situations and cause transferrin downregulation independently from hepcidin. We also studied association of the two variants to ferritin. HFE C282Y was previously reported to be associated with ferritin in a single study[35]. In our whole cohort it was borderline significant, but became significant at GW levels (p=7.49E-10) with a strong effect (ß=0.556, SE=0.090) in subset 1, strengthening the importance of excluding, in this type of analysis, acquired conditions that modify iron metabolism and particularly hepcidin and ferritin concentrations. In addition, the effect of HFE association on ferritin in subset 1 was increased (p=1.64E-10, ß=0.576, SE=0.090) after adjusting for hepcidin concentrations. This finding further confirmed the mutual control between the two variables and suggests that the positive effect of HFE C282Y on total body iron (and thus on ferritin) is in part antagonised by its negative effect on hepcidin. HFE C282Y increases transferrin saturation and cell iron uptake. However, the concomitant hepcidin downregulation favours iron release from macrophages. On one side this translates into the vicious cycle of further enhancing transferrin saturation, but on the other side, if the secreted ferritin is related to macrophage iron content[36], it would reduce serum ferritin (figure 3). In conclusion, our study shows a complex interplay between hepcidin and ferritin and points to the high transferrin saturation in C282Y HFE haemochromatosis as the major cause of iron loading through increased cell iron uptake, despite increased iron release due to the low hepcidineferroportin interaction. The results also show a new association between HFE and TMPRSS6 variants with hepcidin/ferritin ratio, that could represent an index of potential clinical utility to assess adequate hepcidin secretion.

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REFERENCES

1. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. 2010 Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24e38.

2. Ganz T. 2011 Hepcidin and iron regulation, ten years later. Blood 2011;117:4425e33.

3. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. 2004 IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271e6.

4. Camaschella C. 2005 Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. Blood 2005;106:3710e17.

5. Fernandes A, Preza GC, Phung Y, De Domenico I, Kaplan J, Ganz T, Nemeth E. 2009 The molecular basis of hepcidin-resistant hereditary hemochromatosis. Blood 2009;114:437e43.

6. Du X, She E, Gelbart T, Truksa J, Lee P, Xia Y, Khovananth K, Mudd S, Mann N ,Moresco EM, Beutler E, Beutler B. 2008 The serine protease TMPRSS6 is required to sense iron deficiency. Science 2008;320:1088e92.

7. Folgueras AR, de Lara FM, Pendas AM, Garabaya C, Rodriguez F, Astudillo A ,Bernal T, Cabanillas R, Lopez-Otin C, Velasco G. 2008 Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. Blood 2008;112:2539e45.

8. Finberg KE, Heeney MM, Campagna DR, Aydinok Y, Pearson HA, Hartman KR ,Mayo MM, Samuel SM, Strouse JJ, Markianos K, Andrews NC, Fleming MD. 2008 Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008;40:569e71.

9. Silvestri L, Guillem F, Pagani A, Nai A, Oudin C, Silva M, Toutain F, Kannengiesser C ,Beaumont C, Camaschella C, Grandchamp B. 2009 Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. Blood 2009;113:5605e8.

10. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. 2008 The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab 2008;8:502e11.

11. Finberg KE. 2009 Iron-refractory iron deficiency anemia. Semin Hematol 2009;46:378e86.

12. Tanaka T, Roy CN, Yao W, Matteini A, Semba RD, Arking D, Walston JD, Fried LP, Singleton A, Guralnik J, Abecasis GR, Bandinelli S, Longo DL, Ferrucci L. 2010 A genomewide association analysis of serum iron concentrations. Blood 2010;115:94e6.

13. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP ,Hottenga JJ, Henders AK, Campbell MJ, Wallace L, Frazer IH, Heath AC, de Geus EJ ,Nyholt DR, Visscher PM, Penninx BW, Boomsma DI, Martin NG, Montgomery GW ,Whitfield JB. 2009 Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009;41:1173e5.

14. Benyamin B, McRae AF, Zhu G, Gordon S, Henders AK, Palotie A, Peltonen L ,Martin NG, Montgomery GW, Whitfield JB, Visscher PM. 2009 Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60e5.

15. Chambers JC, Zhang W, Li Y, Sehmi J, Wass MN, Zabaneh D, Hoggart C, Bayele H, McCarthy MI, Peltonen L, Freimer NB, Srai SK, Maxwell PH, Sternberg MJ, Ruokonen A, Abecasis G, Jarvelin MR, Scott J, Elliott P, Kooner JS. 2009 Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet 2009;41:1170e2.

16. Ganesh SK, Zakai NA, van Rooij FJ, Soranzo N, Smith AV, Nalls MA, Chen MH ,Kottgen A, Glazer NL, Dehghan A, Kuhnel B, Aspelund T, Yang Q, Tanaka T, Jaffe A, Bis JC, Verwoert GC, Teumer A, Fox CS, Guralnik JM, Ehret GB, Rice K, Felix JF, Rendon A ,Eiriksdottir G, Levy D, Patel KV, Boerwinkle E, Rotter JI, Hofman A, Sambrook JG ,Hernandez DG, Zheng G, Bandinelli S, Singleton AB, Coresh J, Lumley T, Uitterlinden AG, Vangils JM, Launer LJ, Cupples LA, Oostra BA, Zwaginga JJ, Ouwehand WH ,Thein SL, Meisinger C, Deloukas P, Nauck M, Spector TD, Gieger C, Gudnason V, van Duijn CM, Psaty BM,

Ferrucci L, Chakravarti A, Greinacher A, O'Donnell CJ, Witteman JC, Furth S, Cushman M, Harris TB, Lin JP. 2009 Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. Nat Genet 2009;41:1191e8.

17. Kamatani Y, Matsuda K, Okada Y, Kubo M, Hosono N, Daigo Y, Nakamura Y ,Kamatani N. 2010 Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet 2010;42:210e15.

18. Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, Willenborg C ,Wright B, Chen L, Li M, Salo P, Voight BF, Burns P, Laskowski RA, Xue Y, Menzel S ,Altshuler D, Bradley JR, Bumpstead S, Burnett MS, Devaney J, Doring A, Elosua R ,Epstein SE, Erber W, Falchi M, Garner SF, Ghori MJ, Goodall AH, Gwilliam R ,Hakonarson HH, Hall AS, Hammond N, Hengstenberg C, Illig T, Konig IR, Knouff CW ,McPherson R, Melander O, Mooser V, Nauck M, Nieminen MS, O'Donnell CJ ,Peltonen L, Potter SC, Prokisch H, Rader DJ, Rice CM, Roberts R, Salomaa V ,Sambrook J, Schreiber S, Schunkert H, Schwartz SM, Serbanovic-Canic J, Sinisalo J ,Siscovick DS, Stark K, Surakka I, Stephens J, Thompson JR, Volker U, Volzke H ,Watkins NA, Wells GA, Wichmann HE, Van Heel DA, Tyler-Smith C, Thein SL ,Kathiresan S, Perola M, Reilly MP, Stewart AF, Erdmann J, Samani NJ, Meisinger C ,Greinacher A, Deloukas P, Ouwehand WH, Gieger C. 2009 A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 2009;41:1182e90.

19. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H. 2008 Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.

20. Traglia M, Sala C, Masciullo C, Cverhova V, Lori F, Pistis G, Bione S, Gasparini P ,Ulivi S, Ciullo M, Nutile T, Bosi E, Sirtori M, Mignogna G, Rubinacci A, Buetti I ,Camaschella C, Petretto E, Toniolo D. Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One 2009;4:e7554.

21. Sala C, Ciullo M, Lanzara C, Nutile T, Bione S, Massacane R, d'Adamo P, Gasparini P ,Toniolo D, Camaschella C. 2008 Variation of hemoglobin levels in normal Italian populations from genetic isolates. Haematologica 2008;93:1372e5.

22. Piperno A. 1998 Classification and diagnosis of iron overload. Haematologica 1998;83:447e55.

23. Campostrini N, Castagna A, Zaninotto F, Bedogna V, Tessitore N, Poli A, Martinelli N, Lupo A, Olivieri O, Girelli D. 2010 Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. J Biomed Biotechnol 2010;2010:329646.

24. Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. 2008 Immunoassay for human serum hepcidin. Blood 2008;112:4292e7.

25. Kroot JJ, Hendriks JC, Laarakkers CM, Klaver SM, Kemna EH, Tjalsma H, Swinkels DW. 2009 (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem 2009;389:124e9.

26. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM. 2007 GenABEL: an R library for genomewide association analysis. Bioinformatics 2007;23:1294e6.

27. Piperno A, Girelli D, Nemeth E, Trombini P, Bozzini C, Poggiali E, Phung Y, Ganz T ,Camaschella C. 2007 Blunted hepcidin response to oral iron challenge in HFE-related hemochromatosis. Blood 2007;110:4096e100.

28. van Dijk BA, Laarakkers CM, Klaver SM, Jacobs EM, van Tits LJ, Janssen MC ,Swinkels DW. 2008 Serum hepcidin levels are innately low in HFE-related haemochromatosis but differ between C282Y-homozygotes with elevated and normal ferritin levels. Br J Haematol 2008;142:979e85.

29. Lin L, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. 2007 Iron transferring regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. Blood 2007;110:2182e9.

30. Piperno A, Galimberti S, Mariani R, Pelucchi S, Ravasi G, Lombardi C, Bilo G ,Revera M, Giuliano A, Faini A, Mainini V, Westerman M, Ganz T, Valsecchi MG, Mancia G, Parati G. 2010 Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project. Blood 2010;117:2953e9.

31. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T ,Kaplan J. 2004 Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090e3.

32. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD ,Pietrangelo A, Vukicevic S, Lin HY, Babitt JL. 2009 BMP6 is a key endogenous regulator of
hepcidin expression and iron metabolism. Nat Genet 2009;41:482e7.

33. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of
the bone morphogenetic protein BMP6 induces massive iron overload. Nat Genet 2009;41:478e81.

34. De Domenico I, Vaughn MB, Paradkar PN, Lo E, Ward DM, Kaplan J. 2011 Decoupling ferritin synthesis from free cytosolic iron results in ferritin secretion. Cell Metab 2011;13:57e67.

35. Oexle K, Ried JS, Hicks AA, Tanaka T, Hayward C, Bruegel M, Gogele M, Lichtner P ,Muller-Myhsok B, Doring A, Illig T, Schwienbacher C, Minelli C, Pichler I, Fiedler GM ,Thiery J, Rudan I, Wright AF, Campbell H, Ferrucci L, Bandinelli S, Pramstaller PP ,Wichmann HE, Gieger C, Winkelmann J, Meitinger T. 2011 Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferring receptor (sTfR) levels. Hum Mol Genet 2011;20:1042e7.

36. Cohen LA, Gutierrez L, Weiss A, Leichtmann-Bardoogo Y, Zhang DL, Crooks DR ,Sougrat R, Morgenstern A, Galy B, Hentze MW, Lazaro FJ, Rouault TA, Meyron-Holtz EG. 2010 Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. Blood 2010;116:1574e84.

Chapter 7

A meta-analysis of genome-wide association studies for serum hepcidin

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Abstract

Serum hepcidin concentration is regulated by iron status, inflammation, erythropoiesis and numerous other factors, but underlying processes are incompletely understood. To obtain better insights, we aimed to identify common genetic determinants of serum hepcidin in the general population.. We meta-analyzed genome-wide association results on serum hepcidin from three European population-based studies (total N up to 6,096), the only three cohorts worldwide with both hepcidin measurements and genome-wide single nucleotide polymorphism (SNP) data. We measured six genetic variants that were among the top findings in up to 3,826 additional independent samples. Our study revealed one interesting locus (linkage disequilibrium region from *EML6* to *SPTBN1 (*alias *ELF)*, lead SNP rs354202) potentially associated with serum hepcidin concentration (discovery beta (SE)= -0.17 (0.03), p=7.0E-08; *in silico* replication beta (SE)= -0.15 (0.10), p=0.12; discovery and *in silico* replication combined beta (SE)= -0.17 (0.03), p=2.1E-08). The ELF protein is essential in TGF-β signaling by son of mothers against decapentaplegic (SMAD) proteins in mice, and the bone morphogenetic protein-SMAD pathway is central in hepcidin regulation. The known common variants rs1800562 (p.Cys282Tyr) in *HFE* and rs855791 (p.Ala736Val) in *TMPRSS6* showed strong associations with the ratio hepcidin/ferritin.

Our findings for rs354202 and serum hepcidin concentration warrant follow up in additional association studies and functional studies. We recommend extension of this study once additional cohorts become available to increase power to identify common variants with small effects on serum hepcidin.

Introduction

Iron is an essential trace element for fundamental metabolic processes in humans[1,2]. Iron deficiency limits hemoglobin synthesis and leads to anemia, whereas an excess of free iron is toxic because it catalyzes the production of free radicals resulting in tissue damage[1,2]. In addition, iron imbalances have been associated with other diseases, *e.g.* diabetes mellitus[3,4], inflammation[5] and diseases of aging[6]. Hence, the iron balance in the human body is tightly controlled, with hepcidin as key regulator of systemic iron homeostasis[7,8]. Hepcidin controls the absorption, storage and tissue distribution of iron by binding to the cellular iron exporter ferroportin and inducing its internalization and degradation[9]. In this way, hepcidin regulates the uptake of dietary iron from the intestine and the release of iron from macrophages involved in recycling of iron from senescent erythrocytes[7,8].

In the last few years, several genome-wide association studies have revealed genetic variants associated with iron status in the general population, including common variants in the hereditary hemochromatosis gene (*HFE*), transferrin gene (*TF*), transferrin receptor 2 gene (*TFR2*) and transmembrane serine protease 6 gene (*TMPRSS6*). On the contrary, little is known about genetic determinants of hepcidin. Mutations in hepcidin antimicrobial peptide (*HAMP*), the hepcidin encoding gene, lead to strongly decreased hepcidin levels and a severe juvenile form of the iron storage disorder hereditary hemochromatosis (HH), but *HAMP* mutations are very rare[10]. In addition, mutations in *HFE*, *TFR2* and *TMPRSS6* have been related to hepcidin expression[10-16]. Until now, however, no *common* genetic variants for hepcidin have been identified. The only published genome-wide association study (GWAS) on serum hepcidin in the Val Borbera genetic isolate was underpowered to find genome-wide significant associations with hepcidin[17]. In addition, the single nucleotide polymorphisms (SNP) rs1800562 (p.Cys282Tyr) in *HFE* and rs855791 (p.Ala736Val) in *TMPRSS6*, which were thought to be associated with hepcidin as an explanation for their effects on iron, ferritin, transferrin and transferrin saturation (TS), did not show association with serum hepcidin in recent studies by our groups[17,18]. Nevertheless, these variants did show association with the ratios hepcidin/ferritin and hepcidin/TS[17,18], which express the dependence of hepcidin concentration on stored and circulating iron, respectively[1,7,8,19].

The aim of the current study was to identify common genetic determinants of serum hepcidin in the general population. We studied hepcidin as well as the ratios hepcidin/ferritin and hepcidin/TS. We performed a meta-analysis using data from the only three cohorts worldwide that have, to the best of our knowledge, both hepcidin measurements and genome-wide SNP data: the Nijmegen Biomedical Study (NBS) (Nijmegen, The Netherlands), Prevention of REnal and Vascular ENd-stage Disease (PREVEND) (Groningen, The Netherlands) and Val Borbera (VB) (Milan, Italy). This was followed by replication of six top hits in additional independent samples of the NBS and PREVEND.

Methods

Study populations

In this meta-analysis, we combined genome-wide association results for hepcidin and the ratios hepcidin/ferritin and hepcidin/TS based on up to 6,096 individuals from three population-based cohorts (Supplemental Table 1). Data for replication were obtained from up to 3,826 additional independent samples from two of the three cohorts. Information on laboratory methods, genotype methods, imputation, quality control, and phenotypes is shown in Supplemental Tables 2-4. All three studies were approved by appropriate ethical committees, and all participants gave informed consent.

Genome-wide association analysis

Genome-wide association analyses were performed in each cohort separately according to a set protocol. A subset analysis was performed in which individuals with ferritin <30 ng/mL and CRP ≥10 mg/L were excluded as to remove individuals with iron deficiency and clinical inflammation, which are acquired conditions known to alter iron metabolism[20]. Hepcidin and the ratios hepcidin/ferritin and hepcidin/TS were log-transformed and thereafter adjusted for age and squared age, separately for males and females. For NBS, time of blood sampling was used as an additional covariate (three categories: before 12 PM, between 12 and 5 PM and after 5 PM). For VB, principal components were used to adjust for family structure. Sex-specific residuals were calculated and merged into one variable. Outliers, defined as values that differed more than four times the SD from the mean, were excluded. The association between the single nucleotide variants and the trait was tested using genotype probabilities and an additive model on the standardized residuals (Z score).

Meta-analysis

The GWAS results from the three cohorts were combined in a fixed-effects meta-analysis using METAL[21]. The standard-error based approach was used, which weighs effect size estimates using the inverse of the corresponding standard errors. Variants with a minor allele frequency <1% and a SNPtest info value or MACH RSQR <0.4 were excluded prior to the meta-analysis. Genomic control correction was applied to the individual cohorts.

Replication

Five SNPs were measured with single SNP assays in additional independent samples from PREVEND (N=2,876) and NBS (N=1018). Single-SNP genotyping in PREVEND samples was performed by KBiosciences (KBiosciences, Herts, UK) utilizing the SNPline system. Single-SNP genotyping in NBS samples was carried out by deCODE Genetics using the Centaurus (Nanogen) platform[22]. The quality of each Centaurus SNP assay was evaluated by genotyping each assay on

the CEU samples and comparing the results with the HapMap data. All assays had mismatch rate <0.5%. One SNP for which the genotyping assay failed was carried forward to *in silico* replication in the additional samples from the NBS that were genotyped with the HumanOmniExpress-12v1-1_B (N=524).

Results

Combination of GWAS results from three cohorts (Supplemental Tables 1-4) revealed two loci that were genome-wide significantly associated (p<5E-08) with serum hepcidin (Table 1 and Supplemental Figures 1-8). The first one (rs118031191 on chromosome 10, nearest gene *FOXI2*) showed genome-wide significant association in all individuals, but not in the subset (p=1.4E-05). The second locus showed genome-wide significant association in the subset (four SNPs on chromosome 2 in *EML6* with lead SNP rs354202). This latter signal covers a region that also includes *SPTBN1*, which encodes spectrin, beta, non-erythrocytic 1 and is the left flanking gene of *EML6* approximately 50 kbp away (Supplemental Figure 8). Conditional analysis in NBS data showed no additional independent signals at the chromosome 2 locus after adjustment for rs354202 (Supplemental Figures 9-10).

The ratio hepcidin/ferritin in all individuals and in the subset showed genome-wide significant association with the previously known genes *HFE* and *TMPRSS6* (Supplemental Table 5). No new statistically significant loci for hepcidin/ferritin were identified; the new locus with the lowest p-value in the subset (rs1594673 on chromosome 5, nearest gene *PRELID2*) also appeared in the top results for serum hepcidin in the subset (Table 1). The associations of the ratio hepcidin/ferritin with rs354202 and rs118031191 in all individuals and in the subset were far from significant (rs354202: p=6.7E-03 and 1.6E-02, respectively; rs118031191: p=3.8E-02 and 1.9E-02). However, directions of the effect estimates were the same as for the association with hepcidin [beta (SE) rs354202: -0.09 (0.03) in all individuals and -0.09 (0.04) in the subset; beta (SE) rs118031191: -0.14 (0.07) in all individuals and -0.18 (0.08) in the subset].

The ratio hepcidin/TS was genome-wide significantly associated with the *TMPRSS6* locus in the subset, but not with *HFE* (Supplemental Table 6). No novel significant loci were found for hepcidin/TS. SNP rs354202 showed the most significant novel signal in the subset (p=1.6E-07) and was also among the top results for hepcidin/TS in all individuals (p=3.0E-6). Directions of the effect estimate were the same as for the association with hepcidin [beta (SE) -0.15 (0.03) in all individuals and -0.18 (0.04) in the subset]. SNP rs118031191 was also among the top results for hepcidin/TS in all individuals (p=2.4E-07), but not in the subset (p=3.4E-05). Directions of the effect estimate were the same as for the association with hepcidin [beta (SE) -0.35 (0.07) in all individuals and -0.32 (0.08) in the subset].

Six SNPs were brought forward to replication. SNPs rs354202, rs118031191, rs56281245 and rs12289793 were selected based on p<1E-06 for association to hepcidin in all individuals and/or in the subset. Two additional SNPs were selected, although not present in the top with p<1E-06,

because they showed hepcidin association p-values close to 1E-06, their MA results were based on three cohorts, they lie inside genes, and their MAF is >10%. These were rs1835473 (p =1.9E-06 for hepcidin in all individuals), which lies in the gene *PKIB* encoding protein kinase (cAMP-dependent, catalytic) inhibitor beta, and rs12441903 (p=4.3E-06 for hepcidin in all individuals), which lies in the gene LRRK1 encoding leucine-rich repeat kinase 1. SNPs rs117568227 and rs141939445 showed p<1E-06 but were not selected because the MA results were based on only one or two cohorts, they lie in intergenic regions, and have MAF~1%. Replication analysis revealed no significant associations at p=0.05 (Table 2). The p-value for discovery and replication analyses combined became stronger for rs354202 only.

Discussion

This is the first meta-analysis of GWAS for serum hepcidin, which is based on analysis of up to 9,917 individuals. It revealed a potentially interesting locus on chromosome 2 with lead SNP rs354202.

SNP rs354202 is located on chromosome 6 in an intron of *EML6*, encoding echinoderm microtubule associated protein like 6. The linkage disequilibrium region of rs354202 stretches from *EML6* to *SPTBN1* [alias *ELF* (embryonic liver fodrin)], encoding spectrin, beta, non-erythrocytic 1. This gene is a member of a family of beta-spectrin genes, which are involved in linking the plasma membrane to the actin cytoskeleton. The ELF protein was shown to be essential in TGF-β signaling by son of mothers against decapentaplegic (SMAD) proteins in mice[23]. Central in hepcidin regulation is the bone morphogenetic protein-SMAD pathway[24], and the ELF protein is thus a plausible candidate to influence hepcidin expression. In addition, a recent genome-wide RNA interference screen provided a large number of putative hepatic hepcidin regulators; results also pointed to adaptor proteins as hepcidin activators[25]. Based on current knowledge, spectrins could be considered as adaptor proteins[26], but results of the RNA screen did not specifically point to β-spectrins. Furthermore, rs354202 showed association with hepcidin in both all study individuals and in the subset, suggesting that this signal is not driven by extreme iron deficiency or inflammation. It also showed strong associations with the ratio hepcidin/TS, but not with the ratio hepcidin/ferritin. Finally, the direction of effect of rs354202 on all traits was the same, *i.e.* the A allele of rs354202 is associated with a decrease in hepcidin, hepcidin/ferritin and hepcidin/TS.

We confirmed the association of the ratio hepcidin/ferritin with common variants in *HFE* and *TMPRSS6*. We previously reported on the associations of rs1800562 in *HFE* and rs855791 in *TMPRSS6* with the ratio hepcidin/ferritin via independent studies both in the VB and NBS population[17,18]. As expected, we further substantiated these associations here and found an even stronger signal. The association signal of the ratio hepcidin/TS with common variants in *HFE* and *TMPRSS6* was less strong. Of note, the association of rs1800562 in *HFE* with the ratio hepcidin/TS, previously found in the NBS[18], disappeared upon meta-analysis of results of NBS,

PREVEND and VB in all individuals (p=0.13), but still showed a relatively weak signal in the subset (p=3.7E-04). Also rs855791 in *TMPRSS6* showed a stronger signal for association with the ratio hepcidin/TS in the subset compared to analysis based on all individuals. The stronger signal of rs1800562 and rs855791 with the ratio hepcidin/ferritin compared to the ratio hepcidin/TS indicates that these SNPs have a larger influence on hepcidin response to body iron stores than on hepcidin response to circulating iron.

Recently, a meta-analysis on iron status in up to 48,972 subjects was completed by the Genetics of Iron Status Consortium, which also incorporated the three cohorts included in the current study (Benyamin et al 2014). This meta-GWAS identified 12 SNPs that were significantly associated with one or more of the iron parameters, *i.e.* serum iron, ferritin, transferrin, and TS. The top findings of the current study do not show any overlap with the 12 SNPs of the iron status meta-GWAS. This is unexpected, as hepcidin and iron metabolism are clearly intertwined[1,7,8,19], as also indicated by the strong and positive correlation between serum hepcidin and serum ferritin in the NBS and the VB population[17,27].

The fact that our meta-analysis revealed only one locus that potentially affects serum hepcidin suggests that there are no common variants that explain a large proportion of phenotypic variation in serum hepcidin. Indeed, with our N of 6,096 we had 80% and 99.4% chance of detecting (at alpha 5E-08) a variant that explains 0.62% and 1% of hepcidin variance, respectively. For comparison, the well-known iron-related SNPs rs1800562 in *HFE* and rs855791 in *TMPRSS6* explain ~1% of serum iron variation. In addition, (narrow-sense) heritability of hepcidin was previously estimated to be 9.8% (non-significant) in the VB population and genome-wide SNP explained variance was estimated at ~37% (SE~20%) in the NBS (data not shown), suggesting that a large part of hepcidin variability is caused by variation in environmental factors. Future studies that aim to detect common variants with small effects on serum hepcidin could increase power by further enlarging sample size and/or reducing the hepcidin variability by adjustment for non-genetic factors associated with serum hepcidin, like we did for age, gender[17,27], and diurnal rhythm[7,28], but also for *e.g.* alcohol consumption[29], and pregnancy[30]. Studies into rare variants using exome or whole genome sequencing and gene-gene and/or gene-environment interactions could further increase insights into the genetic etiology of hepcidin.

In conclusion, our study revealed one interesting locus (lead SNP rs354202) potentially affecting serum hepcidin concentration. Furthermore, our results indicate that there are no common genetic variants that explain more than 1% of phenotypic hepcidin variation. We recommend to measure rs354202 in additional independent samples in order to confirm its association with serum hepcidin and to follow-up this locus with fine mapping and functional studies to obtain insight into the underlying mechanism of association.

Table 1.

						All						Subset					
SNP	CHR	BP (Build 37)	In gene/ nearest gene	A1*	A2	Freq A1	Beta	SE	p	Direction#	N	Freq A1	Beta	SE	p	Direction#	N
rs12477708	2	54905508	EML6	A	G	0.1	0.1	0	2.83E-05	+++	6096	0.1	0.2	0	1.69E-07	+++	5051
rs80098840	2	54918152	EML6	A	G	0.89	-0.1	0	5.73E-06	---	6096	0.89	-0.2	0	1.51E-07	---	5051
rs76949049	**2**	**54965697**	**EML6**	**T**	**C**	**0.1**	**0.2**	**0**	**1.14E-06**	**+++**	**6096**	**0.1**	**0.2**	**0**	**2.15E-08**	**+++**	**5051**
rs354202	**2**	**54970943**	**EML6**	**A**	**G**	**0.9**	**-0.2**	**0**	**7.02E-08**	**---**	**6096**	**0.9**	**-0.2**	**0**	**1.21E-08**	**---**	**5051**
rs354204	2	54971385	EML6	A	G	0.86	-0.1	0	4.92E-06	---	6096	0.86	-0.2	0	5.30E-08	---	5051
rs9973793	2	54998516	EML6	T	C	0.13	0.1	0	2.11E-05	+++	6096	0.13	0.2	0	2.84E-07	+++	5051
rs2033823	**2**	**55057740**	**EML6**	**T**	**C**	**0.9**	**-0.1**	**0**	**1.11E-05**	**---**	**6096**	**0.9**	**-0.2**	**0**	**2.64E-08**	**---**	**5051**
rs13420395	**2**	**55058720**	**EML6**	**A**	**G**	**0.1**	**0.1**	**0**	**1.32E-05**	**+++**	**6096**	**0.1**	**0.2**	**0**	**2.79E-08**	**+++**	**5051**
rs7592363	2	55060479	EML6	T	C	0.1	0.1	0	1.39E-05	+++	6096	0.1	0.2	0	6.11E-08	+++	5051
rs6747033	2	55061294	EML6	C	G	0.89	-0.1	0	6.65E-06	---	6096	0.89	-0.2	0	1.10E-07	---	5051
rs56281245	5	145007639	PRELID2‡	T	C	0.95	0.2	0.1	6.00E-04	+++	6096	0.95	0.3	0.1	6.66E-07	+++	5051
rs11388147	7	71647721	CALN1	G	GA	0.27	-0.2	0	9.63E-07	?--	3279	0.26	-0.1	0	7.98E-05	?--	2695
rs118031191	**10**	**129582469**	**FOXI2‡**	**A**	**G**	**0.03**	**-0.4**	**0.1**	**1.59E-08**	**---**	**6096**	**0.03**	**-0.3**	**0.1**	**1.41E-05**	**---**	**5051**
rs12289793	11	21348000	NELL1	A	G	0.79	0.1	0	1.60E-04	+++	6096	0.79	0.1	0	9.91E-07	+++	5051
rs117568227	12	66447376	LLPH‡$	A	G	0.01	-1	0.2	7.82E-07	?-?	1479	0.01	-1.2	0.2	5.85E-07	?-?	1206
rs150188223	13	42844491	AKAP11	T	C	0.01	-0.6	0.2	1.41E-03	-??	1800	0.01	-1	0.2	5.10E-07	??-	1489
rs141939445	20	36896818	KIAA1755‡$	T	C	0.99	0.7	0.2	6.87E-06	+?+	3279	0.99	0.9	0.2	1.98E-07	?++	2695

Analyses were performed for all individuals with a hepcidin measurement above the detection limit of the hepcidin assay.
*A1 is the effect allele in the association analysis.
#Order of direction: PREVEND, NBS, VB. A question mark (?) indicates that the variant had a minor allele frequency <1%, and/or a SNPtest info value or MACH RSQR <0.4, and/or was not imputed in a cohort.
‡These SNPs lie in intergenic regions.
$Closer than LLPH lies RNA, 5S ribosomal pseudogene 362 (RNA5SP362).

Table 2.

						Replication					Discovery + Replication				
SNP	Population	A1*	A2	Freq A1 PREVEND	Freq A1 NBS	Beta	SE	p	Direction#	N	Beta	SE	p	Direction†	N
rs12289793	All	A	G	0.78	0.72	0	0	0.4	++	3770	0.1	0	7.01E-04	+++	9866
	Subset	A	G	0.78	0.74	0	0	0.3	++	3072	0.1	0	1.49E-05	+++	8123
rs1835473	All	A	G	0.68	0.7	0	0	0.3	++	3754	0.1	0	1.48E-05	+++	9850
	Subset	A	G	0.68	0.7	0	0	0.5	+-	3059	0.1	0	2.27E-03	++-	8110
rs56281245	All	T	C	0.95	0.95	0.1	0.1	0.2	++	3798	0.1	0	8.40E-04	+++	9894
	Subset	T	C	0.95	0.96	0	0.1	0.6	++	3092	0.2	0	3.83E-05	+++	8143
rs118031191	All	A	G	0.03	0.03	0	0.1	1	-+	3821	-0.2	0.1	9.12E-05	--+	9917
	Subset	A	G	0.03	0.03	0	0.1	1	-+	3115	-0.2	0.1	2.60E-03	--+	8166
rs12441903	All	A	G	0.89	0.87	-0	0	0.3	--	3816	-0.1	0	3.13E-05	---	9912
	Subset	A	G	0.89	0.87	-0	0	0.4	--	3108	-0.1	0	6.29E-05	---	8159
rs354202‡	All	A	G	NA	0.89	-0.2	0.1	0.1	-	524	-0.2	0	2.11E-08	--	6620
	Subset	A	G	NA	0.88	-0.1	0.1	0.3	-	444	-0.2	0	9.58E-09	--	5495

HWE p-values in PREVEND and NBS, respectively, were for rs12289793: p=0.71 and 0.89; for rs1835473 p=0.001 and 0.90; for rs56281245 p=0.12 and 0.90; for rs118031191 p=0.04 and 0.29; for rs12441903 p=0.04 and 0.95; and for rs354202 p=0.83 (NBS only).
*A1 is the effect allele in the association analysis.
#Order of direction: NBS, PREVEND.
†Order of direction: discovery MA, NBS, PREVEND.
‡Result of of *in silico* replication for rs354202 in additional NBS samples.

REFERENCES

1. Hentze MW, Muckenthaler MU, Galy B, Camaschella C (2010) Two to tango: regulation of Mammalian iron metabolism. Cell 142: 24-38.

2. Ganz T (2013) Systemic iron homeostasis. Physiol Rev 93: 1721-1741.

3. Jiang R, Manson JE, Meigs JB, Ma J, Rifai N, et al. (2004) Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 291: 711-717.

4. Montonen J, Boeing H, Steffen A, Lehmann R, Fritsche A, et al. (2012) Body iron stores and risk of type 2 diabetes: results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study. Diabetologia 55: 2613-2621.

5. Cherayil BJ (2010) Iron and immunity: immunological consequences of iron deficiency and overload. Arch Immunol Ther Exp (Warsz) 58: 407-415.

6. Altamura S, Muckenthaler MU (2009) Iron toxicity in diseases of aging: Alzheimer's disease, Parkinson's disease and atherosclerosis. J Alzheimers Dis 16: 879-895.

7. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW (2011) Hepcidin in human iron disorders: diagnostic implications. Clin Chem 57: 1650-1669.

8. Ganz T, Nemeth E (2012) Hepcidin and iron homeostasis. Biochim Biophys Acta 1823: 1434-1443.

9. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, et al. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306: 2090-2093.

10. Swinkels DW, Janssen MC, Bergmans J, Marx JJ (2006) Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. Clin Chem 52: 950-968.

11. Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, et al. (2002) Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 29: 361-366.

12. Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C (2005) Hepcidin is decreased in TFR2 hemochromatosis. Blood 105: 1803-1806.

13. van Dijk BA, Laarakkers CM, Klaver SM, Jacobs EM, van Tits LJ, et al. (2008) Serum hepcidin levels are innately low in HFE-related haemochromatosis but differ between C282Y-homozygotes with elevated and normal ferritin levels. Br J Haematol 142: 979-985.

14. Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, et al. (2003) Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 361: 669-673.

15. Finberg KE, Heeney MM, Campagna DR, Aydinok Y, Pearson HA, et al. (2008) Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 40: 569-571.

16. De Falco L, Sanchez M, Silvestri L, Kannengiesser C, Muckenthaler MU, et al. (2013) Iron refractory iron deficiency anemia. Haematologica 98: 845-853.

17. Traglia M, Girelli D, Biino G, Campostrini N, Corbella M, et al. (2011) Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 48: 629-634.

18. Galesloot TE, Geurts-Moespot AJ, den Heijer M, Sweep FC, Fleming RE, et al. (2013) Associations of common variants in HFE and TMPRSS6 with iron parameters are independent of serum hepcidin in a general population: a replication study. J Med Genet 50: 593-598.

19. Fleming RE, Ponka P (2012) Iron overload in human disease. N Engl J Med 366: 348-359.

20. Nai A, Pagani A, Silvestri L, Campostrini N, Corbella M, et al. (2011) TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. Blood 118: 4459-4462.

21. Willer CJ, Li Y, Abecasis GR (2010) METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 26: 2190-2191.

22. Kutyavin IV, Milesi D, Belousov Y, Podyminogin M, Vorobiev A, et al. (2006) A novel endonuclease IV post-PCR genotyping system. Nucleic Acids Res 34: e128.

23. Tang Y, Katuri V, Dillner A, Mishra B, Deng CX, et al. (2003) Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. Science 299: 574-577.

24. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, et al. (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38: 531-539.

25. Mleczko-Sanecka K, Roche F, da Silva AR, Call D, D'Alessio F, et al. (2014) Unbiased RNAi screen for hepcidin regulators links hepcidin suppression to the proliferative Ras/RAF and the nutrient-dependent mTOR signaling pathways. Blood.

26. Machnicka B, Czogalla A, Hryniewicz-Jankowska A, Boguslawska DM, Grochowalska R, et al. (2014) Spectrins: a structural platform for stabilization and activation of membrane channels, receptors and transporters. Biochim Biophys Acta 1838: 620-634.

27. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, et al. (2011) Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 117: e218-225.

28. Schaap CC, Hendriks JC, Kortman GA, Klaver SM, Kroot JJ, et al. (2013) Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin Chem 59: 527-535.

29. Bridle K, Cheung TK, Murphy T, Walters M, Anderson G, et al. (2006) Hepcidin is down-regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. Alcohol Clin Exp Res 30: 106-112.

30. van Santen S, Kroot JJ, Zijderveld G, Wiegerinck ET, Spaanderman ME, et al. (2013) The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. Clin Chem Lab Med 51: 1395-1401.

Chapter 8

Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis

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Abstract

Variation in body iron is associated with or causes diseases, including anaemia and iron overload. Here, we analyse genetic association data on biochemical markers of iron status from 11 European-population studies, with replication in eight additional cohorts (total up to 48,972 subjects). We find 11 genome-wide-significant ($P<5x10^{-8}$) loci, some including known iron-related genes (HFE, SLC40A1, TF, TFR2, TFRC, TMPRSS6) and others novel (ABO, ARNTL, FADS2, NAT2, TEX14). SNPs at ARNTL, TF, and TFR2 affect iron markers in HFE C282Y homozygotes at risk for hemochromatosis. There is substantial overlap between our iron loci and loci affecting erythrocyte and lipid phenotypes. These results will facilitate investigation of the roles of iron in disease.

Introduction

Absorption, transport and storage of iron are tightly regulated, as expected for an element, which is both essential and potentially toxic. Iron deficiency is the leading cause of anaemia[1], and it also compromises immune function[2] and cognitive development[3]. Iron overload damages the liver and other organs in hereditary hemochromatosis[4], and in thalassaemia patients with both transfusion and non-transfusion-related iron accumulation[5]. Excess iron has harmful effects in chronic liver diseases caused by excessive alcohol, obesity or viruses[6]. There is evidence for involvement of iron in neurodegenerative diseases[7–9] and in Type 2 diabetes[10,11]. Variation in transferrin saturation, a biomarker of iron status, has been associated with mortality in patients with diabetes[12] and in the general population[13]. All these associations between iron and either clinical disease or pathological processes make it important to understand the causes of variation in iron status. Importantly, information on genetic causes of variation can be used in Mendelian randomization studies to test whether variation in iron status is a cause or consequence of disease[14,15].

We have used biomarkers of iron status (serum iron, transferrin, transferrin saturation and ferritin), which are commonly used clinically and readily measurable in thousands of individuals, and carried out a meta-analysis of human genome-wide association study (GWAS) data from 11 discovery and eight replication cohorts. These phenotypes show significant heritability in normal adults[16,17], and previous population-based studies have identified relevant single-nucleotide polymorphisms (SNPs) and gene loci (HFE, TF, TFR2 and TMPRSS6 (refs 18,19)) for iron status biomarkers. HFE and TMPRSS6 have also been shown to affect red cell count, haemoglobin and erythrocyte indices[20], most likely by affecting iron availability[20–22].

Our aims were to identify additional loci affecting markers of iron status in the general population and to relate the significant loci to information on gene expression to identify relevant genes. We also made an initial assessment of whether any such loci affect iron status in HFE C282Y homozygotes, who are at genetic risk of HFE-related iron overload (hereditary hemochromatosis type 1, OMIM #235200).

Combination of results from discovery and replication stages of our analysis shows significant effects on one or more of the iron biomarkers at 11 loci. Those primarily affecting serum iron and transferrin saturation include, or are close to, genes whose products have recognized roles in iron homeostasis; HFE (the haemochromatosis gene), TMPRSS6 (transmembrane protease, serine 6) and TFR2 (transferrin receptor 2). Those mainly affecting serum transferrin, apart from the TF (transferrin) gene itself and TFRC (transferrin receptor), and those mainly affecting ferritin (apart from SLC40A1, solute carrier family 40 (iron- regulated transporter), member 1) are unexpected. There is a significant overlap between the genes or loci affecting iron biomarkers and those known to affect erythrocyte numbers or size, which is reasonable given the importance of iron for erythropoesis. We also find significant overlap between genes or loci affecting iron biomarkers and

known loci affecting plasma lipids or lipoproteins, showing an unexplained link between these areas of metabolism.

Results

SNP and gene associations

The combination of allelic association data from 11 discovery and eight replication cohorts (Supplementary Tables 1–3) showed 11 loci with significant effects on one or more of the iron-related phenotypes (Table 1, Fig. 1, Supplementary Figs 1 and 2, Supplementary Table 4). Four of these (HFE, TF, TFR2, TMPRSS6) were previously known to affect iron biomarker variation in the general population[18,19].

Genes at two newly significant loci, SLC40A1, which codes for the cellular iron exporter ferroportin and TFRC, which codes for the iron importer transferrin receptor 1, are known to be important for cellular iron homeostasis[23]. The other five loci (chromosome 8 at 18.3 Mbp, nearest gene NAT2; chromosome 9 at 136.2 Mbp, nearest gene ABO; chromosome 11 at 13.4Mbp, nearest gene ARNTL; chromosome 11 at 61.6Mbp, nearest gene FADS2; chromosome 17 at 54.1Mbp, nearest gene TEX14) were not previously known to affect any of these phenotypes. These affect either transferrin (NAT2, ARNTL, FADS2) or ferritin (ABO, TEX14).

Conditional analysis on the discovery cohorts (Table 1, Supplementary Fig. 4) showed additional independent signals at the TF locus for transferrin and transferrin saturation and at TMPRSS6 for iron. Gene-based analysis in the discovery cohort (Supplementary Table 5) gave significant results (critical P-value for testing of 17,000 genes $<3x10^{-6}$) for ferritin in a region covering two genes (C15orf43 and SORD) on chromosome 15, where individual SNPs gave only suggestive associations. Allelic associations across this region are also shown in Supplementary Fig. 2. This locus did not show any SNPs with genome-wide significance in the combined discovery+replication data.

In the replication cohorts, the lead SNPs at the 11 significant loci explained 3.4, 7.2, 6.7 and 0.9% of the phenotypic variance for iron, transferrin, saturation and (log-transformed) ferritin, respectively. Allelic association results for all SNPs tested will be available from http://genepi.qimr.edu.au/.

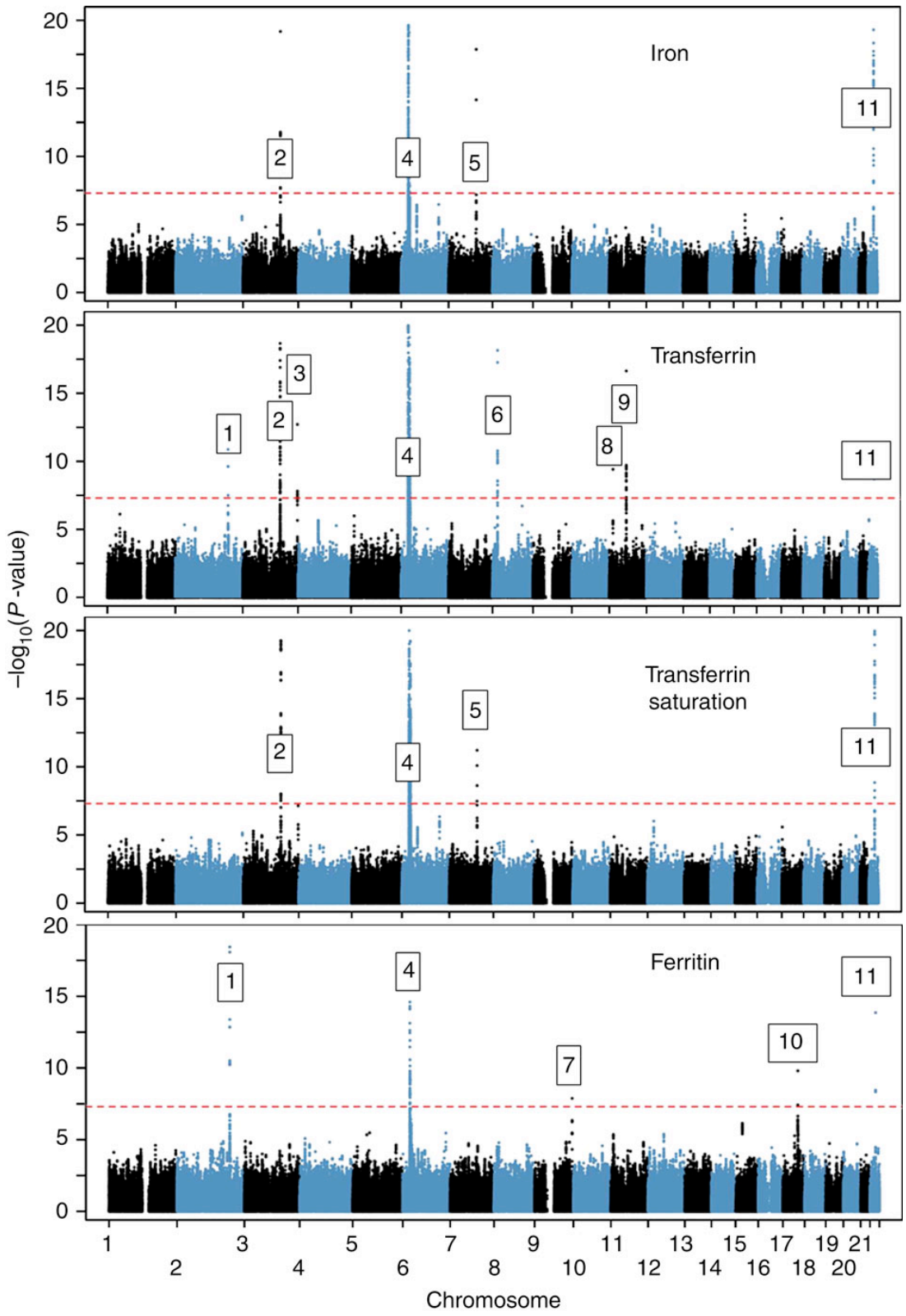

Figure 1. Location of regions showing significant association with one or more of serum iron, transferrin, transferrin saturation and ferritin. The –log(p) values on the y-axes are truncated at 20. For SNPs taken forward for replication, the –log(p) values are from the combined Discovery + Replication datasets. Genes are assigned to the loci as follows: 1 SLC40A1; 2 TF; 3 TFRC; 4 HFE; 5 TFR2; 6 NAT2; 7 ABO; 8 ARNTL; 9 FADS2; 10 TEX14; 11 TMPRSS6.

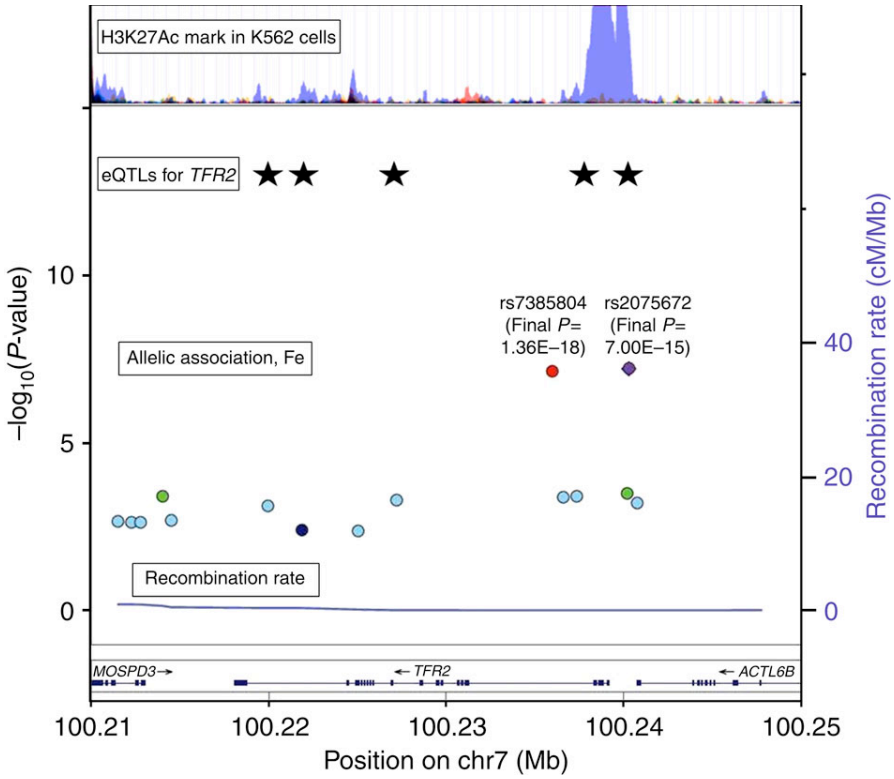

Figure 2. Comparison of results for serum iron with regulatory features at the chromosome 7 (TFR2) locus. From bottom: regional association plot with recombination rate and –log(P.value) for serum iron; documented eQTL locations for TFR2 expression (from left to right: rs10247962, rs4729598, rs7457868, rs4729600, rs1052897); ENCODE data on histone modification. P-values for serum iron at rs7385804 and rs2075672 are shown as text (Final p) for the discovery + replication dataset, but positions for all SNPs on the y-axis are determined by the discovery dataset only.

Table 1. Results from discovery, discovery + replication and conditional analyses.

								Discovery			Discovery + Replication		
CHR	SNP	BP (Build 37)	Nearest Gene(s) †	A1††	A2	Freq A1	Phenotype	Beta	SE	p	Beta	SE	p
2	rs744653	190,378,750	*WDR75 – SLC40A1*	T	C	0.854	Iron	-0.002	0.014	0.902	0.004	0.010	0.702
							Transferrin	0.092	0.014	2.00×10^{-10}	0.068	0.010	1.35×10^{-11}
							Saturation	-0.037	0.014	0.0087	-0.028	0.011	0.0084
							Ferritin (log)	-0.098	0.013	1.20×10^{-13}	-0.089	0.010	8.37×10^{-19}
3	rs8177240	133,477,701	*TF*	T	G	0.669	Iron	-0.073	0.011	2.37×10^{-12}	-0.066	0.007	6.65×10^{-20}
							Transferrin	-0.423	0.011	3.82×10^{-340}	-0.380	0.007	3.29×10^{-615}
							Saturation	0.097	0.011	5.85×10^{-20}	0.100	0.008	7.24×10^{-38}
							Ferritin (log)	0.028	0.010	0.0050	0.021	0.007	0.0039
3	rs9990333	195,827,205	*TFRC*	T	C	0.460	Iron	0.021	0.010	0.030	0.017	0.007	0.014
							Transferrin	-0.067	0.010	3.01×10^{-11}	-0.051	0.007	1.95×10^{-13}
							Saturation	0.049	0.010	7.37×10^{-7}	0.039	0.007	7.28×10^{-8}
							Ferritin (log)	0.002	0.009	0.829	0.001	0.007	0.878
6	rs1800562	26,093,141	*HF E* (C282Y)	A	G	0.067	Iron	0.372	0.020	3.96×10^{-77}	0.328	0.016	2.72×10^{-97}
							Transferrin	-0.550	0.021	1.26×10^{-153}	-0.479	0.016	8.90×10^{-196}
							Saturation	0.577	0.020	1.52×10^{-178}	0.577	0.016	2.19×10^{-270}
							Ferritin (log)	0.211	0.019	1.43×10^{-29}	0.204	0.016	1.54×10^{-38}
6	rs1799945	26,091,179	*HFE* (H63D)	C	G	0.850	Iron	-0.190	0.014	1.65×10^{-42}	-0.189	0.010	1.10×10^{-81}
							Transferrin	0.119	0.014	5.59×10^{-17}	0.114	0.010	9.36×10^{-30}
							Saturation	-0.228	0.014	2.98×10^{-60}	-0.231	0.010	5.13×10^{-109}
							Ferritin (log)	-0.059	0.013	7.38×10^{-6}	-0.065	0.010	1.71×10^{-10}
7	rs7385804	100,235,970	*TFR2*	A	C	0.621	Iron	0.055	0.010	7.19×10^{-8}	0.064	0.007	1.36×10^{-18}

CHR	SNP	BP (Build 37)	Nearest Gene(s) †	A1††	A2	Freq A1	Phenotype	Discovery Beta	Discovery SE	Discovery p	Discovery + Replication Beta	Discovery + Replication SE	Discovery + Replication p
							Transferrin	-0.009	0.011	0.396	-0.003	0.007	0.728
							Saturation	0.054	0.010	1.79×10^{-7}	0.054	0.008	6.07×10^{-12}
							Ferritin (log)	0.022	0.010	0.0255	0.015	0.007	0.039
8	rs4921915	18,272,466	*NAT2*	A	G	0.782	Iron	-0.009	0.012	0.477	0.004	0.009	0.633
							Transferrin	0.082	0.012	1.74×10^{-11}	0.079	0.009	7.05×10^{-19}
							Saturation	-0.034	0.012	0.0041	-0.026	0.009	0.0036
							Ferritin (log)	-0.006	0.011	0.603	0.001	0.009	0.886
9	rs651007	136,153,875	*ABO*	T	C	0.202	Iron	-0.012	0.013	0.358	-0.004	0.009	0.611
							Transferrin	0.017	0.013	0.188	-0.001	0.009	0.916
							Saturation	-0.020	0.013	0.110	-0.006	0.009	0.498
							Ferritin (log)	-0.060	0.012	2.54×10^{-7}	-0.050	0.009	1.31×10^{-8}
11	rs6486121	13,355,770	*ARNTL*	T	C	0.631	Iron	0.001	0.010	0.898	-0.009	0.007	0.202
							Transferrin	-0.056	0.011	1.04×10^{-7}	-0.046	0.007	3.89×10^{-10}
							Saturation	0.026	0.010	0.0132	0.015	0.008	0.048
							Ferritin (log)	0.012	0.010	0.2298	0.006	0.007	0.424
11	rs174577	61,604,814	*FADS2*	A	C	0.330	Iron	0.003	0.011	0.785	0.001	0.007	0.878
							Transferrin	0.068	0.011	1.90×10^{-10}	0.062	0.007	2.28×10^{-17}
							Saturation	-0.023	0.011	0.029	-0.025	0.008	0.0016
							Ferritin (log)	-0.020	0.010	0.040	-0.012	0.007	0.098
17	rs411988	56,709,034	*TEX14*	A	G	0.564	Iron	-0.007	0.010	0.4673	-0.002	0.007	0.770
							Transferrin	0.033	0.010	0.0012	0.014	0.007	0.052
							Saturation	-0.021	0.010	0.036	-0.012	0.007	0.115
							Ferritin (log)	-0.049	0.009	1.28×10^{-7}	-0.044	0.007	1.59×10^{-10}
22	rs855791	37,462,936	*TMPRSS6* (V736A)	A	G	0.446	Iron	-0.187	0.010	4.31×10^{-77}	-0.181	0.007	1.32×10^{-139}
							Transferrin	0.040	0.010	0.00013	0.044	0.007	1.98×10^{-9}
							Saturation	-0.192	0.010	3.50×10^{-80}	-0.190	0.008	6.41×10^{-137}
							Ferritin (log)	-0.051	0.010	5.81×10^{-8}	-0.055	0.007	1.38×10^{-14}
Conditional analysis													
3	rs8177179	133,463,457	*TF*	A	G	0.521	Transferrin	-0.154	0.010	2.74×10^{-49}	-	-	-
3	rs1799852	133,475,722	*TF* ((L247L)	T	C	0.098	Saturation	0.110	0.019	7.13×10^{-9}	-	-	-
22	rs228916	37,505,552	*TMPRSS6*	T	C	0.875	Iron	-0.086	0.016	2.94×10^{-8}	-	-	-

† Where the SNP is a coding variant, the amino acid change is also shown.
†† A1 is the effect allele for each SNP in the association analysis.

Secondary analyses

In view of the known association between ferritin concentration and inflammatory conditions, we repeated the discovery meta-analysis of ferritin including C-reactive protein (CRP, a marker of inflammation) as a covariate. This resulted in a decrease in effect sizes (expressed as standardized regression slopes or betas in an additive-allelic-effect model) for the lead SNPs at significant and suggestive loci, to an average of 73% (s.d. 15%) of the previous betas (Supplementary Fig. 5). The P-values became less significant, partly because of the decrease in effect size and partly because the number of subjects with CRP data was less than the number available for the initial analysis.

To check whether results were similar after excluding people with iron deficiency, we removed subjects with serum ferritin concentration below 30µgl^{-1} and repeated the meta-analyses for all four phenotypes. This decreased effect sizes for transferrin and transferrin saturation, but had negligible effects for SNPs, which were significant or suggestive for ferritin or iron compared with those from the all-subjects analysis (Supplementary Table 6).

We also examined the association between serum transferrin concentration and FADS2 variation. Because this gene is known to be associated with other phenotypes related to lipids and components of the metabolic syndrome, we included high- density lipoprotein cholesterol (HDL-C) as a covariate and repeated the association meta-analysis for transferrin and the most significant SNP at the FADS2 locus, rs174577. (HDL-C was chosen because it was available for a greater proportion of subjects than either triglycerides or glucose, which are also associated with FADS polymorphisms.) This conditional analysis resulted in a 35% reduction in the effect size for this SNP, from ß=0.068±0.011 to 0.044±0.009.

Table 2. Results for *HFE* YY subjects. Combined data from meta-analysis of HEIRS and QIMR adult subjects with *HFE* YY genotype. Results for the most significant SNP at each locus where any SNP shows p < 0.005 for any of the four phenotypes, are shown.

SNP Information						Iron			Transferrin			Saturation			Ferritin		
SNP	CHR	BP	A1	A2	Freq1	Effect	SE	P	Effect	SE	P	Effect	SE	P	Effect	SE	P
rs8177240	3	134,962,864	T	G	0.669	-0.094	0.053	0.077	-0.306	0.051	1.93×10^{-9}	0.017	0.054	0.752	0.022	0.051	0.670
rs7385804	7	100,078,232	A	C	0.621	0.178	0.053	0.00076	0.038	0.054	0.485	0.119	0.054	0.026	-0.037	0.052	0.471
rs6486121	11	13,355,770	T	C	0.631	-0.057	0.053	0.288	0.029	0.053	0.588	-0.058	0.054	0.280	-0.153	0.05	0.0022

Effects on gene expression and regulation

We next checked for data that may help explain the biological role of the significant SNPs or identify the causal variants which they tag, using sources listed in Methods. The synthesis of information from our results and external sources is exemplified in Fig. 2, which shows the alignment of data at the TFR2 locus. The region that includes genome-wide-significant SNPs (after replication) for serum iron contains documented eQTLs for TFR2, and H3K27Ac histone modification sites (documented in data from ENCODE). In this case, there is striking alignment at the region around 100.2 Mbp at one end of the TFR2 gene, which includes the most significant SNPs at this locus, documented eQTLs for this gene, and the histone modification in K562 (erythroleukaemia) cells.

A similar approach was taken for the other significant loci, as summarized in Supplementary Table 7. SNPs identified through the GWAS had significant cis-effects on expression of SLC40A1, TFRC, ARNTL and FADS1/FADS2. At the C15orf43-SORD locus on chromosome 15, rs16976620 (allelic association with ferritin $P = 4.52 \times 10^{-7}$) affected expression of SORD at $P=4.02 \times 10^{-4}$. The chromosome 22 region near TMPRSS6 contains eQTLs for the hepatic expression of TMPRSS6 (ref. 24). However, the chromosome 3 locus near TF contains eQTLs for SRPRB but not for TF; and SNPs at the loci identified as TFR2, ABO and TEX14 are eQTLs for multiple other genes. The ENCODE regulatory data show potential regulatory sequences or histone marks in the regions where we found SNP associations on chromosome 2 near SLC40A1, chromosome 11 near the FADS genes, and at the chromosome 17 locus near TEX14.

Some lead SNPs from our significant loci also showed trans- effects on more distant genes (Supplementary Table 7). Most notably, the three non-synonymous coding SNPs in HFE and TMPRSS6 (rs1800562, rs1799945 and rs855791) had strong effects on expression of ALAS2 (aminolevulinate, delta-, synthase 2), which catalyses the initial step in haem synthesis in erythroid tissues.

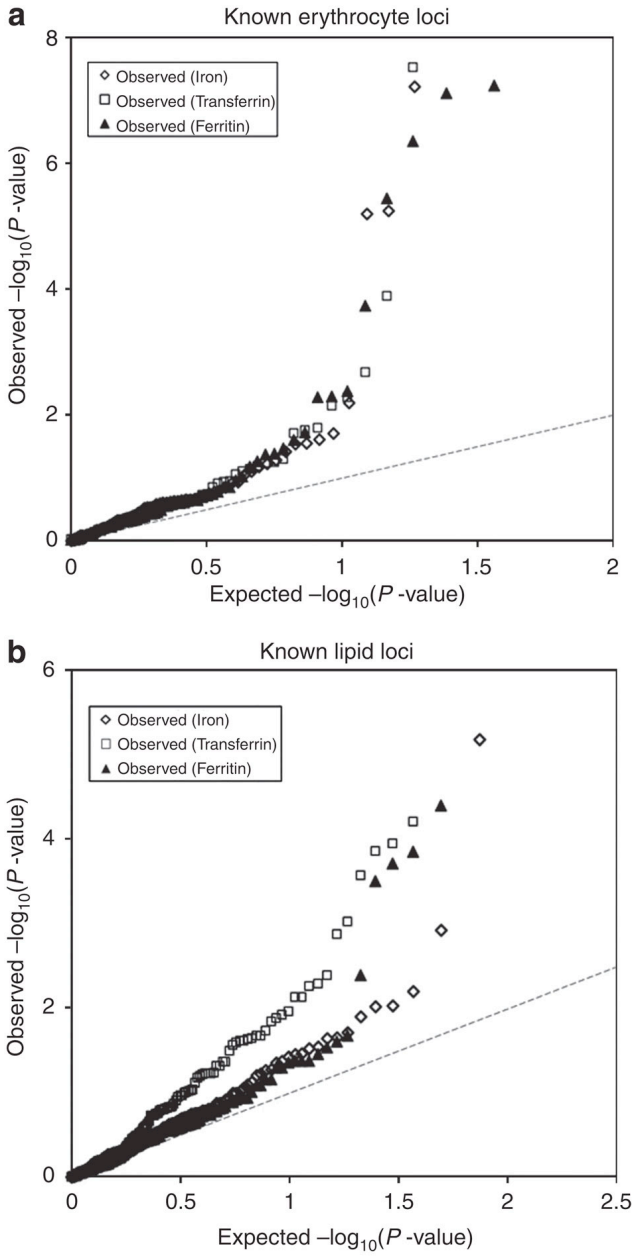

Figure 3. Deviation from the expected distribution of association p-values for iron, transferrin and ferritin at loci previously reported to be significant for (a) erythrocyte phenotypes 25 and (b) plasma lipid phenotypes[26]. For clarity, the y-axes only extend to p < 10-8 or p < 10-6 so that two associations with observed p < 10-8 for erythrocyte loci (at HFE and TMPRSS6) and four associations with p < 10-6 for lipid loci (at ABO, FADS2, HFE and NAT2) are not plotted. The interrupted line in each plot is the line of equivalence, observed = expected.

Overlap with other phenotypes and disease associations

Because of previous data showing that iron-related loci overlap with loci affecting erythrocyte phenotypes, and because several of our significant loci have been reported to affect lipid phenotypes, we compared our results against published meta-analysis data on erythrocytes and lipids. Results are summarized in Supplementary Table 8. Among the 75 significant loci for erythrocyte phenotypes[25], we found associations with one or more of our iron phenotypes after Bonferroni correction for multiple testing at $P<6.7x10^{-4}$ ($P<0.05$ adjusted for testing of 75 SNPs) for ABO, HFE, TFR2, TFRC and TMPRSS6, and additionally for HBS1L (P=9.78x10-7 for transferrin saturation) and PGS1 ($P=1.84x10^{-4}$ for ferritin). For the 157 lipid loci reported by the Global Lipids Genetics Consortium[26], two loci (HFE and HBS1L) gave $P<3.18x10^{-4}$ (Po0.05 adjusted for testing of 157 loci) for iron and saturation, six (FADS1/2/3, GCKR, HFE, NAT2, SNX5 and TRIB1) for transferrin, and six (ABO, HFE, LOC84931, LRP1, PGS1 and TRIB1) for ferritin. Moreover, plots of observed versus expected P-value distributions for the iron phenotypes (Fig. 3) showed that even the erythrocyte and lipid loci not reaching statistical significance do affect iron biomarkers to a greater degree than can be explained by chance.

The SNP association results were also analysed using Ingenuity Pathway Analysis, selecting SNPs, which showed associations at $P<0.01$, <0.001 and <0.0001 for transferrin saturation, and similarly for ferritin. Results for these two phenotypes, chosen as markers of iron availability and iron stores, showed substantial overlap. The $P<0.01$ threshold identified an excess of genes that have been reported to affect or be associated with lung cancer, cardiovascular disease and diabetes; and also with a range of developmental and nerve cell functions (Supplementary Fig. 6).
Results for the $P<0.001$ threshold were similar but showed lesser statistical significance, as expected because of the smaller number of genes included.

Effects on iron status in HFE C282Y homozygotes

We tested whether the lead SNPs at loci that affect iron-related biomarkers in the general population also explain variation in iron status in C282Y homozygotes who are at genetic risk of HFE-related iron overload. These comprised 76 homozygotes from the QIMR Adult cohort (one of the discovery cohorts), plus 277 homozygotes from the HEIRS study[27]. Results are shown in Table 2 for significant associations, and more fully in Supplementary Table 9.
The strong association between rs8177240 in the TF gene and serum transferrin was clearly present in HFE YY homozygotes ($P=1.93x10^{-9}$). The YY group showed association between serum iron and rs7385804 at TFR2 (ß=0.178±0.053, P=0.00076, critical P-value=0.005 after adjusting for testing of ten loci). The standardized beta for this SNP was approximately three times as great in the YY sample as in the overall meta-analysis (0.178±0.053 against 0.055±0.010). There was also a significant association (P=0.0022) between rs6486121 in ARNTL and ferritin. When we checked

for associations between a genetic risk score calculated from the significant and suggestive SNPs in the population-based meta-analysis results, and the biomarker phenotypes in the HEIRS sample, only transferrin showed a significant association and this was stronger among the men than the women (Supplementary Table 10).

Discussion

Our meta-analysis of GWAS on iron-related phenotypes from up to 48,000 people of European descent showed multiple significant associations. Some increased the significance of loci known from previous studies or showed significant associations with additional phenotypes (TF, TFR2, HFE, TMPRSS6); some were at loci containing genes whose products have known roles in iron homeostasis, including the transferrin receptor TFRC and the iron transporter ferroportin (SLC40A1); and others were novel (near to ARNTL, FADS2 and NAT2 for transferrin, ABO and TEX14 for ferritin). Significant associations were found for biomarkers of iron status that reflect both cellular iron metabolism and systemic regulation of iron[23].

There was variation in the phenotypes affected by the significant loci, as summarized in Supplementary Fig. 3. Three of the loci mainly affected serum ferritin (ABO, SLC40A1, TEX14); three others mainly affected serum iron and transferrin saturation (HFE, TFR2, TMPRSS6); and five mainly affected serum transferrin (ARNTL, FADS2, NAT2, TF and TFRC). The loci with the strongest effects on serum iron (HFE, TMPRSS6) had significant, but smaller, effects on serum ferritin and it is likely that this is due to higher circulating concentrations of iron leading over time to higher iron stores and hence higher serum ferritin.

We note that there are factors that can modify the relationships between these biomarker phenotypes and whole-body or tissue-specific iron status. Ferritin has been criticized as a marker of iron stores because it is an acute-phase protein increased by inflammation, but comparisons with independent methods[28,29] have validated it sufficiently for use in epidemiological studies. Moreover, the loci that affected ferritin in this study have not been reported in GWAS for inflammatory biomarkers or CRP[30], and SLC40A1, which showed a highly significant association with ferritin, has strong biological plausibility because it codes for ferroportin. Including CRP as a covariate in the ferritin association analysis changed the effect size similarly for all the significant or suggestive SNPs (Supplementary Fig. 5), whereas effects related to both inflammation and iron status would be expected to alter betas for some SNPs and not others.

We also note that matching of significant loci to genes is subject to uncertainty. For some, the location of the peak association close to a gene with a known and relevant physiological function gives confidence in the gene assignment. For others, data from previous reports or databases on association between SNPs and gene expression will identify a probable gene, but in other cases expression data are consistent with any of several genes or else no relevant data are available. If so,

the name of the nearest gene may be provided for identification of the locus but this may require revision as more information becomes available.

Five confirmed loci contain genes (TF, TFR2, HFE, TMPRSS6, SLC40A1) that were already known to affect iron homeostasis. These genes have been previously identified via monogenic diseases or from functional studies. Interestingly, no association has been identified with genes for several other important players in iron homeostasis such as ferritin, the protein that safely stores excess iron, or hepcidin and hemojuvelin, which are essential in the hepcidin signalling pathway and when mutated cause severe juvenile-onset hemochromatosis (type 2A, 2B). Mutations at the loci identified cause late-onset (HFE, type 1) or less severe (TFR2, type 3 and SLC40A1, type 4A) hemochromatosis.

SNPs at HFE and TMPRSS6 that mainly affect iron and transferrin saturation showed interesting trans-effects on gene expression for ALAS2. As this gene is on the X chromosome and we only analysed GWAS data for autosomes, we do not know whether ALAS2 variation affects our phenotypes. However, ALAS2 activity controls the initial and rate-limiting step in porphyrin synthesis so a co-ordinated effect on both iron and protoporphyrin availability for formation of haem is an interesting possibility.

SLC40A1 is a prime candidate for affecting iron stores, as it codes for ferroportin and mutations in this gene are associated with the autosomal dominant type 4 hemochromatosis, characterized by high ferritin levels. The most significant SNPs near SLC40A1 in our study are about 45 and 60 kbp from the gene, but are known to affect SLC40A1 expression. Variation near SLC40A1 also affects transferrin, probably through an effect on cellular iron availability.

Genome-wide studies of erythrocyte traits known to vary with iron status[20–22,25] have previously found associations with many of these loci: erythrocyte volume (MCV) and haemoglobin content (MCH) with HFE, TFR2, TFRC and TMPRSS6; haematocrit with HFE, TFR2, and TMPRSS6; and erythrocyte count with TFR2 (ref. 25). The results for our iron data at loci known to affect erythrocyte phenotypes are illustrated in Fig. 3a; an unexpectedly high proportion of them affect iron, transferrin and ferritin.

New associations were found for ferritin near ABO and TEX14. The ABO blood group locus has shown significant associations for several phenotypes; rs651007 has a particularly strong effect on E-selectin[31] and has also been found in GWAS on low-density lipoprotein cholesterol[32], coronary artery disease[33] and red blood cell count[25]. The latter is relevant to our ferritin finding, but whether ABO variation primarily affects iron stores and therefore erythrocyte count, or vice versa, is unclear.

TEX14 codes for a testis-expressed protein, but there was no evidence for male–female heterogeneity in the effect on ferritin (pHet for the lead SNP, rs368243, was 0.45). The most significant SNPs are within the TEX14 gene but the suggestive-significance region extends across other genes. Expression data suggest that variation affecting RAD51C may be important, but the

function of this gene (in DNA repair and meiosis) also has no obvious connection with iron status. The same holds for SEPT4, for which rs411988 is an expression QTL. Another gene within the LD block, MTMR4, deserves consideration because it changes SMAD phosphorylation, with possible effects on the BMP-SMAD pathway affecting control of hepcidin[34]. The region on chromosome 15 identified in the gene-based analysis is centred on C15orf43 but also overlaps with SORD (sorbitol dehydrogenase). SORD has no obvious connection with iron status and the function of the protein coded by C15orf43 is unknown, although there is some evidence that it is present in human plasma (http://pax-db.org/#!protein/986968, accessed 2014-03-27). These two loci illustrate the difficulty, which may be encountered in interpreting allelic associations; in some cases, the region containing the most significant results overlaps with several genes, there may be unrecognized regulatory regions with effects on more distant genes, and data on gene expression may not reflect expression in the relevant tissue. For all these reasons, assignment of significant effects to specific genes must often be provisional.

Effects on transferrin were seen for most of the loci, which affect serum iron, including HFE, TF, TFRC, and TMPRSS6. Contrary to the result for TFRC, variation at the other transferrin receptor gene TFR2 did not affect transferrin concentration; this may reflect the different functions of the two receptors. TfRC is involved in cellular iron uptake, which may directly affect the regulation of transferrin expression. TfR2 on the other hand has been reported to be involved in hepatocyte sensing of circulating iron and signalling to hepcidin production, which may subsequently affect circulating levels of iron and the transferrin saturation. TfR2 variation could also affect these iron parameters through its effect on erythropoiesis[35].

Transferrin was also affected by SNPs near ARNTL, NAT2 and FADS2. The role of these in iron homeostasis is uncertain; transferrin is central to iron transport and receptor-mediated uptake by cells but these loci did not affect serum iron or ferritin. ARNTL, and its product BMAL1, is mainly known for interactions with CLOCK genes and generation of circadian rhythm. Notably, serum iron[16,36], liver iron[37], hepcidin[38] and TfR1 gene expression[39] all show circadian variation. The region affecting transferrin on chromosome 8 contains the NAT2 gene, which again has no obvious relevance for iron. It has been shown to affect lipids[32] and cardiovascular risk (see Supplementary Table 8 of ref. 40). The gene product is important for xenobiotic metabolism; NAT2 codes for an N-acetyl transferase, which determines fast- or slow-acetylator status. At FADS2, the significant SNPs for transferrin are intronic but they affect expression of FADS genes. FADS1/2/3 variation affects a wide range of phenotypes including serum lipids[32,41], polyunsaturated fatty acid content of serum phospholipids[42]; fatty acid composition of membranes and phospholipids[43]; fasting glucose and insulin response[44,45] and liver enzymes[46]. The most significant FADS SNPs for lipids are rs174546, rs174547 and rs174548 (refs 32,41,47) and each gave significant or near- significant P-values for transferrin in our data ($P=7.43\times10^{-10}$, 8.47×10^{-10} and 7.29×10^{-8}, respectively). This, together with the decrease in the locus effect on transferrin after inclusion of HDL-C as a covariate,

suggests a common basis for effects on lipids and transferrin. The pathways involved are unknown, but iron homeostasis and lipid metabolism show overlap in the literature[32,48–51] as well as in our data. It has recently been shown, for example, that signalling pathways for the protein kinase mTOR, which regulates energy metabolism and lipid synthesis among other functions[52], affect transcriptional control of hepcidin and therefore potentially affect iron uptake and distribution[53].

Despite the varied functions of these three genes (ARNTL, FADS2, NAT2), which unexpectedly affect transferrin, they have the common feature of significant effects on plasma triglycerides[26]. Detailed comparison of our results against published lipid loci showed that a high proportion of lipid loci (not only for triglycerides) have detectable effects on our iron phenotypes, especially on transferrin (Fig. 2b, Supplementary Table 8). The pleiotropic effects at such loci, connecting iron homeostasis not only with erythropoiesis but also with lipids and possibly with cardiovascular risk, deserve further investigation.

One important clinical question about iron overload is why some HFE C282Y homozygotes develop biochemical evidence of iron overload and clinical symptoms of hemochromatosis, while most do not[54]. A systematic review of longitudinal studies found that 38–76% of homozygous people have increased ferritin and transferrin saturation (biochemical penetrance)[55]. However, clinical symptoms are less common at 2–38% in men and 1– 10% in women[56,57.] We therefore evaluated the effects of our lead SNPs in HFE C282Y homozygotes, combining data from the largest of our Discovery cohorts with available phenotypic information and DNA from participants in the HEIRS study.

Because of limited numbers of C282Y homozygotes (total N available for data analysis was 353), we had limited power to detect relevant effects. Among our results, the association between two SNPs in TFR2 and serum iron seems the most relevant. There is both clinical and experimental evidence for interaction between the gene products of HFE and TFR2. Severe juvenile hemochromatosis occurred in a family carrying mutations in both HFE and TFR2 (ref. 58). In mice, homozygosity for deletion of both Hfe and TfR2 greatly decreases hepcidin levels[59] and causes massive iron overload[60]. These reports are consistent with evidence that TFR2 and HFE proteins interact in control of hepcidin signalling; they may form an iron-sensing complex that modulates hepcidin expression in response to blood levels of diferric transferrin[61,62]. Overall, there was a lack of correlation between effect sizes for lead SNPs at the significant loci identified in the general population, and in the YY homozygotes. Similarly, a predictor based on allele count and effect size for SNPs taken forward for replication and genotyped in the HEIRS subjects did not significantly predict iron, saturation or ferritin in the HEIRS C282Y homozygotes (Supplementary Table 10 and Supplementary Fig. 7). The exception, transferrin, was due to the strong effects at the TF locus.

Previous studies have proposed determinants of HFE clinical or biochemical penetrance. Apart from age, sex and probably alcohol intake[63], the focus has been on genetic modifiers but no

candidate has been convincingly identified[64]. Since iron[23,65] homeostasis involves a complex regulating network , it seems probable that any genetic effects on penetrance are either highly polygenic (in which case large genome-wide studies on HFE C282Y homozygotes will be needed) or result from rare variants, which have not yet been examined in sufficient detail. TFR2 variation as a modifier of HFE C282Y risk has statistical support and biological plausibility but confirmation is needed.

Our results have revealed genes or loci whose effects on iron status were previously unsuspected and which need to be integrated into our understanding of iron homeostasis. Discovery of SNPs that significantly affect iron status, and compilation of genomic scores, will allow Mendelian randomization studies on the multiple conditions associated with variation in iron load and help to clarify a potential causal role of iron in such conditions (for example, Parkinson's[14] or Alzheimer's[66] diseases). However, the existence of pleiotropic effects, with many loci affecting both iron and lipid phenotypes, shows the need for caution in selecting SNPs or scores for such applications.

Methods

Subjects.

We established the Genetics of Iron Status Consortium to coordinate our efforts in understanding the causes and consequences of genetic variation in biochemical markers for iron status, that is, serum iron, transferrin, transferrin saturation and ferritin. Discovery samples consisted of summary data on genome-wide allelic associations between SNP genotypes and iron markers from 23,986 subjects of European ancestry gathered from 11 cohorts in nine participating centres (Supplementary Table 1). Replication samples to confirm suggestive and significant associations were obtained from up to 24,986 subjects of European ancestry in 8 additional cohorts (also in Supplementary Table 1). There was no systematic selection whether a cohort was allocated into the discovery or replication samples. This allocation was based on the availability of data when the analyses were conducted. Information on phenotypic means, methods for phenotype measurement, and genotyping methods for each contributing cohort are shown in Supplementary Tables 2 and 3. Each participating study was approved by the appropriate human research ethics committee, as listed for each study in Supplementary Table 1, and all subjects gave informed consent.

GWAS

Genome-wide association tests, genotype imputation and associated quality control procedures (QCs) were performed in each cohort separately. Within each cohort, QCs were applied to individual samples and SNPs before imputation into HAPMAP II (Release 22, NCBI Build36, dbSNP b126) or, for InterAct, 1,000 Genomes. These include removing individuals based on missingness, relatedness, population and ethnic outliers. Poor-quality SNPs were also removed

based on missingness, minor allele frequency, Hardy–Weinberg equilibrium test and Men- delian errors for family data. These QCs for each cohort are summarized in Supplementary Table 3.

The association between genotyped and imputed SNPs and each iron phenotype was performed using an additive model for allelic effects, on the standardized residuals of the phenotype after adjusting for age, principal component scores and other study specific covariates, for each sex separately. The details of the association analysis and imputation method for each cohort are presented in Supplementary Table 3.

Meta-analysis

We conducted meta-analysis of GWAS results from the discovery cohorts in the Metal package67 using a standard error-based approach, which weights the SNP effect size (standardized regression slope, beta) using the inverse of the corresponding squared s.e. SNPs were included in the meta-analysis if they met the following conditions: imputation quality score either Rsq (which estimates the squared correlation between imputed and true genotypes) for MACH software Z0.3, or the 'info' measure for IMPUTE software 40.5; Hardy–Weinberg Equilibrium Test P-value (pHWE) Z10 □ 6; minor allele frequency Z0.01; genotyping Call Rate Z0.95 and if they survived QCs in all cohorts to avoid disproportionate contribution of a single cohort to the meta-analysis. In total, B2.1 million SNPs met these conditions. A genomic control correction was applied to all cohorts. Heterogeneity of effect sizes between cohorts or between sexes was also assessed using Cochran's Q statistic within Metal. Loci containing SNPs with Po5 □ 10 □ 6 were carried forward for in silico replication in independent samples, again using Metal for the meta-analysis. The threshold P-value for choice of SNPs for replication is conventional and based in part on data for European populations in Duggal et al.68

Power to detect allelic effects was estimated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/Bpurcell/gpc/). Under reasonable assumptions about allele frequencies for causative and marker polymorphisms (QTL increaser allele frequency = 0.2, marker allele frequency = 0.2, linkage disequilibrium between them d'=0.8, a alpha=$5x10^{-8}$), the Discovery dataset with N=24,000 gives 77% power to detect allelic effects which each account for 0.25% of the phenotypic variance.

Gene-based analysis

Gene-based analysis considers all SNPs within a gene as a unit for the association analysis. We performed gene-based analysis on SNP association P-values from the meta-analysis of discovery samples using VEGAS (http://gump.qimr.edu.au/VEGAS/, accessed 2014-03-27) (ref. 69). The significance of gene-based analysis was based on Bonferroni correction of testing ~17,000 genes (that is, P<$3x10^{-6}$).

Conditional analysis

To find independent signals within each significant locus, we performed conditional analysis in each cohort by repeating the association analysis but including the most significant SNP at each significant locus (in the initial meta-analysis) as covariates. We performed meta-analysis of the conditional association results using the same approach as in the main meta-analysis.

Gene expression

The eQTL look-up was based on a meta-analysis of expression data for known disease-associated loci in non-transformed peripheral blood cells, from 5,300 samples from seven cohorts. The original analysis used HapMap2 imputed SNPs and a cis-window of ±250 kb from the transcription start-site. More details can be found in the paper by Westra et al.70

Information on gene expression in macrophages and monocytes was based on results obtained by the Cardiogenics consortium, on 758 samples, as described in the Supplementary Note[33].

Online resources for gene expression and regulation included http:// eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/, http://genenetwork.nl/bloodeqtlbrowser/ and Schadt et al.24 for eQTL data, http://genome.ucsc.edu/ENCODE/ for information on histone modification and http://ecrbrowser.dcode.org/ for comparison of DNA sequences across species.

Bioinformatic analyses. Pathway analysis and assessment of known disease associations or biological functions was performed using Ingenuity Pathway Analysis (IPA; Ingenuity Inc., Redwood City, CA, 94063), selecting SNPs, which showed associations at $P<0.01$,<0.001 and <0.0001 for transferrin saturation, and similarly for ferritin. IPA compares the list of genes associated with the selected SNPs against a proprietary library of gene-disease and gene-function associations and test frequencies of observed and expected occurrences.

Analysis in HFE C282Y homozygotes

Data and DNA samples from HFE C282Y homozygotes in the HEIRS study[27] were obtained from the NIH Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC) (https:// biolincc.nhlbi.nih.gov/home/). HEIRS was a population-based survey of the prevalence and effects of HFE polymorphisms, and subjects were not selected for having a diagnosis or positive family history of hemochromatosis. Selected SNPs (those showing significant or suggestive results in our primary meta-analysis) were genotyped by primer-extension mass spectrometry (MassArray, Sequenom Inc, San Diego CA); all samples were confirmed as being homozygous for the minor allele of rs1800562 by this method. Allelic association results for the QIMR adults and HEIRS C282Y homozygotes were combined by meta-analysis.

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REFERENCES

1. Miller, J. L. Iron deficiency anemia: a common and curable disease. Cold Spring Harb. Perspect. Med. 3, a011866 (2013).

2. Cherayil, B. J. Iron and immunity: immunological consequences of iron deficiency and overload. Arch. Immunol. Ther. Exp. (Warsz) 58, 407–415 (2010).

3. McCann, J. C. & Ames, B. N. An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. Am. J. Clin. Nutr. 85, 931–945 (2007).

4. Swinkels, D. W., Janssen, M. C., Bergmans, J. & Marx, J. J. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. Clin. Chem. 52, 950–968 (2006).

5. Porter, J. B. & Shah, F. T. Iron overload in thalassemia and related conditions: therapeutic goals and assessment of response to chelation therapies. Hematol. Oncol. Clin. North Am. 24, 1109–1130 (2010).

6. Philippe, M. A., Ruddell, R. G. & Ramm, G. A. Role of iron in hepatic fibrosis: one piece in the puzzle. World J. Gastroenterol. 13, 4746–4754 (2007).

7. Liu, B. et al. Iron promotes the toxicity of amyloid beta peptide by impeding its ordered aggregation. J. Biol. Chem. 286, 4248–4256 (2011).

8. Liu, Y. et al. Mutant HFE H63D protein is associated with prolonged endoplasmic reticulum stress and increased neuronal vulnerability. J. Biol. Chem. 286, 13161–13170 (2011).

9. Nandar, W. & Connor, J. R. HFE gene variants affect iron in the brain. J. Nutr. 141, 729S–739S (2011).

10. Kunutsor, S. K., Apekey, T. A., Walley, J. & Kain, K. Ferritin levels and risk of type 2 diabetes mellitus: an updated systematic review and meta-analysis of prospective evidence. Diabetes Metab. Res. Rev. 29, 308–318 (2013).

11. Simcox, J. A. & McClain, D. A. Iron and diabetes risk. Cell Metab. 17, 329–341 (2013).

12. Ellervik, C. et al. Total mortality by elevated transferrin saturation in patients with diabetes. Diabetes Care 36, 2646–2654 (2013).

13. Ellervik, C., Tybjaerg-Hansen, A. & Nordestgaard, B. G. Total mortality by Transferrin saturation levels: two general population studies and a meta- analysis. Clin. Chem. 57, 459–466 (2011).

14. Pichler, I. et al. Serum iron levels and the risk of Parkinson disease: a mendelian randomization study. PLoS Med. 10, e1001462 (2013).

15. Rhodes, S. L. et al. Pooled analysis of iron-related genes in Parkinson's disease: Association with transferrin. Neurobiol. Dis. 62C, 172–178 (2013).

16. Whitfield, J. B. et al. 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 (2000).
17. Traglia, M. et al. Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS ONE 4, e7554 (2009).
18. Benyamin, B. et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat. Genet. 41, 1173–1175 (2009).
19. Pichler, I. et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum. Mol. Genet. 20, 1232–1240 (2011).
20. Ganesh, S. K. et al. Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. Nat. Genet. 41, 1191–1198 (2009).
21. Chambers, J. C. et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat. Genet. 41, 1170–1172 (2009).
22. Soranzo, N. et al. A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat. Genet. 41, 1182–1190 (2009).
23. Hentze, M. W., Muckenthaler, M. U., Galy, B. & Camaschella, C. Two to tango: regulation of mammalian iron metabolism. Cell 142, 24–38 (2010).
24. Schadt, E. E. et al. Mapping the genetic architecture of gene expression in human liver. PLoS Biol. 6, e107 (2008).
25. van der Harst, P. et al. Seventy-five genetic loci influencing the human red blood cell. Nature 492, 369–375 (2012).
26. Global Lipids Genetics C et al. Discovery and refinement of loci associated with lipid levels. Nat. Genet. 45, 1274–1283 (2013).
27. Adams, P. C. et al. Hemochromatosis and iron-overload screening in a racially diverse population. New Engl. J. Med. 352, 1769–1778 (2005).
28. Skikne, B. S., Flowers, C. H. & Cook, J. D. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. Blood 75, 1870–1876 (1990). 29. Olthof, A. W. et al. Correlation between serum ferritin levels and liver iron concentration determined by MR imaging: impact of hematologic disease and inflammation. Magn. Reson. Imaging 25, 228–231 (2007).
30. Dehghan, A. et al. Meta-analysis of genome-wide association studies in 480 000 subjects identifies multiple loci for C-reactive protein levels. Circulation 123, 731–738 (2011).
31. Qi, L. et al. Genetic variants in ABO blood group region, plasma soluble E-selectin levels and risk of type 2 diabetes. Hum. Mol. Genet. 19, 1856–1862 (2010).
32. Teslovich, T. M. et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466, 707–713 (2010).
33. Schunkert, H. et al. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. Nat. Genet. 43, 333–338
(2011).
34. Yu, J. et al. Myotubularin-related protein 4 (MTMR4) attenuates BMP/Dpp
signaling by dephosphorylation of Smad proteins. J. Biol. Chem. 288, 79–88 (2013).
35. Forejtnikova, H. et al. Transferrin receptor 2 is a component of the erythropoietin receptor complex and is required for efficient erythropoiesis. Blood 116, 5357–5367 (2010).

36. Ridefelt, P., Larsson, A., Rehman, J. U. & Axelsson, J. Influences of sleep and the circadian rhythm on iron-status indices. Clin. Biochem. 43, 1323–1328 (2010).

37. Unger, E. L., Earley, C. J. & Beard, J. L. Diurnal cycle influences peripheral and brain iron levels in mice. J. Appl. Physiol. (1985) 106, 187–193 (2009).

38. Schaap, C. C. et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin. Chem. 59, 527–535 (2013).

39. Okazaki, F. et al. Circadian rhythm of transferrin receptor 1 gene expression controlled by c-Myc in colon cancer-bearing mice. Cancer Res. 70, 6238–6246 (2010).

40. Suhre, K. et al. Human metabolic individuality in biomedical and pharmaceutical research. Nature 477, 54–60 (2011).

41. Kathiresan, S. et al. Common variants at 30 loci contribute to polygenic dyslipidemia. Nat. Genet. 41, 56–65 (2009).

42. Lemaitre, R. N. et al. Genetic loci associated with plasma phospholipid n-3 fatty acids: a meta-analysis of genome-wide association studies from the CHARGE Consortium. PLoS Genet. 7, e1002193 (2011).

43. Merino, D. M., Ma, D. W. & Mutch, D. M. Genetic variation in lipid desaturases and its impact on the development of human disease. Lipids Health Dis. 9, 63 (2010).

44. Dupuis, J. et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. Nat. Genet. 42, 105–116 (2010). 45. Ingelsson, E. et al. Detailed physiologic characterization reveals diverse mechanisms for novel genetic Loci regulating glucose and insulin metabolism in humans. Diabetes 59, 1266–1275 (2010).

46. Chambers, J. C. et al. Genome-wide association study identifies loci influencing concentrations of liver enzymes in plasma. Nat. Genet. 43, 1131–1138 (2011).

47. Waterworth, D. M. et al. Genetic variants influencing circulating lipid levels and risk of coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 30, 2264–2276 (2010).

48. Shalev, H., Kapelushnik, J., Moser, A., Knobler, H. & Tamary, H. Hypocholesterolemia in chronic anemias with increased erythropoietic activity. Am. J. Hematol. 82, 199–202 (2007).

49. Saeed, O. et al. Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 32, 299–307 (2012).

50. Finn, A. V. et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J. Am. Coll. Cardiol. 59, 166–177 (2012).

51. Ahmed, U., Latham, P. S. & Oates, P. S. Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis. World J. Gastroenterol. 18, 4651–4658 (2012).

52. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).

53. Mleczko-Sanecka, K. et al. Unbiased RNAi screen for hepcidin regulators links hepcidin suppression to the proliferative Ras/RAF and the nutrient-dependent mTOR signaling pathways. Blood 123, 1574–1585 (2014).

54. van Bokhoven, M. A., van Deursen, C. T. & Swinkels, D. W. Diagnosis and management of hereditary haemochromatosis. BMJ 342, c7251 (2011).

55. Whitlock, E. P., Garlitz, B. A., Harris, E. L., Beil, T. L. & Smith, P. R. Screening for hereditary hemochromatosis: a systematic review for the U.S. Preventive Services Task Force. Ann. Intern. Med. 145, 209–223 (2006).

56. Beutler, E., Felitti, V. J., Koziol, J. A., Ho, N. J. & Gelbart, T. Penetrance of 845G—4 A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet 359, 211–218 (2002).

57. Allen, K. J. et al. Iron-overload-related disease in HFE hereditary hemochromatosis. New Engl. J. Med. 358, 221–230 (2008).

58. Pietrangelo, A. et al. Juvenile hemochromatosis associated with pathogenic mutations of adult hemochromatosis genes. Gastroenterology 128, 470–479 (2005).

59. Tjalsma, H. et al. Mass spectrometry analysis of hepcidin peptides in experimental mouse models. PLoS ONE 6, e16762 (2011).

60. Wallace, D. F. et al. Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology 50, 1992–2000 (2009).

61. Goswami, T. & Andrews, N. C. Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing. J. Biol. Chem. 281, 28494–28498 (2006).

62. Gao, J. et al. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab. 9, 217–227 (2009).

63. Scotet, V. et al. Hereditary hemochromatosis: effect of excessive alcohol consumption on disease expression in patients homozygous for the C282Y mutation. Am. J. Epidemiol. 158, 129–134 (2003).

64. Weiss, G. Genetic mechanisms and modifying factors in hereditary hemochromatosis. Nat. Rev. Gastroenterol. Hepatol. 7, 50–58 (2010).

65. Casanovas, G., Banerji, A., d'Alessio, F., Muckenthaler, M. U. & Legewie, S. A multi-scale model of hepcidin promoter regulation reveals factors controlling systemic iron homeostasis. PLoS Comput. Biol. 10, e1003421 (2014).

66. Crespo, A. C. et al. Genetic and biochemical markers in patients with Alzheimer's disease support a concerted systemic iron homeostasis dysregulation. Neurobiol. Aging 35, 777–785 (2014).

67. Willer, C. J., Li, Y. & Abecasis, G. R. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 26, 2190–2191 (2010).

68. Duggal, P., Gillanders, E. M., Holmes, T. N. & Bailey-Wilson, J. E. Establishing an adjusted p-value threshold to control the family-wide type 1 error in genome wide association studies. BMC Genomics 9, 516 (2008).

69. Liu, J. Z. et al. A versatile gene-based test for genome-wide association studies. Am. J. Hum. Genet. 87, 139–145 (2010).

70. Westra, H. J. et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. Nat. Genet. 45, 1238–1243 (2013).

Chapter 9

Serum Iron Levels and the Risk of Parkinson Disease: A Mendelian Randomization Study

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Abstract

Background

Although levels of iron are known to be increased in the brains of patients with Parkinson disease (PD), epidemiological evidence on a possible effect of iron blood levels on PD risk is inconclusive, with effects reported in opposite directions. Epidemiological studies suffer from problems of confounding and reverse causation, and mendelian randomization (MR) represents an alternative approach to provide unconfounded estimates of the effects of biomarkers on disease. We performed a MR study where genes known to modify iron levels were used as instruments to estimate the effect of iron on PD risk, based on estimates of the genetic effects on both iron and PD obtained from the largest sample meta-analyzed to date.

Methods and Findings

We used as instrumental variables three genetic variants influencing iron levels, HFE rs1800562, HFE rs1799945, and TMPRSS6 rs855791. Estimates of their effect on serum iron were based on a recent genome-wide meta-analysis of 21,567 individuals, while estimates of their effect on PD risk were obtained through meta-analysis of genome-wide and candidate gene studies with 20,809 PD cases and 88,892 controls. Separate MR estimates of the effect of iron on PD were obtained for each variant and pooled by meta-analysis. We investigated heterogeneity across the three estimates as an indication of possible pleiotropy and found no evidence of it. The combined MR estimate showed a statistically significant protective effect of iron, with a relative risk reduction for PD of 3% (95% CI 1%–6%; p = 0.001) per 10 μg/dl increase in serum iron.

Conclusions

Our study suggests that increased iron levels are causally associated with a decreased risk of developing PD. Further studies are needed to understand the pathophysiological mechanism of action of serum iron on PD risk before recommendations can be made.

Introduction

Iron is involved in fundamental biochemical activities, such as oxygen delivery, mitochondrial respiration, and DNA synthesis in almost all cell types. In the brain, iron is a cofactor for a large number of enzymes, including key enzymes of neurotransmitter biosynthesis, such as the tyrosine hydroxylase, which represents the rate-limiting enzyme of dopamine synthesis[1]. However, iron is also potentially toxic as an excess of free iron contributes to the generation of reactive oxygen species and can favor oxidative tissue damage[1]. In the brains of patients with Parkinson disease (PD), increased levels of iron in the substantia nigra (SN) and the lateral globus pallidus have been observed, and yet the mechanisms responsible for this phenomenon are not completely understood[2,3]. PD is characterized by the rather selective loss of dopaminergic neurons[4] and the presence of α-synuclein-enriched Lewy body inclusions in the SN[5], and several studies have demonstrated that free iron in the SN can enhance the aggregation of α-synuclein and may thus promote the formation of Lewy bodies[1].

Limited epidemiological evidence on the relationship between peripheral blood levels of iron and PD risk is available. A recent meta-analysis of ten studies, with a total of 520 PD cases and 711 controls, showed a trend for lower serum iron levels in PD patients compared with controls, although the difference in iron levels was not statistically significant (standardized mean difference: −0.45; 95% CI −0.98 to 0.08; p = 0.09)[6]. However, the very large degree of heterogeneity observed across studies (I2: 93%; p<0.0001) makes it difficult to interpret these findings.

A major limitation of observational studies is the difficulty in distinguishing between causal and spurious associations due to problems of confounding and reverse causation. Mendelian randomization (MR) is an approach based on the use of genes as instrumental variables, which has been proposed to assess causality and provide estimates of the effect of modifiable intermediate phenotypes on disease unaffected by classical confounding or reverse causation, whenever randomized clinical trials are not feasible[7]. Genes are randomly allocated at conception, so that genetic effects on the intermediate phenotype cannot be affected by classical confounding, such as lifestyle factors, or reverse causation, as in the situation where the phenotype level is influenced by the presence of the disease[8]. For this reason, demonstration that a genetic polymorphism known to modify the phenotype level also modifies the disease risk represents indirect evidence of a causal association between phenotype and disease.

The MR estimate of the effect of the intermediate phenotype on the disease is derived from the estimates of the associations of the polymorphism with both intermediate phenotype and disease. MR, as any other instrumental variable approach, has low statistical power and therefore requires very large sample sizes[9]. The recent availability of large collections of genome-wide data on intermediate phenotypes, such as blood biomarkers, and disease traits within international consortia

represents a great opportunity to exploit the potentials of this approach, and indeed MR studies have become increasingly popular over the last few years.

The validity of the MR approach relies on the crucial assumption that the polymorphism acts on the disease only through the intermediate phenotype of interest and not through others (assumption of no pleiotropy)[8]. Evaluating the possibility of pleiotropic effects of the polymorphism is therefore fundamental when using MR, and yet pleiotropy can only be excluded with confidence if the function of the gene and its polymorphisms is completely known, which is rarely the case. This problem can be addressed by using multiple instruments (polymorphisms in multiple genes influencing the same intermediate phenotype), since in the absence of pleiotropy, similar MR estimates should be obtained regardless of the instrument used, so that differences across MR estimates beyond what can be expected by chance can indicate the presence of pleiotropy[10].

In this study, we provide evidence on the presence, direction, and magnitude of a causal effect of serum iron levels on PD risk by performing a MR study, based on iron data in 21,567 individuals from the general population and PD data from 20,809 PD cases and 88,892 controls. We used three polymorphisms as instruments in order both to increase statistical power by combining their MR estimates and to investigate the possible presence of pleiotropy.

Methods

Mendelian Randomization Approach

The selection of the genes modifying iron levels to be used as instruments in our MR study was based on published results showing that polymorphisms in the hemochromatosis (HFE, ENSG00000010704) gene and the transmembrane protease 6 (TMPRSS6, ENSG00000187045) gene have the strongest effects on serum iron in the general population of European ancestry[11]. The choice of the polymorphisms within these two genes was based on the findings of a recent large meta-analysis of genome-wide association (GWA) studies on iron levels in the general population (unpublished data). We selected the polymorphisms with the strongest statistical evidence, two for the HFE gene, rs1800562 (C282Y) and rs1799945 (H63D), which are not in linkage disequilibrium (HapMap CEU $r2<0.01$) and therefore represent independent signals of association, and one for the TMPRSS6 gene, rs855791 (V736A) (Figure 1).

Our MR approach was based on the use of aggregate results for both the gene–iron and gene–PD associations: for each polymorphism, we performed a meta-analysis of studies investigating its effect on iron levels and a meta-analysis of studies investigating its effect on PD risk, with no studies contributing to both meta-analyses (see next sections). Three separate MR estimates of the effect of iron on PD were obtained for the three polymorphisms, and they were subsequently pooled by meta-analysis to provide a single MR estimate. Heterogeneity between the three MR estimates was investigated to detect the possible presence of pleiotropy.

Data on Gene Associations with Iron

Estimates of the effect sizes of the three polymorphisms in HFE and TMPRSS6 on total serum iron levels was based on the findings of a recent GWA meta-analysis on iron parameters performed by the Genetics of Iron Status (GIS) Consortium (Table 1) (unpublished data). The GIS meta-analysis includes ten cohorts from eight participating research groups. The individual datasets included in the meta-analysis are described in Table S1.

Data on Gene Associations with PD Risk

To estimate the association of the three polymorphisms with PD risk, we performed a meta-analysis of both candidate gene and GWA studies (Table 1).

Candidate gene studies were identified using PDGene (http://www.pdgene.org), a database providing a regularly updated synopsis of genetic association studies performed in PD[12]. These studies provided data for the two polymorphisms in HFE, rs1800562 and rs1799945. A total of nine studies were included in our analysis for both rs1800562[13-20] and rs1799945 [13-17,19-21] (Tables 1 and S2).

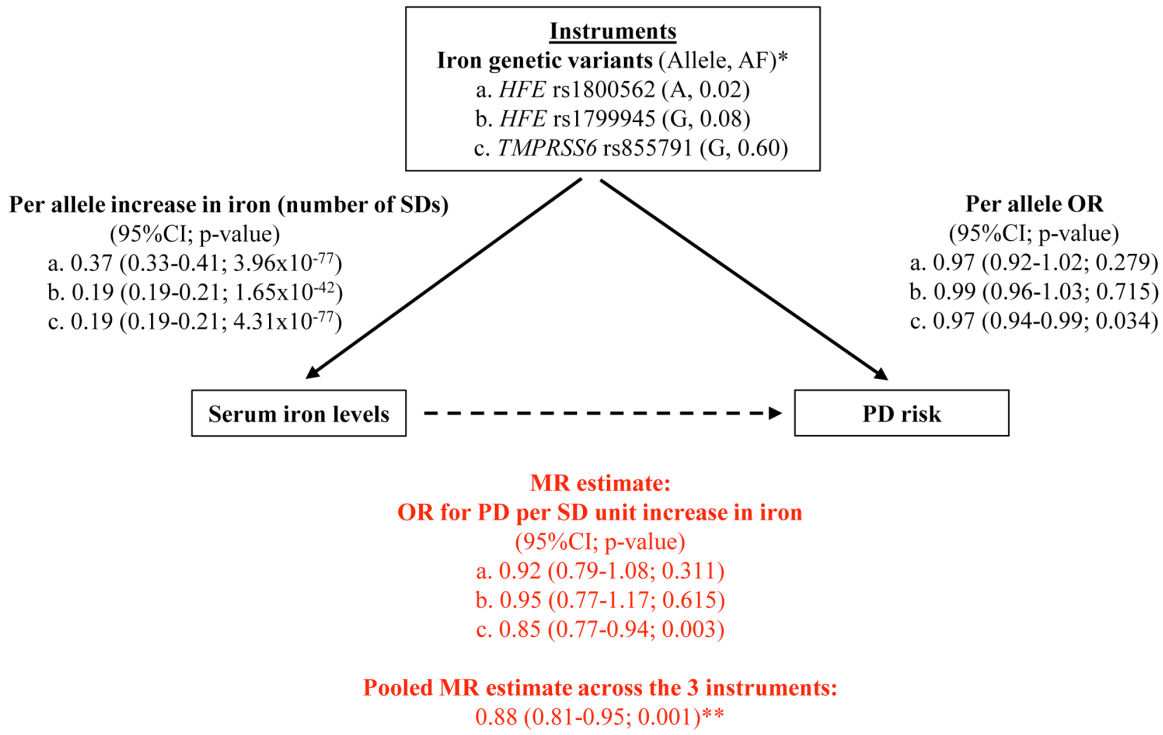

Figure 1. Graphical representation of the MR approach, with all estimates used to derive the final MR estimate. *Reported is the allele that increases iron levels, together with its frequency (AF). **This corresponds approximately to an OR per unit mg/dl increase in iron of 0.997 (95%CI 0.994–0.999), that is 0.3% (0.1%–0.6%) relative reduction in PD risk per 1 mg/dl increase in iron.

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Three large international GWA studies recently published, the PD GWAS Consortium[22], the 23andMe study[23], and the International Parkinson's Disease Genomics Consortium (IPDGC)[24,25] provided data for all three polymorphisms (Table 1). The PD GWAS Consortium includes data from five studies: PROGENI/GenePD[26], NIA Phase I[27], NIA Phase II[28], HIHG[29], and NGRC[30]. The 23andMe data come from a slightly expanded version of the cohort used in[23], including more than 4,000 PD cases and 60,000 controls. From the IPDGC, four GWA studies were included in our analysis, together with five studies genotyped with a custom genotyping array (Immunochip Illumina iSelect array); the USA-NIA and the USA-dbGAP studies were not included because of overlap with the PD GWAS dataset, and the Icelandic study was not available for analysis.
A detailed description of the individual datasets is reported in Text S1 and in Table S2.

Table 1.

Data Source	*n* studies	Type of Study	Maximum Sample Size
Gene–iron association			
GIS Consortium[a]	10	GWA	21,567
Gene–PD association			
PDGene database [13]–[21]	9	Candidate gene studies (*HFE* rs1800562 and *HFE* rs1799945)	2,384 cases; 6,908 controls
PD GWAS Consortium [22]	5	GWA	4,238 cases; 4,239 controls
23andMe [23][b]	1	GWA	4,127 cases; 62,037 controls
IPDGC [24],[25][c]	4	GWA	4,258 cases; 10,152 controls
IPDGC [24],[25]	5	Immunochip genotyping	5,802 cases; 5,556 controls

Details on individual datasets are reported in Text S1 and in Tables S1 and S2.
[a]Unpublished data. The original sample size was 22,444, but genotype and phenotype data were available only for 21,567.
[b]23andMe: slightly expanded version of the cohort used in [23].
[c]IPDGC: USA-NIA and USA-dbGAP studies were not included in our analysis due to overlap with PD GWAS Consortium; the Icelandic dataset was not available for analysis.

Statistical Analyses

GIS meta-analysis results for the gene–iron association were expressed in terms of Z-score, that is the number of standard deviations (SDs) above the mean iron level associated with each copy of the allele.

Study results for the candidate gene studies investigating the gene–PD risk association were obtained either from the PDGene website or directly from the original papers[18-20]. For two studies, estimates of the associations of interest were not provided, but they could be calculated from the data reported, by performing a per-genotype analysis based on an additive genetic model[19], or a per-allele analysis when genotype data were not available[20]. For the gene–PD meta-analysis, estimates of the (log) odds ratio (OR) were combined across studies using an inverse-variance-weighted fixed-effect model and assuming an additive genetic model, consistently with the gene–iron meta-analysis.

As for the instrumental variable analysis, an MR estimate of the effect of iron on PD risk was obtained for each of the three instruments separately, and the three estimates were combined using an inverse-variance-weighted fixed-effect meta-analysis. We evaluated the presence and magnitude of heterogeneity across the three instruments with the I2 statistics, a measure defined as the percentage of total variation in study estimates explained by heterogeneity rather than sampling error[31]. MR estimates were derived using the Wald-type estimator[32]:

$$\log \mathrm{OR}_{\mathrm{PD/iron}} = \log \mathrm{OR}_{\mathrm{PD/allele}} / \mathrm{beta}_{\mathrm{iron/allele}}$$

where log ORPD/iron is the (log) increase of PD risk by SD unit increase in iron (MR estimate), log ORPD/allele is the (log) increase in PD risk per allele (gene–PD association), and betairon/allele is the number of SDs above the mean iron level per allele (gene–iron association). The standard error of the MR estimate was derived using the Delta method[33,34] . The MR estimate is presented in terms of OR, by exponentiating the log ORPD/iron.

We evaluated the strength of each instrument using the F statistics, which is a function of the magnitude and precision of the genetic effect on the biomarker (iron):

$$\mathrm{F} = \mathrm{R}^2(n-2)/(1-\mathrm{R}^2)$$

where R2 is the variance of iron blood levels explained by the genetic variant and n is the sample size for the gene–iron association. We also evaluated the overall F statistics for the three combined instruments assuming that their effects were independent, as are expected to be given that the three gene variants are not in linkage disequilibrium.

A sensitivity analysis was performed to investigate the possible impact on our findings of population stratification in any of the studies included in the gene–iron or gene–PD analyses, by excluding studies which had not adjusted for population stratification.

All analyses were performed using Stata 10 (StataCorp LP).

Results

Gene Association with Iron

The GIS meta-analysis for iron levels included 21,567 individuals from Europe and Australia (Table S1). The effect on iron levels, expressed as number of SDs from the mean, was 0.37 (95% CI 0.33–0.41; p = 4.0×10−77) for each copy of the A allele of HFE rs1800562, 0.19 (95% CI 0.17–0.21; p = 1.7×10−42) for the G allele of HFE rs1799945, and 0.19 (95% CI 0.17–0.21; p = 4.3×10−77) for the G allele of TMPRSS6 rs855791 (Figure 1; Table S3). With a SD for serum iron

levels of 37.6 µg/dl, these figures correspond to an increase in iron per allele of approximately 13.9, 7.1. and 7.1 µg/dl, respectively. HFE rs1800562, HFE rs1799945, and TMPRSS6 rs855791 explained 1.7%, 0.9%, and 1.7% of iron total variance, respectively (Table S3).

The F statistics was very high for all genetic variants, as can be expected given the sample size of more than 21,000 individuals[35]: 382, 199, and 379 for HFE rs1800562, HFE rs1799945, and TMPRSS6 rs855791, respectively. The F statistics for all combined instruments was 987.

Gene Association with PD Risk

All datasets available for the analysis of the effects of the three genetic polymorphisms on PD risk (Table S2) were checked for the presence of overlapping studies, and duplicates were removed. The meta-analysis, which included a total of 20,809 PD cases and 88,892 controls from Europe and North America (Table S2), revealed a significant association for TMPRSS6 rs855791 with PD risk, with an OR of 0.97 (95% CI 0.94–0.99; p = 0.034) per copy of the G allele. As shown in the Forest plot of the meta-analysis for this polymorphism (Figure S3), there was no statistical evidence of heterogeneity across studies, with a heterogeneity test p-value of 0.86 and an I2 of 0% (95% CI 0%–85%). In particular, although the 23andMe study was based on self-reported disease status and therefore differed from the rest, its results were consistent with those of the other PD studies. The association with PD risk for the two polymorphisms in HFE was not statistically significant, with an OR of 0.97 (95% CI 0.92–1.02; p = 0.281) for the A allele of rs1800562 and 0.99 (95% CI 0.96–1.03; p = 0.715) for the G allele of rs1799945 (Figures 1, S1, and S2; Table S4). This might be explained by the much lower statistical power for the two HFE variants compared with the TMPRSS6 variant due to their lower minor allele frequency (1,000 Genomes project: 0.02 and 0.08 versus 0.40), as suggested by their wide confidence intervals.

Mendelian Randomization Estimate of Iron Association with PD Risk

The meta-analysis of the three MR estimates resulted in a statistically significant combined estimate of 0.88 (95% CI 0.82–0.95; p = 0.001), representing the OR for PD per SD unit increase in iron (Figure 1). Again, with a SD for iron levels of 37.6 µg/dl, this corresponds approximately to an OR of 0.997 (95% CI 0.994–0.999) per 1 µg/dl increase in iron, that is a 0.3% (95% CI 0.1%–0.6%) relative risk reduction. The Forest plot in Figure 2 shows how the meta-analysis result was driven by the TMPRSS6 rs855791 variant, and that there was no statistical evidence of heterogeneity across instruments (p = 0.54; I2: 0%, 95% CI 0%–90%), suggesting that the assumption of no pleiotropy might hold.

The sensitivity analysis investigating the impact of population stratification excluded the nine studies from PDGene, which had not reported any adjustment for population stratification, while there were no exclusions from the GIS consortium on iron since all studies had adjusted for

population stratification (Table S2). The result of the sensitivity analysis was similar to that of the main analysis, with a combined MR estimate of 0.91 (95% CI 0.83–0.99; p = 0.032) (Figure S4).

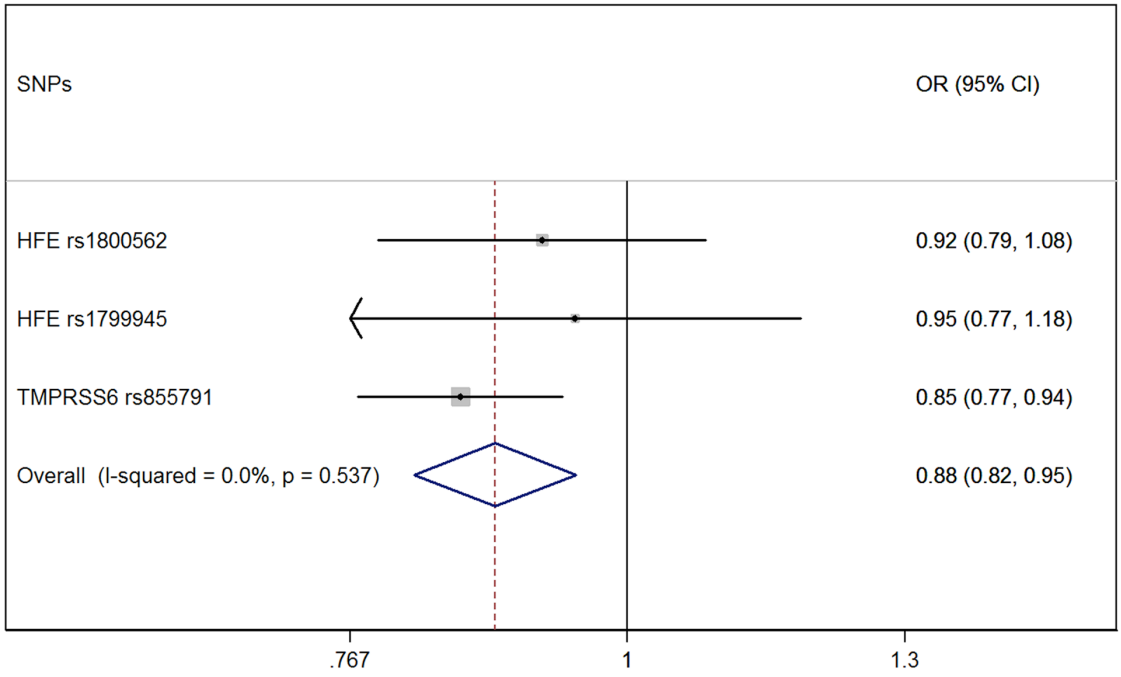

Figure 2. Forest plot of the MR estimates from the three instruments. The size of the squares is proportional to the precision of the MR estimates for each polymorphism, with the horizontal lines indicating their 95% confidence intervals. The combined MR estimate is represented by the centre of the diamond, with the lateral tips indicating its 95% confidence interval. The solid vertical line is the line of no effect. doi:10.1371/journal.pmed.1001462.g002

Discussion

Our study shows a protective effect of serum iron levels on PD, with a 3% (95% CI 1%–6%; p = 0.001) relative reduction in PD risk per 10 µg/dl increase in iron. If we hypothesise increasing serum iron levels of one SD unit (38 µg/dl in our study) in a population of Caucasians older than 60, where PD risk is around 1%[36], a corresponding relative risk reduction of 12% would translate to a decrease in PD cases from 100/10,000 to 88/10,000. Since genotype influences on serum iron levels represent differences that generally persist throughout adult life, the estimate of our MR study reflects an effect of iron over the course of a lifetime. These findings are important since evidence on the association between serum iron levels and PD risk collected so far has been controversial. Although iron is generally thought of as a risk factor for PD, in line with the well-known phenomenon of iron accumulation in the brain of PD patients[2,3], epidemiological studies have shown effects of iron in opposite directions. A recent meta-analysis of epidemiological studies suggests a possible protective role of serum iron levels on PD risk, but its findings are difficult to interpret owing to the very large degree of heterogeneity across studies[6].

Epidemiological studies suffer from confounding and reverse causation, which are intrinsic to their observational nature, so that they can hardly provide conclusive evidence on the causality of an observed association. Tobacco smoking and coffee drinking, which have been suggested as protective factors for PD[37,38], represent two potential confounders for the association between iron and PD, since both might have an effect on iron levels. Nicotine might decrease the availability of free reactive iron[39], and coffee is known to inhibit the intestinal absorption of iron[40,41]. Reverse causation could also produce spurious associations in epidemiological studies if the phenotype level can be influenced by the presence of the disease. An example is that of monoamine oxidase (MAO) inhibitors used to treat PD. MAO inhibitors may have iron-chelating effects and thus reduce iron blood levels, which could lead to spurious epidemiological evidence of a difference in iron levels between PD cases and controls[42]. Although causality is usually assessed by use of randomized clinical trials, the MR approach represents a valuable alternative whenever these are not feasible[7]. It is based on the concept that genetic variation modifying the concentration of a biomarker should also affect the disease risk if (and only if) the biomarker is directly and causally involved in the disease pathogenesis. Being genes randomly allocated at conception, their effects on biomarkers are unaffected by classical confounding factors and reverse causation[8].

The protective effect of higher serum iron levels on PD risk found in our study may seem somewhat counterintuitive at first sight. However, there are several reports in the literature in line with our findings. A recent study showed a negative correlation between SN echogenicity, a marker for increased SN iron content[43], and serum iron levels in PD patients[44]. A case-control study suggested an increased risk of PD in men who reported multiple recent blood donations and thus experienced depleted systemic iron stores[45], and another study showed an association of anemia experienced early in life with increased PD risk, with the authors hypothesizing that anemia could be a surrogate marker for iron deficiency[46]. Finally, in dietary iron-restricted mice impaired motor behavior and a marked decrease of striatal dopamine levels was observed, which was explained with the fact that iron is essential for the activity of tyrosine hydroxylase, the rate-limiting enzyme in the dopamine synthesis[47]. Consistent with these findings, a recent study performed in Japan found an association between higher iron intake and reduced PD risk[48].

The underlying mechanisms of the protective effect of iron on PD risk observed in our study remains unclear, as does the mechanism that regulates the relationship between serum and brain iron levels. Low peripheral iron levels may reduce the functioning of neuronal enzymes or receptors, since iron is a crucial cofactor of tyrosine hydroxylase[49], plays a role in the synthesis of monoamine neurotransmitters, and is involved in dopaminergic neurodevelopment[50]. Furthermore, low iron levels may decrease neuronal iron storage in the form of ferritin[51], which was found to be inappropriately low in SN neurons in PD[1]. A reduction in ferritin could decrease neuronal iron utilization by decreasing the pool of iron available for neuronal enzymes[47], thus leading to the

accumulation of free iron in SN[1]. Similar large-scale MR studies investigating other markers of iron metabolism, such as ferritin and transferrin, could contribute to our understanding of the role of peripheral iron homeostasis in the pathophysiology of PD.

To our knowledge, this is the first MR study aimed at estimating the magnitude of the effect of serum iron levels on PD risk. Previous case-control studies have tried to assess causality and direction of the association by investigating the effect on PD risk of genes involved in iron metabolism and homeostasis, although their findings are somewhat inconsistent with only some supporting the hypothesis of a causal association. Among the many genes evaluated, which include FTL, FTH1, TF, TFRC, IREB2, LTF, CP, FXN, HFE[52], HPX, HAMP, HFE2[53], and FTMT[54], only the G258S polymorphism in the TF gene showed a statistically significant association with PD[17], although the finding was not replicated in a subsequent study[55], and a haplotype in the SLC11A2 gene was found to occur more frequently in PD[56]. However, all these previous studies were relatively small and therefore underpowered to detect modest genetic effects on PD risk. Our MR study used three polymorphisms in the HFE and TMPRSS6 genes as instruments. Evidence on their association with PD risk was obtained through meta-analysis of several candidate gene studies and three large GWA studies, including a total of more than 20,000 patients and 88,000 controls, which represents the largest PD case-control sample with genetic data meta-analyzed to date. Similarly, estimates of the effect of the three polymorphisms on serum iron levels were based on results from a recent GWA meta-analysis including more than 21,000 individuals. Unlike similar MR investigations that have combined multiple instruments into a single allele score using individual data analyses from all contributing studies, our analyses required only aggregate results for the effect of each genetic variant on both biomarker and disease. This may have practical importance, since it allows inclusion of results from ongoing genetic consortia without requiring further analyses, as well as inclusion of previous findings from published reports. However, methodological work will be needed to assess the relative benefits of the two approaches under different scenarios.

The crucial aspect of a MR study, and more generally of any study based on an instrumental variable approach, is the choice of the gene (instrument) that needs to have a strong effect on the intermediate phenotype of interest. We used three polymorphisms as instrumental variables, since the use of multiple instruments influencing the intermediate phenotype of interest can increase the statistical power of the MR analysis[10]. The instrument strength was high for all of them, as shown by their very large F-statistic values. Two of them, rs1800562 (C282Y) and rs1799945 (H63D), are non-synonymous polymorphisms in HFE, a gene with well known effects in the modulation of iron blood levels[57]. The third non-synonymous polymorphism, rs855791 (V736A), is located in TMPRSS6, a gene whose role in iron regulation was demonstrated more recently[58]. The two variants in the HFE gene are responsible for most cases of hereditary hemochromatosis[59,60], and they are associated with iron overload when present in the homozygous (C282Y/C282Y) or

compound heterozygous (C282Y/H63D) state. The C282Y variant prevents the altered HFE protein from reaching the cell surface and interacting with the transferrin receptor (TfR)[61,62]. As a result, iron regulation is disrupted. The exact functional effect of the H63D variant is as yet unclear, but some evidence suggests that it may alter an intramolecular salt bridge, possibly affecting the interaction of the HFE protein with the TfR[63]. The TMPRSS6 V736A variant was found associated with iron-deficiency anemia[64]. Furthermore, the A allele has been shown to inhibit hepcidin more efficiently than the V allele in in vitro experiments, and to affect hepcidin levels in healthy individuals[65]. Interestingly, TMPRSS6 rs855791 was by far the most influential and was the one driving the result of the meta-analysis of MR estimates from the three instruments. The wide confidence intervals of the MR estimates for HFE rs1800562 and rs1799945 suggest that the power of their MR analysis was very limited due to their low allele frequency. This illustrates the importance of balancing the strength of the effect on the intermediate phenotype with allele frequency and statistical power when choosing the instruments for a MR study.

A potential source of bias specific to MR studies is pleiotropy, whereby the HFE or TMPRSS6 genotypes could influence PD risk through another mechanism that is independent of their effect on serum iron levels. Although we cannot completely exclude pleiotropic effects of the three polymorphisms used in our study because of incomplete knowledge of the underlying biology, we can indirectly investigate the presence of such effects through the simultaneous use of the three polymorphisms as multiple instruments. In a MR study, if all instruments are valid, their MR estimates should differ only as a result of sampling error[10], so that there should be no heterogeneity in the meta-analysis of MR estimates. In our meta-analysis of MR estimates there was no evidence of heterogeneity, although the statistical power to detect heterogeneity is limited when only three estimates are included in the meta-analysis[66]. As more evidence on genes influencing iron blood levels becomes available, MR studies investigating the effects of iron on the risk of PD and other diseases will be able to include many more genetic variants as instruments. This will ensure that pleiotropy can be ruled out with greater confidence. Selection of genes to be used as instruments requires careful consideration, since inclusion of variants with small genetic effects on the biomarker may introduce a “weak instrumental variable” bias[35]. Another potential issue in MR investigations is developmental canalization, the ability to produce the same phenotype regardless of genetic (or environmental) variation. If a genetic polymorphism is expressed during fetal development, compensatory processes may influence development in a way that can protect against the effect of the polymorphism[8]. Although canalization of genetic effects needs to be considered when interpreting MR findings, this problem is very difficult to investigate. Finally, one could speculate that the observed association of the subject's iron-related genotype with PD risk might actually reflect an intrauterine effect of iron due to a similar iron-related maternal genotype. Some

evidence suggests that maternal iron deficiency could result in an altered iron status of the newborn, with possible negative effects on the neurophysiologic development[67].

Despite all the possible limitations discussed above, MR offers a valuable approach to derive causal effect estimates whenever randomized trials are very difficult to perform, as in the case of iron and PD. A trial investigating the long-term effect of changes in a subject's iron status, obtained by some means, on the risk of developing PD would require not only a very long follow-up but also a huge sample size, given the low frequency of the disease and the magnitude of the effect that might realistically be expected.

In our study, the MR analysis to combine the OR of the gene–PD association with the effect of the gene–iron association was based on a Wald-type estimator, which works under a "rare disease assumption" that is appropriate in the case of PD. However, the use of a Wald-type estimator for the MR analysis of binary outcomes represents only an approximate method and may produce biased MR estimates[32]. Although such bias has been recently shown to be small, typically within 10% of the MR estimate[68], methods in this area are still under active development.

In summary, our MR study suggests a causal association between increased serum iron levels and decreased risk of developing PD, suggesting that disrupted iron metabolism may be an important factor in the pathogenesis of PD. However, further research is needed to elucidate the pathophysiological mechanism of action underlying our findings. The effect of dietary iron or drugs capable of altering the balance between serum iron and iron storage compartments, might prove to be suitable to test in experimental models. The development of such disease models is therefore necessary before any public health or clinical recommendation can be made for primary prevention in subjects at high risk of developing PD.

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REFERENCES

1. Crichton RR, Dexter DT, Ward RJ (2011) Brain iron metabolism and its perturbation in neurological diseases. J Neural Transm 118: 301–314.

2. Zecca L, Youdim MB, Riederer P, Connor JR, Crichton RR (2004) Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci 5: 863–873.

3. Dusek P, Jankovic J, Le W (2012) Iron dysregulation in movement disorders. Neurobiol Dis 46: 1–18.

4. Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55: 259–272.

5. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, et al. (1997) Alpha-synuclein in Lewy bodies. Nature 388: 839–840.

6. Mariani S, Ventriglia M, Simonelli I, Donno S, Bucossi S, et al. (2013) Fe and Cu do not differ in Parkinson's disease: a replication study plus meta-analysis. Neurobiol Aging 34: 632–633.

7. Davey Smith G, Ebrahim S (2005) What can mendelian randomisation tell us about modifiable behavioural and environmental exposures? BMJ 330: 1076–1079.

8. Davey Smith G, Ebrahim S (2003) 'Mendelian randomization': Can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 32: 1–22.

9. Pierce BL, Ahsan H, Vanderweele TJ (2011) Power and instrument strength requirements for mendelian randomization studies using multiple genetic variants. Int J Epidemiol 40: 740–752.

10. Palmer TM, Lawlor DA, Harbord RM, Sheehan NA, Tobias JH, et al. (2012) Using multiple genetic variants as instrumental variables for modifiable risk factors. Stats Methods Med Res 21: 223–242.

11. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, et al. (2009) Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 41: 1173–1175.

12. Lill CM, Roehr JT, McQueen MB, Kavvoura FK, Bagade S, et al. (2012) Comprehensive research synopsis and systematic meta-analyses in parkinson's disease genetics: The PDGene database. PLoS Genet 8: e1002548. doi: 10.1371/journal.pgen.1002548.

13. Greco V, De Marco EV, Rocca FE, Annesi F, Civitelli D, et al. (2011) Association study between four polymorphisms in the HFE, TF and TFR genes and Parkinson's disease in southern Italy. Neurol Sci 32: 525–527.

14. Halling J, Petersen MS, Grandjean P, Weihe P, Brosen K (2008) Genetic predisposition to parkinson's disease: CYP2D6 and HFE in the Faroe Islands. Pharmacogenet Genomics 18: 209–212.

15. Guerreiro RJ, Bras JM, Santana I, Januario C, Santiago B, et al. (2006) Association of HFE common mutations with Parkinson's disease, Alzheimer's disease and mild cognitive impairment in a portuguese cohort. BMC Neurol 6: 24.

16. Dekker MC, Giesbergen PC, Njajou OT, van Swieten JC, Hofman A, et al. (2003) Mutations in the hemochromatosis gene (HFE), Parkinson's disease and parkinsonism. Neurosci Lett 348: 117–119.

17. Borie C, Gasparini F, Verpillat P, Bonnet AM, Agid Y, et al. (2002) Association study between iron-related genes polymorphisms and Parkinson's disease. J Neurol 249: 801–804.

18. Buchanan DD, Silburn PA, Chalk JB, Le Couteur DG, Mellick GD (2002) The Cys282Tyr polymorphism in the HFE gene in Australian Parkinson's disease patients. Neurosci Lett 327: 91–94.

19. Aamodt AH, Stovner LJ, Thorstensen K, Lydersen S, White LR, et al. (2007) Prevalence of haemochromatosis gene mutations in Parkinson's disease. J Neurol, Neurosurg Psychiatry 78: 315–317.

20. Biasiotto G, Goldwurm S, Finazzi D, Tunesi S, Zecchinelli A, et al. (2008) HFE gene mutations in a population of Italian Parkinson's disease patients. Parkinsonism Relat Disord 14: 426–430.

21. Akbas N, Hochstrasser H, Deplazes J, Tomiuk J, Bauer P, et al. (2006) Screening for mutations of the HFE gene in Parkinson's disease patients with hyperechogenicity of the substantia nigra. Neurosci Lett 407: 16–19.

22. Pankratz N, Beecham GW, DeStefano AL, Dawson TM, Doheny KF, et al. (2012) Meta-analysis of Parkinson's disease: Identification of a novel locus, RIT2. Ann Neurol 71: 370–384.

23. Do CB, Tung JY, Dorfman E, Kiefer AK, Drabant EM, et al. (2011) Web-based genome-wide association study identifies two novel loci and a substantial genetic component for Parkinson's disease. PLoS Genet 7: e1002141. doi:10.1371/ journal.pgen.1002141

24. International Parkinson Disease Genomics Consortium, Nalls MA, Plagnol V, Hernandez DG, Sharma M, et al. (2011) Imputation of sequence variants for identification of genetic risks for Parkinson's disease: A meta-analysis of genome- wide association studies. Lancet 377: 641–649.

25. International Parkinson's Disease Genomics Consortium (IPDGC), Wellcome Trust Case Control Consortium 2 (WTCCC2) (2011) A two-stage meta-analysis identifies several new loci for Parkinson's disease. PLoS Genet 7: e1002142. doi:10.1371/journal.pgen.1002142

26. Pankratz N, Wilk JB, Latourelle JC, DeStefano AL, Halter C, et al. (2009) Genomewide association study for susceptibility genes contributing to familial Parkinson disease. Hum Genet 124: 593–605.

27. Fung HC, Scholz S, Matarin M, Simon-Sanchez J, Hernandez D, et al. (2006) Genome-wide genotyping in Parkinson's disease and neurologically normal controls: first stage analysis and public release of data. Lancet Neurol 5: 911– 916.

28. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, et al. (2009) Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet 41: 1308–1312.

29. Edwards TL, Scott WK, Almonte C, Burt A, Powell EH, et al. (2010) Genome- wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Ann Hum Genet 74: 97–109.

30. Hamza TH, Zabetian CP, Tenesa A, Laederach A, Montimurro J, et al. (2010) Common genetic variation in the HLA region is associated with late-onset sporadic Parkinson's disease. Nat Genet 42: 781–785.

31. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ 327: 557–560.

32. D Didelez V, Meng S (2010) Assumptions of IV methods for observational epidemiology. Stat Sci 25: 22–40

33. Bautista LE, Smeeth L, Hingorani AD, Casas JP (2006) Estimation of bias in nongenetic observational studies using ''mendelian triangulation''. Ann Epide- miol 16: 675–680.

34. Thomas DC, Lawlor DA, Thompson JR (2007) Re: Estimation of bias in nongenetic observational studies using ''mendelian triangulation'' by Bautista et al. Ann Epidemiol 17: 511–513.

35. Pierce BL, Ahsan H, Vanderweele TJ (2011) Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. Int J Epidemiol 40: 740–752.

36. Tanner CM, Goldman SM (1996) Epidemiology of Parkinson's disease. Neurol Clin 14: 317–335.

37. Ritz B, Ascherio A, Checkoway H, Marder KS, Nelson LM, et al. (2007) Pooled analysis of tobacco use and risk of Parkinson disease. Arch Neurol 64: 990–997.

38. Hernan MA, Takkouche B, Caamano-Isorna F, Gestal-Otero JJ (2002) A meta- analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease.
Ann Neurol 52: 276–284.

39. Linert W, Bridge MH, Huber M, Bjugstad KB, Grossman S, et al. (1999) In
vitro and in vivo studies investigating possible antioxidant actions of nicotine: Relevance to Parkinson's and Alzheimer's diseases. Biochim Biophy Acta 1454: 143–152.

40. Morck TA, Lynch SR, Cook JD (1983) Inhibition of food iron absorption by coffee. Am J Clin Nutr 37: 416–420.

41. Zijp IM, Korver O, Tijburg LB (2000) Effect of tea and other dietary factors on iron absorption. Crit Rev Food Sci Nutr 40: 371–398.

42. Kupershmidt L, Amit T, Bar-Am O, Youdim MB, Weinreb O (2012) Neuroprotection by the multitarget iron chelator M30 on age-related alterations in mice. Mech Ageing Dev 133: 267–274.

43. Berg D, Roggendorf W, Schroder U, Klein R, Tatschner T, et al. (2002) Echogenicity of the substantia nigra: Association with increased iron content and marker for susceptibility to nigrostriatal injury. Arch Neurol 59: 999–1005.

44. Walter U, Witt R, Wolters A, Wittstock M, Benecke R (2012) Substantia nigra echogenicity in Parkinson's disease: Relation to serum iron and C-reactive protein. J Neural Transm 119: 53–57.

45. Logroscino G, Chen H, Wing A, Ascherio A (2006) Blood donations, iron stores, and risk of Parkinson's disease. Mov Disord 21: 835–838.

46. Savica R, Grossardt BR, Carlin JM, Icen M, Bower JH, et al. (2009) Anemia or low hemoglobin levels preceding Parkinson disease: a case-control study. Neurology 73: 1381–1387.

47. Levenson CW, Cutler RG, Ladenheim B, Cadet JL, Hare J, et al. (2004) Role of dietary iron restriction in a mouse model of Parkinson's disease. Exp Neurol 190: 506–514.

48. Miyake Y, Tanaka K, Fukushima W, Sasaki S, Kiyohara C, et al. (2011) Dietary intake of metals and risk of Parkinson's disease: a case-control study in Japan. J Neurol Sci 306: 98–102.

49. Ramsey AJ, Hillas PJ, Fitzpatrick PF (1996) Characterization of the active site iron in tyrosine hydroxylase. Redox states of the iron. J Biol Chem 271: 24395– 24400.

50. Beard J, Erikson KM, Jones BC (2003) Neonatal iron deficiency results in irreversible changes in dopamine function in rats. J Nutr 133: 1174–1179. Levenson CW, Tassabehji NM (2004) Iron and ageing: an introduction to iron regulatory mechanisms. Ageing Res Rev 3: 251–263.

51. Rhodes SL, Ritz B (2008) Genetics of iron regulation and the possible role of iron in Parkinson's disease. Neurobiol Dis 32: 183–195.

52. Castiglioni E, Finazzi D, Goldwurm S, Pezzoli G, Forni G, et al. (2010) Analysis of nucleotide variations in genes of iron management in patients of Parkinson's disease and other movement disorders. Parkinsons Dis 2011: 827693. Castiglioni E, Finazzi D, Goldwurm S, Levi S, Pezzoli G, et al. (2010) Sequence variations in mitochondrial ferritin: Distribution in healthy controls and different types of patients. Genet Test Mol Biomarkers 14: 793–796.

53. Ezquerra M, Campdelacreu J, Munoz E, Tolosa E (2005) Association study of the G258S transferrin gene polymorphism and Parkinson's disease in the Spanish population. J Neurol 252: 1269–1270.

54. He Q, Du T, Yu X, Xie A, Song N, et al. (2011) DMT1 polymorphism and risk of Parkinson's disease. Neurosci Lett 501: 128–131.

55. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, et al. (2003) The discovery of the new haemochromatosis gene. 1996. J Hepatol 38: 704–709. Du X, She E, Gelbart T, Truksa J, Lee P, et al. (2008) The serine protease TMPRSS6 is required to sense iron deficiency. Science 320:

1088–1092. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, et al. (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 13: 399–408.

56. Bradley LA, Johnson DD, Palomaki GE, Haddow JE, Robertson NH, et a. (1998). Hereditary haemochromatosis mutation frequencies in the general population. J Med Screen 5: 34–36.

57. Waheed A, Parkkila S, Zhou XY, Tomatsu S, Tsuchihashi Z, et al. (1997) Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. Proc Natl Acad Sci U S A 94: 12384–12389.

58. Feder JN, Tsuchihashi Z, Irrinki A, Lee VK, Mapa FA, et al. (1997) The hemochromatosis founder mutation in HLA-H disrupts beta2-microglobulin interaction and cell surface expression. J Biol Chem 272: 14025–14028. Lebro´n JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, et al. (1998) Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell 93: 111–123.

59. An P, Wu Q, Wang H, Guan Y, Mu M, et al. (2012) TMPRSS6, but not TF, TFR2 or BMP2 variants are associated with increased risk of iron-deficiency anemia. Hum Mol Genet 21: 2124–2131.

60. Nai A, Pagani A, Silvestri L, Campostrini N, Corbella M, et al. (2011) TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. Blood 118: 4459–4462.

61. Fleiss JL (1993) The statistical basis of meta-analysis. Stat Methods Med Res 2: 121–145.

62. Tamura T, Goldenberg RL, Hou J, Johnston KE, Cliver SP, Ramey SL, et al. (2002) Cord serum ferritin concentrations and mental and psychomotor development of children at five years of age. J Ped 186: 458–463.

63. Harbord RM, Didelez V, Palmer TM, Meng S, Sterne JA, et al. (2013) Severity of bias of a simple estimator of the causal odds ratio in mendelian randomization studies. Stat Med 32: 1246-1258.

Chapter 10

General discussion and perspectives

The genetic architecture of iron homeostasis parameters

The studies described in this thesis aimed at highlighting novel and causative variants in loci that have a role in hepcidin-iron pathway as regulators of iron homeostasis.

Iron is a key element for humans' homeostasis involved in many cellular and tissue processes. The storage and distribution of iron are tightly regulated by a complex pathway in which the liver hormone hepcidin plays a critical role.

Iron is absorbed by the enterocytes in duodenum and derived by the recycling of exhausted macrophages, bound to transferrin and exported to the bloodstream depending on the needs of iron-consuming cells, tissues and organs. The distribution of iron in the bloodstream is strictly regulated by transmembrane protein ferroportin-hepcidin interaction and genetic disorders that affect this fundamental interaction are known to damage body iron homeostasis.

The clarification of the role of known loci and the findings of novel loci involved in the regulation of iron homeostasis can help to clarify the effect of unbalanced iron on humans and the onset of genetic iron disorders that causes iron overload (hereditary hemocromatosis or HH) and iron deficiency (iron-refractory iron deficiency anemia or IRIDA). Having measured hepcidin level in the entire population of Val Bobera we could study several novel aspects of the biology of hepcidin as well as of the hepcidin isoforms present in serum. Hep-25 processing can result in the generation of two amino-terminal truncated isoforms, hepcidin-22 (hep-22) and hepcidin-20 (hep-20). Only Hep-20 can be measured by Mass Specrometry (MS). in serum.. The truncated forms have lost the ability to bind ferroportin and they may be degradation products of hep-25. Recent studies however showed a strong antimicrobial activity of hep-20 in respect to hep-25 and relatively high levels of hep-20 were detected in heterogeneous pathological conditions like acute myocardial infarction (AMI)[1], anemia of chronic disease (ACD)[2] and chronic kidney disease (CKD)[3-5]. Our study is the first description of hep-20 levels of in a large normal population: we showed that Hep-20 is detectable mainly in older individuals that show high transferrin saturation and ferritin levels. It correlates in a gender specific manner: with age, haemoglobin and C-Reactive Protein in males and with age, BMI, ferritin, C-Reactive Protein and creatinine in females. We could show a negative correlation between ferritin and hep-20 suggesting that hep-20 is not only a product of degradation of hep-25 but instead an active regulation of hep-25 degradation according to body iron need. The proteases responsible has to be identified.

We also focused on the variation of hepcidin level in different human genetic disorders in which iron could be unbalanced. The correlation between iron parameters and metabolic syndrome (MetS) is quite accepted but pathophysiological link between iron and MetS remains unclear. To better understand their relationship the levels of hepcidin have been analysed in MetS cases with at least three pathological conditions that define metabolic syndrome (MetS) affection (abdominal obesity, high fasting plasma, elevated serum triglycerides, low serum HDL cholesterol or high blood pressure) and in controls collected in Val Borbera population to characterize the levels by

sex and age. As known serum ferritin levels are often elevated in MetS in a condition known as dysmetabolic hyperferritinemia or DHF[6] and are sometimes associated with a true mild-to-moderate hepatic iron overload (dysmetabolic iron overload syndrome or DIOS)[7]. In Val Borbera MetS cases show significantly higher serum levels of both ferritin and hepcidin as compared to controls linearly with the increase of the five MetS features for both sexes. We observed an independent influence of hepcidin in women: MetS affection is associated with hepcidin adjusted for ferritin and age only in females and the increase of hepcidin in MetS cases is higher in females with lower ferritin levels, in particular with iron deficiency. The strong association between hepcidin and ferritin, as well as their parallel behaviour observed as a function of increasing number of MetS features provides the first evidence that hyperhepcidinemia may occur mainly in response to mild-to-moderate increase of body iron stores. The high levels of hepcidin in MetS suggest future investigations on the possible role of this hormone in promoting the cardiovascular complications of MetS.

The most common genetic disorders of iron homeostasis hemochromatosis (HH) and iron-refractory-iron deficiency anemia (IRIDA) are caused by the two known loci HFE and TMPRSS6 and their genetic variants are known to affect serum iron concentration[8], transferrin saturation[9,10], haemoglobin (Hb) concentrations and erythrocyte traits[11-14] in normal populations. We performed genome-wide association studies on Val Borbera population with the aim to clarify whether the association of HFE and TMPRSS6 to hematological traits could be iron mediated or dependent on a direct effect of the variants on erythropoiesis.

Val Borbera cohort replicated the association of HFE and TMPRSS6 to iron and erythrocytes parameters. Considering the total effect of iron, transferrin saturation and ferritin on erythrocytes parameters, HFE association was abolished and that of TMPRSS6 greatly reduced whereas the association of HFE and TMPRSS6 to iron parameters did not change significantly when hepcidin is used as covariate. These results suggest that associations to erythroid traits are mostly dependent on the amount of iron available but the association to iron parameters is not only mediated by hepcidin.

The genetic characterization on hepcidin trait highlights also remarkable effects of environmental factors as acquired iron deficiency and inflammation: when multiple interactions between envinronmental factors, iron parameters and hepcidin were taken into account, the HFE and TMPRSS6 variants were associated with ferritin and with hepcidin normalised to ferritin levels.

The levels of iron, ferritin, transferrin, hemoglobin, hepcidin, hepcidin adjusted for transferrin saturation and ferritin were further measured in homozygotes for the Caucasian major allele 736^{A} and the minor allele 736^{V} of rs855791, the causative variants in TMPRSS6 catalytic domain, to assess their inhibitor effect on hepcidin *in vitro* and *in vivo*. The levels of hepcidin, normalized hepcidin on ferritin and on transferrin saturation are significantly higher in homozygotes 736^{A} in respect to 736^{V} in Val Borbera healthy individuals after exclusion of samples with acquired

confounding factors. No difference was found for ferritin, transferrin and Hb levels but only for transferrin and iron as expected. The data show that TMPRSS6 rs855791 has a functional role in determining the protease activity and regulating hepcidin expression both in vitro and in normal subjects, suggesting that it influences hepcidin response to the increase of both circulating and total body iron.

Other human common pathologies as chronic renal diseases present concomitant alteration of iron metabolism as anemia due to erythropoietin deficiency, blood losses and inflammation and as hyperferritinemia due to low transferrin saturation. Probably these alterations are due to TMPRSS6-dependent upregulation of hepcidin, whose elevated levels are ascertained in patients. To clarify whether TMPRSS6 variant rs855791 influences iron metabolism and anemia during chronic inflammation and renal failure a case-controls study in patients with chronic hemodialysis (CHD) has been done in Val Borbera. The results showed differences in hep-25 levels between cases and controls after acquired iron deficient individuals exclusion and lower levels in CHD patients stratified for carrying HFE mutations. The combined presence of HFE and A736V TMPRSS6 mutation shows that the A736V polymorphism influenced serum hepcidin in patients positive for HFE mutations. This suggests that the 736^{V} variant with defective proteolytic activity determining increased hepcidin transcription may abrogate the inhibitory effect of HFE mutations on hepcidin. In patients without acute inflammation and severe iron deficiency 736V TMPRSS6 variant is also associated with higher requirement of erythropoietin (Epo) for anemia. The evaluation of the impact of HFE and TMPRSS6 genotype on CHD patients in prospective studies may be useful to optimize anemia management and personalized therapies.

These findings clarified how HFE and TMPRSS6 modulate the hepcidin and iron levels but highlighted the need to uncover the missing fraction of genetic variability to reconstruct the biological processes of iron homeostasis and the mechanism of the onset of iron-related disorders.

No novel significant candidate genes were highlighted by genome-wide association analysis performed due to the low statistical power of Val Borbera sample to detect unknown genetic factors for hepcidin and iron parameters.

Thanks to the collaboration with other two Dutch groups (Nijmegen Biomedical Study or NBS and Prevention of REnal and Vascular ENd-stage Disease or PREVEND) a meta-analysis of GWAS for serum hepcidin levels has been performed in the three cohorts (the only available worldwide with both phenotype and genotypes) with a total sample size of 6,096 individuals. Of European origin Thanks to our previous findings, the study focused on hepcidin and hepcidin adjusted for ferritin and transferrin saturation levels. Data for replication were obtained from up to 3,826 additional independent samples from the Dutch cohorts. Combination of GWAS results from three cohorts revealed two loci that were associated with serum hepcidin at genome-wide significance ($p<5x10^{-8}$): rs118031191 on chromosome 10, nearest gene FOXI2 on the whole dataset and rs354202 on chromosome 2 in the EML6 gene, encoding echinoderm microtubule associated protein like 6 and

near SPTBN1 (alias ELF) a member of a family of beta-spectrin genes, which are involved in linking the plasma membrane to the actin cytoskeleton. The ELF protein was shown to be essential in TGF-β signaling by son of mothers against decapentaplegic (SMAD) proteins in mice[15]. Central in hepcidin regulation is the bone morphogenetic protein-SMAD pathway[16], and the ELF protein is a plausible candidate to influence hepcidin expression.

The associations of the causative variants rs1800562 in HFE and rs855791 in TMPRSS6 to the hepcidin/ferritin ratio as previously independently reported in Val Borbera[17] and in NBS[18] are high also in the current analysis. In hepcidin adjusted for transferrin saturation the association is less strong. This difference indicates that these SNPs have a larger influence on hepcidin response to body iron stores than on hepcidin response to circulating iron.

To focus on the missing genetic factors that affect the parameters commonly used to determine the clinical iron metabolism status (serum ferritin, transferrin, iron and transferrin saturation) the Australian Genetic Iron Status (GIS) consortium planned and performed the largest meta-analysis on 48,000 individuals of European ancestry with the aim to identify additional loci affecting markers of iron status in the general population.

The study showed more significant associations or pleiotropic effects of the previous population-based findings in several loci (HFE, TF, TFR2 and TMPRSS6) and five novel associated loci at significative levels.

Three of the loci mainly affected serum ferritin (ABO, SLC40A1, TEX14), three others mainly affected serum iron and transferrin saturation (HFE, TFR2, TMPRSS6) and five mainly affected serum transferrin (ARNTL, FADS2, NAT2, TF and TFRC).

The ABO blood group locus has shown significant associations for several phenotypes: on low-density lipoprotein cholesterol[19], coronary artery disease[20] and red blood cell count[21] but whether ABO variation primarily affects iron stores and therefore erythrocyte count, or vice versa, is unclear.

The second associated genomic region contains TEX14 and other genes. TEX14 codes for a testis-expressed protein, but there was no evidence for male–female heterogeneity in the effect on ferritin. The most significant SNPs are within the TEX14 gene but the suggestive-significance region extends across other genes in the same LD block. Expression data suggest that variation affects RAD51C and SEPT4 but the connection with iron status is unclear and MTMR4 that changes SMAD phosphorylation, with possible effects on the BMP-SMAD pathway affecting control of hepcidin. The gene-based analysis identified a further region on chromosome 15 centred on C15orf43 and SORD (sorbitol dehydrogenase) with no obvious connection with iron status.

Transferrin is affected by SNPs near ARNTL, NAT2 and FADS2. The role of these in iron homeostasis is uncertain. ARNTL is known to interact with CLOCK gene for the generation of circadian rhythm and iron, hepcidin and TfR1 gene expression showed circadian variations.

NAT2 encodes a N-acetyl transferase and its genomic region is associated to lipids affections and cardiovascular risk and FADS2 variations affect several phenotypes as lipids fatty acids, fasting glucose and liver enzyme.

In particular the top hit rs174548 on FADS2 for transferrin decrease its effect using HDL as covariate and all these genes affect plasma triglycerides highlighting a common effects on iron and lipids metabolisms and a possible involvement in cardiovascular diseases. The substantial overlap between iron loci and loci affecting erythrocyte and lipid phenotypes could integrate our understanding on iron homeostasis.

The large sample from GIS consortium and from PDGene database have been involved in an epidemiological study to dissect the knowledge about Parkinson diseases (PD) and its correlation with iron. As previously shown in literature the levels of iron increase in brain of Parkinson patients but today the studies on serum iron are inconclusive. This Mendelian randomization study provided unconfounded estimates of the effects of iron on PD using the known variants in HFE and TMPRSS6. A meta-analysis on 20,809 PD cases and 88,892 controls from GIS and candidate gene studies showed a protective effect of serum iron with 3% of reduction in PD risk of onset. The molecular mechanism is not completely clear: low iron levels may decrease the iron storage as ferritin in neurons as found in substantia nigra (SN) neurons in PD patients and this may provoke the decrease of iron available for neuronal enzymes and the accumulation of iron in SN. These findings assess that the disorders of iron metabolism could be important in the pathogenesis of Parkinson diseases.

All the results obtained on hepcidin and iron parameters contribute to the understanding of the molecular mechanism of iron homeostasis and iron associated diseases highlighting the strong correlation of hepcidin and ferritin and the link between iron homeostasis and lipid metabolism that could have implication on the onset of cardiovascular disorders.

These findings confirm that Val Borbera genetic isolate represents a suitable model for genetic study on common diseases and for replication of known associated loci but the reduced number of samples represents a limitation to highlight strong associations in novel loci for iron parameters and hepcidin.

Insight into low-frequency and rare variants and prospects for genetic studies.

The Val Borbera and other Italian cohorts participate in the Italian network of Genetic Isolates (INGI) including about 6,000 individuals. Among other projects, the INGI cohorts are involved in a international collaboration with Wellcome Trust Sanger Institute in UK, to identify and determine the role and the genetic impact of rare and low-frequency variants on common diseases.

The approach used is the innovative whole generation sequencing (WGS) at low coverage (4X-10X) to discover population-specific and Italian specific markers (SNPs and INDELs) as well as to identify rare variants that may be enriched in the isolated cohorts.

To investigate the distribution of rare and novel variants in INGI population a first group of 225 samples from Val Borbera and 250 from Friuli Venezia Giulia cohorts have been sequenced at the Wellcome Trust Sanger Institute and variants have been identified using an ad hoc pipeline. The quality controls of the sequences concerned the read depth of the calling *per situ* in the overall samples and the analysis of the overlapping dataset between WGS and GWAS genotypes: the read depth is congruent with the coverage and homogeneous along the genome and the sequences and genotypes showed an overall concordance of about 99.5%. After validation the final set of called variants show an enrichment in variants with MAF <=5% as expected in genetic isolates: 59% in Val Borbera and 52% in Friuli Venezia Giulia (Figure 10).

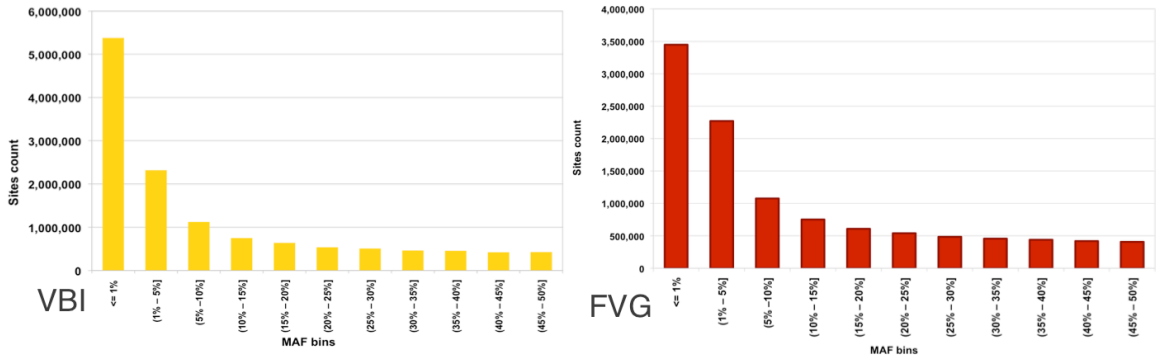
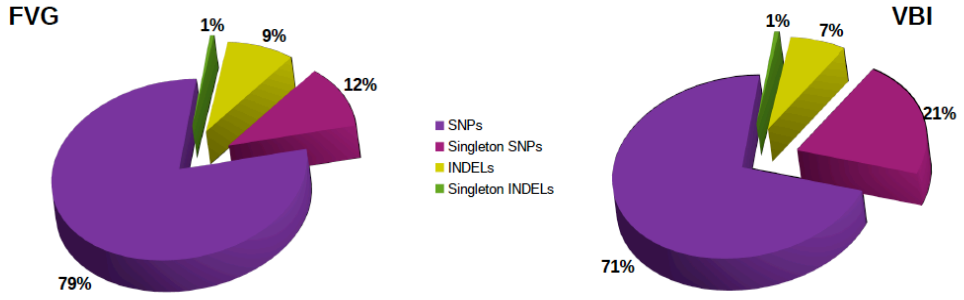


Figure 10. Minor allele frequency distribution in Val Borbera (VBI) and in Friuli Venezia Giulia (FVG) cohorts on a sample of about 500 whole genome sequences at low coverage. Thanks to Massimiliano Cocca et al [in preparation]

The distribution of variants in the two cohorts is shown in Figure 11. Val Borbera is enriched in singleton SNPs respect to FVG cohorts (21% vs 12%) and it shows a higher number of SNPs although VBI sample size is slightly smaller (225 vs 250 individuals).



	FVG			VBI		
	Autosomal	Autosomal+ChrX	Multiallelic	Autosomal	Autosomal+ChrX	Multiallelic
Total Variants	12,385,318	12,783,550	298,283	14,897,511	15,343,148	271,256
#SNPs	11,209,533	11,568,618	40,082	13,726,771	14,141,052	34,406
#INDELs	1,175,785	1,214,932	258,201	1,170,740	1,202,096	236,850

Figure 11. SNPs and INDELs distributions observed in Friuli Venezia Giulia (FVG) and in Val Borbera (VBI). Thanks to Massimiliano Cocca et al [in preparation]

The final set of FVG and VBI called variants has been compared to the complete set of called variants in all the population collected by 1000 Genome Project[22] (TGP).

In Figure 12 the distribution in ten different categories of frequency of the number of called variants shared between FVG and TGP and between VBI and TGP showed a large number of INGI variants shared with 1000Genome populations ranged between 43%-95% in FVG and 35%-95% in VBI. The large part of low-frequency variants (MAF <0.5%) called in FVG or VBI are unique and never called in 1000 Genomes populations. These data highlighted that INGI cohorts are enriched in novel population-specific rare variants.

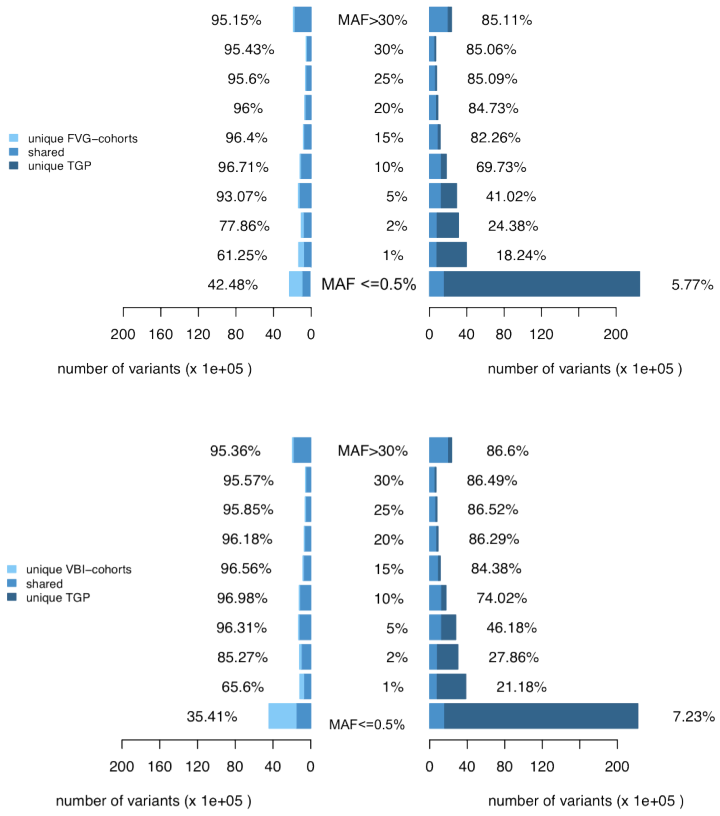


Figure 12. Number of unique variants in 10 different bin of frequency in Friuli Venezia Giulia (FVG) and in Val Borbera (VBI) cohorts and shared with the variants called in all the cohorts collected in 1000 Genome Project (TGP). Thanks to Massimiliano Cocca et al [in preparation]

These preliminary results are very promising and will allow the design of an Italian specific panel of variants based on about 1,000 INGI samples to be used as a reference enriched in lower frequency high quality variants in imputations of the entire Italian isolates cohorts and of other Italian general population such as the INCIPE dataset from University of Verona. Accordingly, we expect to definitely improve the genetic association studies on the large set of phenotypes collected as risk factors for common diseases, and in particular in Val Borbera to focus on the genetics of hepcidin and iron parameters to highlight significant novel loci involved in iron homeostasis pathway.

REFERENCES

1. Suzuki H, Toba K, Kato K, Ozawa T, Tomosugi N, Higuchi M, et al. 2009 Serum hepcidin-20 is elevated during the acute phase of myocardial infarction. Tohoku J Exp Med 2009;218:93-8.

2. Tomosugi N, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H, et al. 2006 Detection of serum hepcidin in renal failure and inflammation by using ProteinChip system. Blood 2006;108:1381-7.

3. Tessitore N, Girelli D, Campostrini N, Bedogna V, Solero PG, Castagna A, et al. 2010 Hepcidin is not useful as a biomarker for iron needs in haemodialysis patients on maintenance erythropoiesis-stimulating agents. Nephrol Dial Transplant 2010;25:3996-4002.

4. Campostrini N, Castagna A, Zaninotto F, Bedogna V, Tessitore N, Poli A, et al. 2010 Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. J Biomed Biotechnol 2010;2010:329646.

5. Peters HP, Laarakkers CM, Swinkels DW, Wetzels JF. 2010 Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010;25:848-53.

6. Dongiovanni P, Fracanzani AL, Fargion S, Valenti L 2011 Iron in fatty liver and in the metabolic syndrome: a promising therapeutic target. J Hepatol 55: 920–932

7. Riva A, Trombini P, Mariani R, Salvioni A, Coletti S, et al. 2008 Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome. World J Gastroenterol 14: 4745–4752

8. Tanaka T, Roy CN, Yao W, Matteini A, Semba RD, Arking D, Walston JD, Fried LP, Singleton A, Guralnik J, Abecasis GR, Bandinelli S, Longo DL, Ferrucci L. 2010 A genomewide association analysis of serum iron concentrations. Blood 2010;115:94e6.

9. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP ,Hottenga JJ, Henders AK, Campbell MJ, Wallace L, Frazer IH, Heath AC, de Geus EJ ,Nyholt DR, Visscher PM, Penninx BW, Boomsma DI, Martin NG, Montgomery GW ,Whitfield JB. 2009 Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009;41:1173e5.

10. Benyamin B, McRae AF, Zhu G, Gordon S, Henders AK, Palotie A, Peltonen L ,Martin NG, Montgomery GW, Whitfield JB, Visscher PM. 2009 Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60e5.

11. Chambers JC, Zhang W, Li Y, Sehmi J, Wass MN, Zabaneh D, Hoggart C, Bayele H, McCarthy MI, Peltonen L, Freimer NB, Srai SK, Maxwell PH, Sternberg MJ, Ruokonen A, Abecasis G, Jarvelin MR, Scott J, Elliott P, Kooner JS. 2009 Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nat Genet 2009;41:1170e2.

12. Ganesh SK, Zakai NA, van Rooij FJ, Soranzo N, Smith AV, Nalls MA, Chen MH ,Kottgen A, Glazer NL, Dehghan A, Kuhnel B, Aspelund T, Yang Q, Tanaka T, Jaffe A, Bis JC, Verwoert GC, Teumer A, Fox CS, Guralnik JM, Ehret GB, Rice K, Felix JF, Rendon A ,Eiriksdottir G, Levy D, Patel KV, Boerwinkle E, Rotter JI, Hofman A, Sambrook JG ,Hernandez DG, Zheng G, Bandinelli S, Singleton AB, Coresh J, Lumley T, Uitterlinden AG, Vangils JM, Launer LJ, Cupples LA, Oostra BA, Zwaginga JJ, Ouwehand WH, Thein SL, Meisinger C, Deloukas P, Nauck M, Spector TD, Gieger C, Gudnason V, van Duijn CM, Psaty BM, Ferrucci L, Chakravarti A, Greinacher A, O'Donnell CJ, Witteman JC, Furth S, Cushman M, Harris TB, Lin JP. 2009 Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. Nat Genet 2009;41:1191e8.

13. Kamatani Y, Matsuda K, Okada Y, Kubo M, Hosono N, Daigo Y, Nakamura Y ,Kamatani N. 2010 Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet 2010;42:210e15.

14. Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, Willenborg C ,Wright B, Chen L, Li M, Salo P, Voight BF, Burns P, Laskowski RA, Xue Y, Menzel S ,Altshuler D, Bradley JR, Bumpstead S, Burnett MS, Devaney J, Doring A, Elosua R ,Epstein SE, Erber W, Falchi M, Garner SF, Ghori MJ, Goodall AH, Gwilliam R ,Hakonarson HH, Hall AS, Hammond N, Hengstenberg C, Illig T, Konig IR, Knouff CW ,McPherson R, Melander O, Mooser V, Nauck M, Nieminen MS, O'Donnell CJ ,Peltonen L, Potter SC, Prokisch H, Rader DJ, Rice CM, Roberts R, Salomaa V ,Sambrook J, Schreiber S, Schunkert H, Schwartz SM, Serbanovic-Canic J, Sinisalo J ,Siscovick DS, Stark K, Surakka I, Stephens J, Thompson JR, Volker U, Volzke H ,Watkins NA, Wells GA, Wichmann HE, Van Heel DA, Tyler-Smith C, Thein SL ,Kathiresan S, Perola M, Reilly MP, Stewart AF, Erdmann J, Samani NJ, Meisinger C ,Greinacher A, Deloukas P, Ouwehand WH, Gieger C. 2009 A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 2009;41:1182e90.

15. Tang Y, Katuri V, Dillner A, Mishra B, Deng CX, et al. (2003) Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. Science 299: 574-577.

16. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, et al. (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38: 531-539.

17. Traglia M, Girelli D, Biino G, Campostrini N, Corbella M, et al. (2011) Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. J Med Genet 48: 629-634.

18. Galesloot TE, Geurts-Moespot AJ, den Heijer M, Sweep FC, Fleming RE, et al. (2013) Associations of common variants in HFE and TMPRSS6 with iron parameters are independent of serum hepcidin in a general population: a replication study. J Med Genet 50: 593-598.

19. Teslovich, T. M. et al. 2010 Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466, 707–713.

20. Schunkert, H. et al. 2011 Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. Nat. Genet. 43, 333–338.

21. van der Harst, P. et al. 2012 Seventy-five genetic loci influencing the human red blood cell. Nature 492, 369–375.

22. The 1000 Genomes Project. 2012 Consortium An integrated map of genetic variation.

Appendix

Supplemental data of Chapter 2

SUPPLEMENTAL TABLES AND FIGURES.

Supplementary Table S1. Main characteristics of subjects stratified according to hepcidin isoforms detectable.

	GROUP 1 Hep-20 undetectable Hep-25 undetectable N=142 (9%)	GROUP 2 Hep-20 detectable Hep-25 undetectable N=30 (1.9%)	GROUP 3 Hep-20 undetectable Hep-25 detectable N=581 (36.8%)	GROUP 4 Hep-20 detectable Hep-25 detectable N=824 (52.3%)	*P*
Male sex (%)	20.4	30	44.8	49.5	<0.001
Age (years)	50.2 ± 20.0	53.6 ± 19.8	53.7 ± 17.1	58.1 ± 17.5	<0.001
BMI	24.1 ± 4.1	25.9 ± 5.8	25.9 ± 4.2	26.3 ± 4.6	<0.001
s-iron (µg/dl)	83.23 ± 37.79	64.80 ± 27.54	99.75 ± 34.70	99.72 ± 32.03	<0.001
Transferrin (mg/dl)	270.21 ± 51.85	281.80 ± 57.89	242.23 ± 41.08	235.60 ± 35.99	<0.001
Transferrin Saturation %	23.20 ± 13.20	17.02 ± 7.91	29.83 ± 11.49	30.42 ± 10.96	<0.001
Ferritin* (ng/ml)	20 (17-24)	15 (11-20)	66 (61-72)	92 (87-98)	<0.001
Hb (g/dl)	13.5 ± 1.6	13.5 ± 1.6	14.5 ± 1.3	14.5 ± 1.4	<0.001
CRP* (mg/l)	0.14 (0.12-0.16)	0.17 (0.12-0.22)	0.17 (0.15-0.18)	0.17 (0.16-0.18)	0.297
Creatinine* (µmol/l)	0.81 (0.77-0.84)	0.82 (0.76-0.88)	0.84 (0.83-0.86)	0.87 (0.85-0.88)	0.001

* : variables not normally distributed are expressed as geometric means with 95% CIs

Supplementary Table S2. Main characteristic of subjects stratified according to hepcidin-20 detectable.

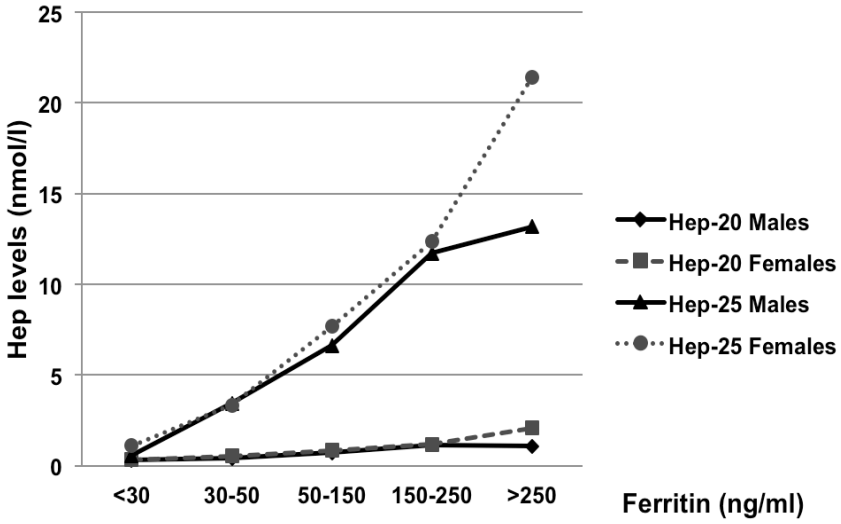
	Hepcidin-20 undetectable N=723	Hepcidin-20 detectable N=854	*P*
Male sex (%)	40	48.8	<0.001
Age (years)	53.0 ± 17.8	58.0± 17.6	<0.001
BMI	25.6 ± 4.2	26.3 ± 4.6	0.001
s-iron (µg/dl)	96.19 ± 35.91	98.49 ± 32.51	0.246
Transferrin (mg/dl)	247.75 ± 44.79	237.23 ± 37.90	<0.001
Transferrin Saturation %	28.52 ± 12.12	29.95 ±11.14	0.015
Ferritin* (ng/ml)	52 (48-57)	86 (81-92)	<0.001
Hb (g/dl)	14.4 ± 1.4	14.5 ± 1.4	0.039
CRP* (mg/l)	0.16(0.15-0.17)	0.17(0.16-0.18)	0.225
Creatinine* (µmol/l)	0.83(0.82-0.85)	0.87(0.85-0.88)	0.001
Hep-25* (nmol/l)**	3.11 (2.72-3.56)	7.36 (6.83-7.94)	<0.001
Hep-25* (nmol/l)***	7.05 (6.60-7.54)	8.48 (8.04-8.94)	<0.001

* : variables not normally distributed are expressed as geometric means with 95% CIs.

**: geometric mean of hep-20 and hep-25 with 95% CIs calculated on whole population (1,577 subjects).

***: geometric mean of hep-25 with 95% CIs calculated on 1,405 subjetcs (with hepc-25 detectable).

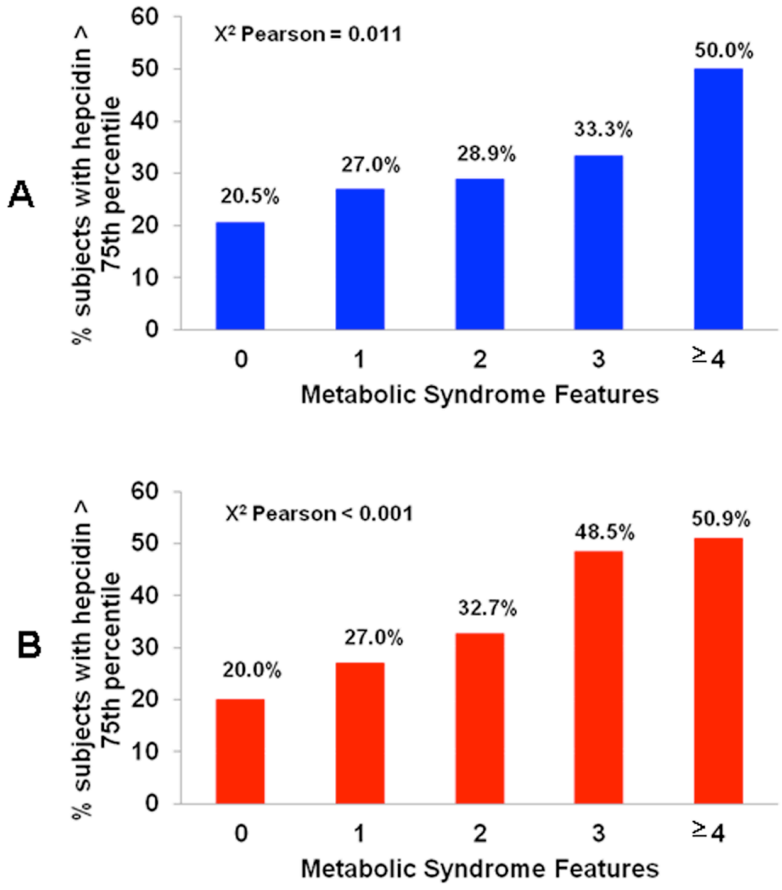
Supplementary Figure S1. Behaviour of hep-20 and hep-25 according to iron status.



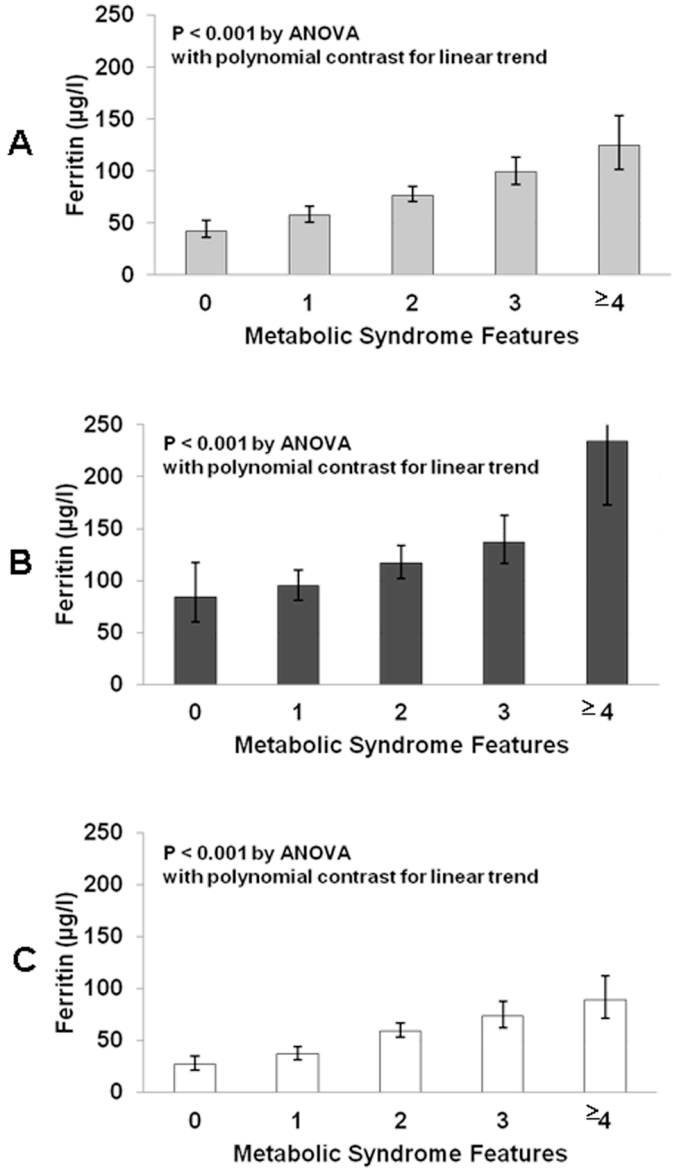
Supplemental data of Chapter 3

SUPPLEMENTAL TABLES AND FIGURES.

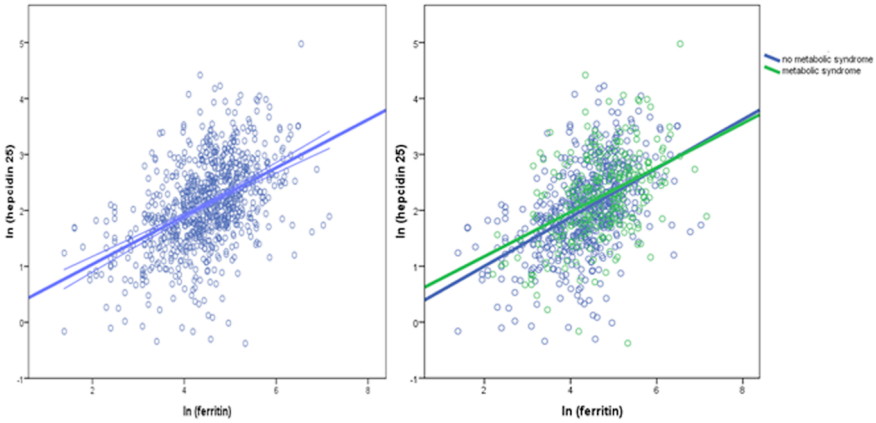
Supplementary Figure S1. Percentage of subjects with hepcidin levels in the top quartile. (A) Males and (B) Females.



Supplementary Figure S2. Serum ferritin levels in the Val Borbera population according to increasing number of MetS features. (A) whole population, (B) males and (C) females.



Supplementary Figure S3. Correlation between hepcidin-25 and ferritin.



Whole study population

Standardized beta-coefficient = 0.480, *P*<0.001

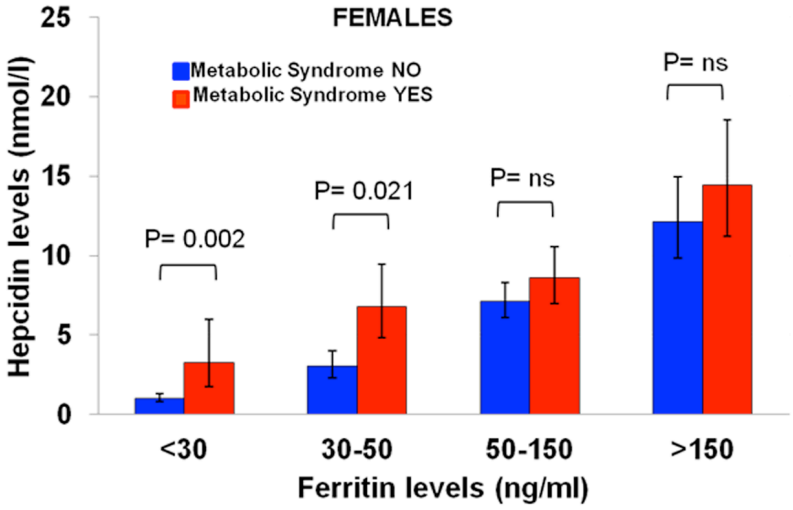
Population without metabolic syndrome

Standardized beta-coefficient = 0.483, *P*<0.001

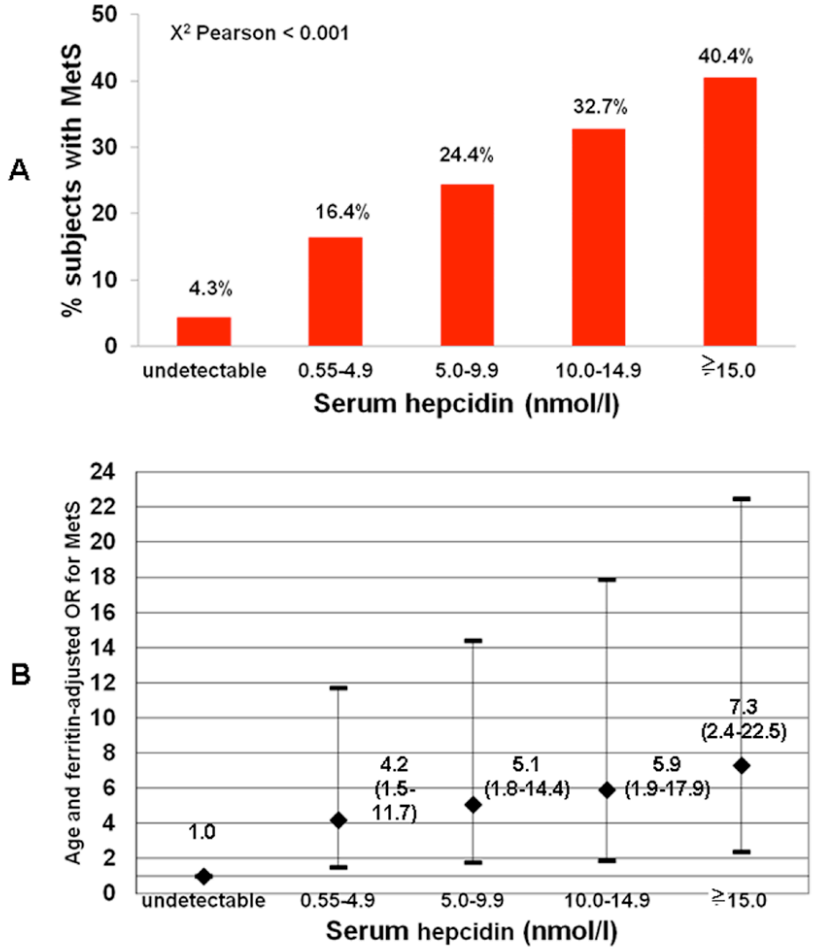
Population with metabolic syndrome

Standardized beta-coefficient = 0.434, *P*<0.001

Supplementary Figure S4. Hepcidin levels in females according to ferritin levels and presence/absence of MetS.



Supplementary Figure S5. Prevalence of MetS in females according to hepcidin levels (A), and the relative ORs for MetS, adjusted for age and ferritin (B).



Supplementary Table S1. Prevalence of MetS features in the VB population.

	All (n = 1,391)	Male (n = 616)	Female (n = 775)	*P*
Abdominal Obesity (%)	58.1	43.5	69.7	< 0.001
Abnormal plasma glucose or Diabetes (%)	16	19.4	13.3	0.002
High Triglycerides (%)	15.3	19	12.4	0.001
Low HDL Cholesterol (%)	14.8	12	17	0.009
Hypertension(%)	68.3	75.8	62.8	< 0.001
Metabolic Syndrome (%)	21.9	21.3	22.3	0.636

Supplementary Table S2. Associations with hepcidin at univariate analyses.

	Male		Female	
	β-coefficient	*P*	β-coefficient	*P*
Age (years)	-0.034	0.394	0.24	< 0.001
Metabolic Syndrome	0.049	0.226	0.245	< 0.001
S-Ferritin	0.559	< 0.001	0.585	< 0.001

Supplementary Table S3. Predictors of hepcidin in males and females, considering the individual MetS features as covariates.

	Male		Female	
	β-coefficient	*P*	β-coefficient	*P*
S-Ferritin	0.588	< 0.001	0.638	< 0.001
Abnormal Glycemia or Diabetes	0.01	0.817	0.08	0.023
Abdominal Obesity	-0.04	0.348	0.012	0.737
High Triglycerides	-0.049	0.256	-0.044	0.231
Low HDL-C	0.008	0.854	0.059	0.101
Hypertension	0.01	0.821	0.001	0.979
Age (years)	-0.007	0.875	-0.108	0.013

Supplemental data of Chapter 4

SUPPLEMENTAL TEXT, TABLES AND FIGURES.

Cell culture and plasmids

Cell-culture media and reagents were from Invitrogen (Carlsbad, CA) and from Sigma-Aldrich (St Louis, MO). HeLa and Hep3B cells were cultured respectively in Dulbecco modified Eagle medium (DMEM) and in Earl's minimal essential medium (EMEM) supplemented with 2 mM L-glutamine, 200 U/mL penicillin, 200 mg/mL streptomycin, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 95% humidifier air and 5% CO2.

The TMPRSS6 variant, encoding alanine at position 736 ($MT2^{736A}$) was obtained by mutagenesis of $MT2^{736V}$ encoding plasmid by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol.

Luciferase assay

Hep3B cells were transiently transfected with 0.25 μg of pGL2-basic reporter vector (Promega, Madison, WI, USA) containing the 2.9 Kb fragment of the human hepcidin promoter [23] in combination with pRL-TK Renilla luciferase vector (Promega) and 0.01 or 0.002 μg of cDNA encoding $MT2^{736V}$ or $MT2^{736A}$ expressing vectors and with 0.05 μg of HJV expressing vector, as described [6,23]. The luciferase activity was determined according to the instructions of the manufacturer of the assay kit (Promega Dual Luciferase Reporter Assay). Relative luciferase activity was calculated as the ratio of firefly (reporter) to renilla luciferase (transfection control) activity and expressed as a multiple of the activity of cells transfected with the reporter alone. Experiments were performed in triplicate.

Cell-surface protein quantification by binding assay

Quantification of cell-surface expression of MT2 was performed as described, with minor modifications [6]. In brief, 10^4 HeLa cells were seeded in 48-well plates and transfected with 0.4 mg of plasmid DNA complexed with 1 ml of Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 12 hours, the medium was replaced and, 24 hours later, cells were fixed with 4% paraformaldehyde for 45 minutes at room temperature. Cells were washed with PBS, blocked with 5% nonfat milk in PBS, and incubated with rabbit anti-FLAG antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) and then with the relative secondary HRP antibody at 37 °C. For total MT2 expression, cells were permeabilized with 0.1% Triton X-100 in PBS, prior to blocking and incubation with anti-FLAG. Peroxidase activity was measured with an HSR substrate (o-phenylenediamine dihydrochloride). Surface MT2 was calculated for each sample as the absorbance ratio of unpermeabilized and permeabilized cells after substraction of background absorbance.

Western blot

HeLa cells, seeded in 100-mm-diameter dishes up to 70–80% confluency, were transiently transfected with 10 μg of HJV expressing vector in the presence of 1 μg of $MT2^{736V}$ or $MT2^{736A}$ expressing vectors using the liposomal transfection reagent Lipofectamine 2000 (Invitrogen) in 3 ml of OptiMem (Invitrogen) according to the manufacturer's instructions. After 18 hours the medium was replaced with 4 ml of OptiMem and 24 hours later media were collected and concentrated using 5 kDa molecular weight cutoff ultrafiltration (Amicon Ultra; Millipore, Billerica, MA). Cells were lysed in lysis buffer (200 mM Tris-HCl [pH 8]; 1 mM EDTA; 100 mM NaCl; 10% Glycerol; 0,5% NP-40). Proteins were quantified by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Equal amount of total proteins (50 μg) were subjected to 10% SDS-PAGE and then transferred to Hybond C membrane (Amersham Biosciences Europe GmbH, Freiburg, Germany) by standard western blotting technique. Blots were blocked with 2% ECL Advance Blocking Agent (Amersham Biosciences) in TBS (0.5 M Tris-Hcl [pH 7.4] and 0.15 M NaCl) containing 0.1% Tween-20 (TBST), incubated 2 hours with rabbit anti-HJV (1:1000) or rabbit anti-FLAG (1:1000). After washing with TBST, blots were incubated 1 hour with relevant HRP-conjugated secondary antisera and developed using a chemiluminescence detection kit (ECL, Amersham Biosciences).

Pi-PLC cleavage of membrane HJV

A total of 106 HeLa cells, transiently transfected with HJV- and matriptase-2- expressing constructs or the empty vector were incubated in DMEM plus

0.3 U/ml phosphatidylinositol-specific phospholipase C (Pi-PLC) at 37 °C in

a 5% CO2 incubator. After 2 hrs, the supernatants were collected. Proteins were precipitated with cold acetone and resuspended in Laemmli sample buffer. Samples were then boiled for 10 min and loaded on a 10% SDS-PAGE.

Supplementary Table S1. Serum hepcidin and iron parameters levels according to AA, AV, VV in VB whole cohort and subset. All mean values are corrected for sex, age, squared age and their interaction by ANOVA (95%CI).

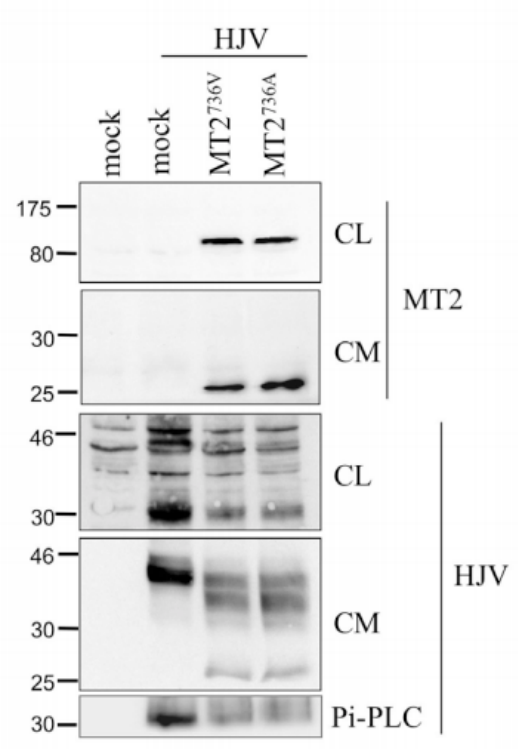
	Whole Cohort							Subset 1						
Trait	AA(N=213)		AV(N=309)		VV (N=133)		p°	AA(N=180)		AV(N=256)		VV (N=109)		p°
	Mean	95% CI	Mean	95% CI	Mean	95% CI		Mean	95% CI	Mean	95% CI	Mean	95% CI	
Hepcidin	9,93	8.72-11.15	10,54	9.53-11.55	11,31	9.78-12.85	NS	10,51	9.12-11.89	11,13	9.97-12.30	12,69	10.91-14.46	*
Hep/Ft	136,22	117.34-155.10	143,28	127.58-158.97	152,36	128.48-176.24	NS	104,77	90.54-118.99	113,26	101.36-125.36	129,25	111.02-147.48	*
Hep/TS	0,35	0.23-0.41	0,38	0.34-0.43	0,42	0.34-0.49	NS	0,34	0.28-0.41	0,39	0.33-0.44	0,45	0.36-0.53	*
Iron	106,63	102.35-110.91	100,85	97.30-104.41	92,11	86.70-97.52	****	110,44	105.85-115.02	104,2	100.28-108.02	94,27	88.39-100.15	****
$Log_{10(Ft)}$	1,92	1.87-1.97	1,89	1.85-1.93	1,87		1.81-1N	2,03	1.99-2.07	1,99	1.96-2.03	1,99	1.94-2.03	NS
TS	32,53	31.06-33.99	30,53	29.31-31.76	27,76	25.89-29.62	****	34,49	32.89-36.07	32,01	30.67-33.35	29,11	27.07-31.14	****

° Pairwise comparison between AA and VV homozygotes
* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0005
Abbreviations: CI: Confidence Interval; NS: not significant; Hep: Hepcidin; Ft: Ferritin; TS: Transferrin Saturation

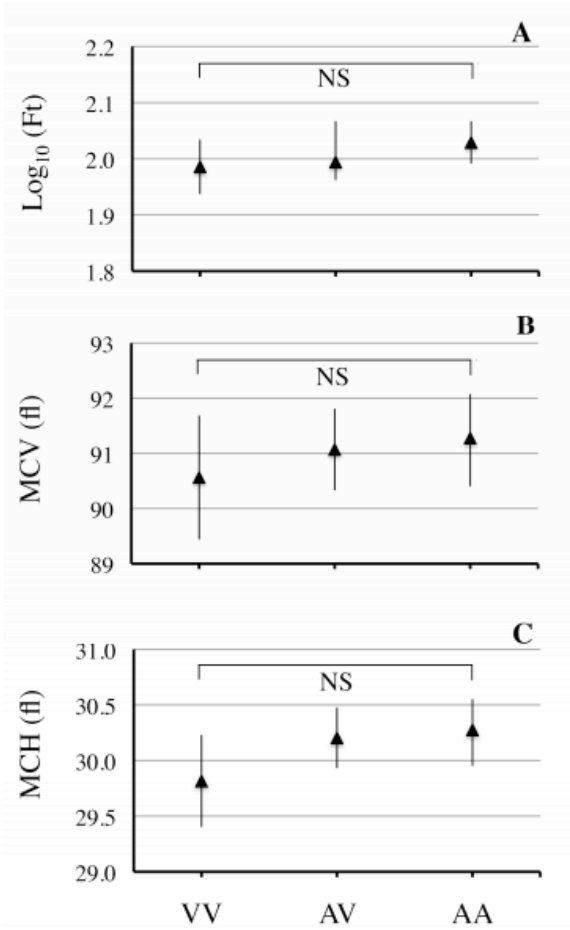
Supplementary Table S2. Frequency of TMPRSS6 rs855791 alleles in different populations

Population	Individual Group	Sample Count	G Frequency (Alanine)	A Frequency (Valine)	Ref
Lolipop and NFBCI966	European	6316	0.57	0.43	14
AFD EUR Panel	European	46	0.587	0.413	NCBI dbSNP
HapMap-CEU	European	120	0.625	0.375	NCBI dbSNP
HapMap-TSI	European	176	0.528	0.472	NCBI dbSNP
ValBorbera Project	European	1545	0.555	0.445	11
Australian families	Australian	2516	0.58	0.42	9
BioBank Japan Project	Japanese	14700	0.45	0.55	22
HapMap-JPT	Japanese	90	0.411	0.589	NCBI dbSNP
Lolipop and NFBCI966	Indian Asian	9685	0.47	0.53	14
AFD CHN Panel	Asian	48	0.396	0.604	NCBI dbSNP
HapMap-CHB	Asian	82	0.463	0.537	NCBI dbSNP
HapMap-LWK	African	180	0.928	0.072	NCBI dbSNP
HapMap-MKK	African	286	0.920	0.080	NCBI dbSNP
HapMap-ASW	African American	98	0.816	0.184	NCBI dbSNP
AFD AFR Panel	African American	44	0.841	0.159	NCBI dbSNP
HapMap-YRI	Sub-Saharan African	120	0.833	0.117	NCBI dbSNP

Supplementary Figure S1. In vitro characterization of the processing and of the cleavage activity of matriptase-2 variants. HeLa cells were transiently transfected with the *TMPRSS6* cDNA encoding $MT2^{736V}$ or $MT2^{736A}$, the empty vector (mock) and HJV. Cellular MT2 and serine protease domain of $MT2^{736V}$ and $MT2^{736A}$ released in the culture supernatant (upper panel) and cellular and membrane HJV and HJV fragments released in the cell culture media by the two MT2 variants (lower panel) were analyzed using western blot. CL= Cell lysate, CM= supernatant, Pi-PLC= Pi- PLC supernatant. Scales refer to relative molecular weight in kilodaltons.



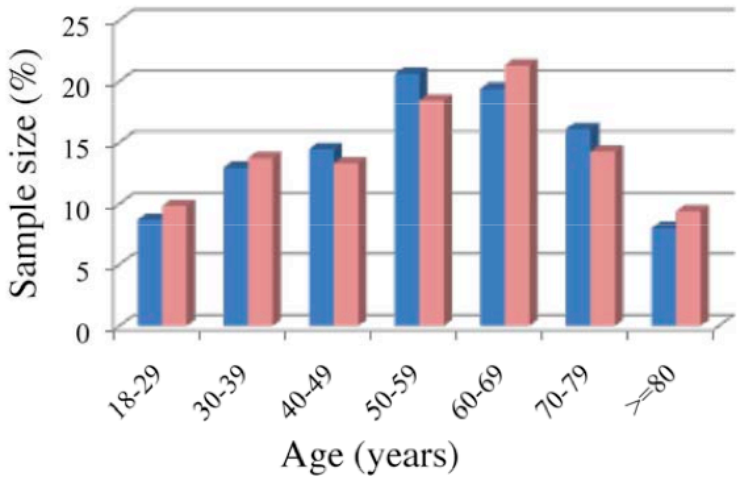
Supplementary Figure S2. Log10(ferritin) (A), MCV (B) and MCH (C) mean levels in AA, AV and VV groups of individuals from subset 1. All mean values are corrected for sex, age, squared age and their interaction by ANOVA (95%CI). P-values are refer to the comparison between AA and VV homozygotes. NS: not significant. CI = Confidence Intervals.



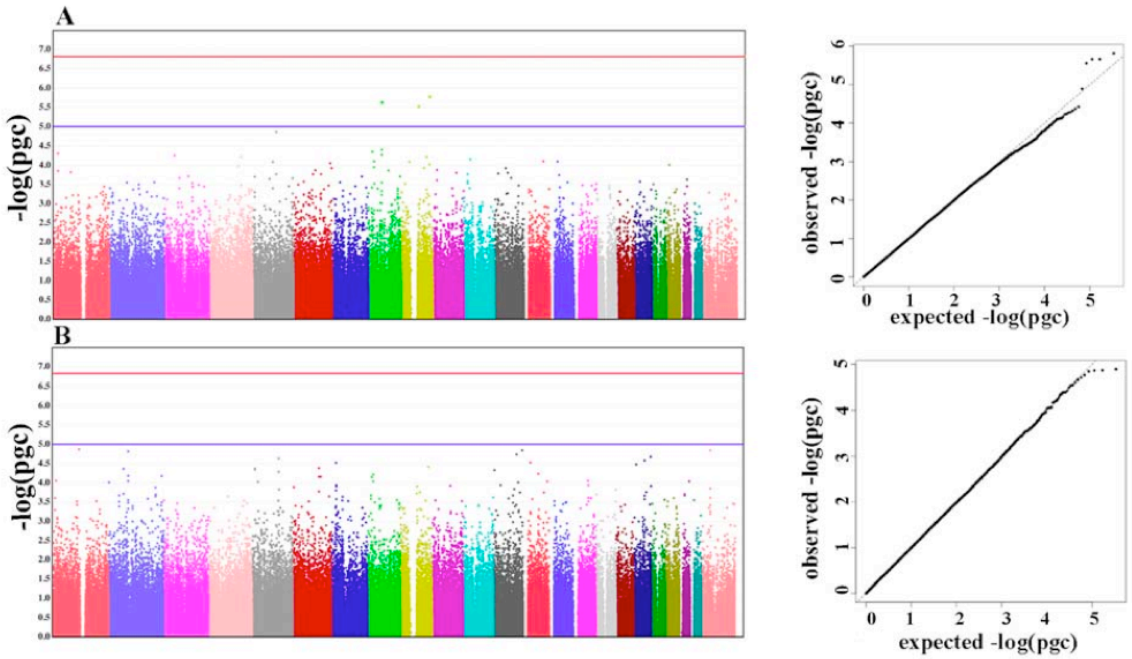
Supplemental data of Chapter 6

SUPPLEMENTAL TABLES AND FIGURES.

Supplementary Figure S1. Age and sex distribution of the VB individuals included in the whole series. In blue are males, in pink are females.



Supplementary Figure S2. A. Manhattan and Q-Q plots of hepcidin/ferritin ratio GWAS in the whole cohort. B. Manhattan and Q-Q plots of hepcidin/ferritin ratio GWAS in subset 1. Red continuous line indicates Bonferroni threshold (p=1.5E-7), blue continuous line indicates suggestive threshold (p=1E-5). Pvalues are corrected by genomic control and indicated as 'pgc'.



Supplementary Table S1. Characteristics of the population by sex.

Trait	Units	Males			Females			p°
		N	Mean	sd	N	Mean	sd	
Weight	Kg	727	78.5	12.4	927	64	12.1	****
Waist	cm	712	93	10.3	912	88	12.2	****
BMI	kg/m2	727	26.5	3.8	926	25.5	4.8	****
Hb	g/dl	728	15.4	1.2	927	13.7	1.1	****
Hct	%	728	46	3.2	927	41.9	3.0	****
MCV	fl	728	91.2	4.8	927	90.6	5.1	**
MCH	pg	728	30.5	1.8	927	29.5	2.0	****
MCHC	%	728	33.5	1.1	927	32.6	1.2	****
Iron	ug/dl	727	105.9	36.1	925	91	30.8	****
Transferrin	mg/dl	727	235.8	36.7	925	248.2	44.9	****
Ferritin	ng/ml	726	160.7	134	923	65.1	62.7	****
Transferrin saturation	%	727	32.3	12.1	925	26.8	10.5	****
Hepcidin	nM	728	11.3	9.5	929	8.5	10.2	****
Hepcidin/ferritin	pmol/ug	726	88.9	79.8	923	179.9	284.8	****
Hepcidin/transferrin saturation	%	727	0.39	0.5	925	0.34	0.5	*
Total cholesterol	mg/dl	727	200.1	40.9	925	209	40.5	*
HDL cholesterol	mg/dl	727	53.4	12.6	925	63.2	14.3	****
LDL cholesterol	mg/dl	727	122.9	34.2	925	126.9	35.7	*
Triglycerides	mg/dl	727	118.9	88.1	925	94.7	50.8	****
CRP	mg/dl	492	0.26	0.42	638	0.27	0.4	NS

° t-test for evaluating mean value differences between males and females.

* p <0.05, ** p <0.01,*** p <0.001. **** p <0.0005.

BMI: Body Mass Index; CRP: C-Reactive Protein; N: Sample size; sd: standard deviation; NS: not significant.

Supplementary Table S2. Serum hepcidin levels by age and sex*

Age class	N	Males	N	Females
18-29	63	11.1 (1.3)	91	4.4 (0.4)
30-39	94	11.8 (0.9)	127	4.7 (0.4)
40-49	105	11.4 (0.8)	123	4.7 (0.4)
50-59	150	10.8 (0.7)	171	11.1 (0.9)
60-69	141	11.8 (0.9)	198	11.6 (0.7)
70-79	117	11 (0.9)	132	10.2 (0.9)
>=80	58	10.6 (1.3)	87	8.6 (1.8)

*Values are presented as means, nM; Standard errors are in brackets.

Supplementary Table S3. Sex-specific correlation analysis of serum hepcidin

Trait	Males	Females
Weight	-0.0238	0.1064*
Waist	0.0156	0.1736*
BMI	0.0065	0.1558*
Hb	-0.0792*	0.1050*
Hct	-0.0741*	0.1278*
MCV	0.0338	0.0856*
MCH	0.0143	0.0554
MCHC	-0.0226	-0.0227
Iron	0.0307	0.1090*
Transferrin	-0.1781*	-0.1784*
Ferritin	0.3194*	0.5263*
Transferrin saturation	0.0742*	0.1472*
Triglycerides	0.0353	0.1588*
CRP	0.0851*	0.2512*

* Pearson's r significantly different from zero at the $p<0.05$ level

BMI: Body Mass Index; CRP: C-Reactive Protoein.

Supplementary Table S4. Simple regression analysis of serum hepcidin by sex.

Trait°	Males		Females	
	Beta	p	Beta	p
Weight	-	NS	0.008	***
Waist	-	NS	0.008	**
BMI	-	NS	0.020	***
Hb	-0.065	**	-	NS
Hct	-	NS	0.024	*
MCV	-	NS	-	NS
MCH	-	NS	-	NS
MCHC	-0.070	**	-	NS
Iron	0.002	**	0.003	**
Transferrin	-	NS	-	NS
Ferritin	0.002	****	0.004	****
Transferrin saturation	0.008	***	-	NS
Total cholesterol	-	NS	0.003	****
HDL cholesterol	-	NS	-	NS
LDL cholesterol	-	NS	0.003	****
Triglycerides	-	NS	0.002	****
CRP	0.366	*	-	NS

° All the models were corrected for age.
* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0005
NS: not significant.
BMI: Body Mass Index; CRP: C-Reactive Protoein.

Supplementary Table S5. Replication of association of rs1800562 and rs855791 to iron parameters.

Trait°	Whole cohort					Subset 1				
	N	Beta	SE	p	R^{2*} (%)	N	Beta	SE	p	R^{2*} (%)
rs1800562 *HFE*^										
Iron	1656	0.442	0.075	3.95E-09	2.2	1128	0.463	0.090	2.46E-07	2.8
Tf	1652	-0.514	0.077	4.95E-11	3.1	1130	-0.521	0.090	8.53E-09	3.1
Tf Sat	1652	0.608	0.077	2.64E-15	4.1	1126	0.636	0.091	2.34E-12	4.8
Hb	1657	0.154	0.076	4.28E-02	0.3	1131	0.111	0.091	2.19E-01	-
Hct	1659	0.042	0.077	5.88E-01	-	1132	-0.016	0.090	8.58E-01	-
MCH	1648	0.334	0.078	1.70E-05	0.9	1125	0.365	0.091	6.44E-05	1.2
MCV	1648	0.191	0.078	1.45E-02	0.3	1126	0.178	0.091	5.11E-02	0.3
MCHC	1659	0.249	0.077	1.13E-03	0.5	1130	0.241	0.090	7.33E-03	0.4
rs855791 *TMPRSS6*$^{\$}$										
Iron	1656	-0.236	0.036	9.41E-11	2.7	1128	-0.271	0.044	6.07E-10	3.5
Tf	1652	-0.001	0.037	9.70E-01	-	1130	0.002	0.044	9.72E-01	-
Tf Sat	1652	-0.215	0.037	3.89E-09	2.1	1126	-0.247	0.044	1.52E-08	2.8
Hb	1657	-0.064	0.037	8.05E-02	-	1131	-0.095	0.044	3.04E-02	0.3
Hct	1659	-0.033	0.037	3.68E-01	-	1132	-0.059	0.044	1.82E-01	-
MCH	1648	-0.187	0.037	4.54E-07	1.5	1125	-0.212	0.044	1.31E-06	1.9
MCV	1648	-0.146	0.037	8.46E-05	0.9	1126	-0.172	0.044	9.03E-05	1.1
MCHC	1659	-0.096	0.037	8.93E-03	0.3	1130	-0.082	0.043	5.71E-02	0.3
rs3811647 *TF*$^{\#}$										
Iron	1656	0.102	0.041	1.21E-02	0.3	1128	0.173	0.049	4.47E-04	1.1
Tf	1652	0.338	0.041	2.10E-16	4.8	1130	0.419	0.049	1.92E-17	7.0

° All the traits were corrected for covariates: sex, age, squared age, interaction between sex and age and interaction between sex and squared age

N: Sample size; SE: Standard Error; Tf: Transferrin; Tf Sat: Transferrin Saturation.

* R^2: Adjusted R square of the linear regression corresponding to the variance of the trait explained by each locus.

^Minor allele is A for rs1800562 *HFE* encoding C282Y (Frequency = 0 .065 in the whole cohort; Frequency = 0.068 in subset 1).

$Minor allele is A for rs855791 *TMPRSS6* encoding A736V. (Frequency = 0.45 in both datasets).

#Minor allele is A for rs3811647 *TF* (Frequency = 0.28 in both datasets).

Supplementary Table S6. Effect of iron parameters in association analysis of rs1800562 and rs855791 to red blood cells traits.

Trait	covariate°	Subset 1					Subset 1				
		N	Beta	SE	p	R^{2*} (%)	N	Beta	SE	p	R^{2*} (%)
		rs1800562 *HFE*^					rs855791 *TMPRSS6*$				
MCH	basic model	1125	0.365	0.091	6.44E-05	1.2	1125	-0.212	0.044	1.31E-06	1.9
MCV	basic model	1126	0.178	0.091	5.11E-02	0.3	1126	-0.172	0.044	9.03E-05	1.1
MCHC	basic model	1130	0.241	0.090	7.33E-03	0.4	1130	-0.082	0.043	5.71E-02	0.3
MCH	Tf Sat	1120	0.158	0.091	8.30E-02	-	1120	-0.159	0.044	2.93E-04	1.0
MCV	Tf Sat	1122	0.065	0.091	4.78E-01	-	1122	-0.136	0.044	2.07E-03	0.6
MCHC	Tf Sat	1126	0.115	0.090	1.99E-01	-	1126	-0.039	0.043	3.74E-01	-
MCH	All°°	1120	0.129	0.091	1.59E-01	-	1120	-0.144	0.044	1.06E-03	0.7
MCV	All°°	1122	0.041	0.091	6.56E-01	-	1122	-0.130	0.044	3.31E-03	0.5
MCHC	All°°	1126	0.103	0.090	2.52E-01	-	1126	-0.024	0.043	5.88E-01	-

° All the traits were corrected for covariates: sex, age, squared age, interaction between sex and age, interaction between sex and squared age in addition to the iron parameters indicated.

N: Sample size; SE: Standard Error; Tf Sat: Transferrin Saturation.

* R^2: Adjusted R square of the linear regression corresponding to the variance of the trait explained by each locus.

°° All: Transferrin Saturation + Ferritin + Iron.

^Minor allele is A for rs1800562 *HFE* encoding C282Y (Frequency = 0 .068 in subset 1).

$Minor allele is A for rs855791 *TMPRSS6* encoding A736V. (Frequency = 0.45 in subset 1).

Supplementary Table S7. Association of rs1800562, rs855791and rs3811647 to iron parameters adjusted for serum hepcidin.

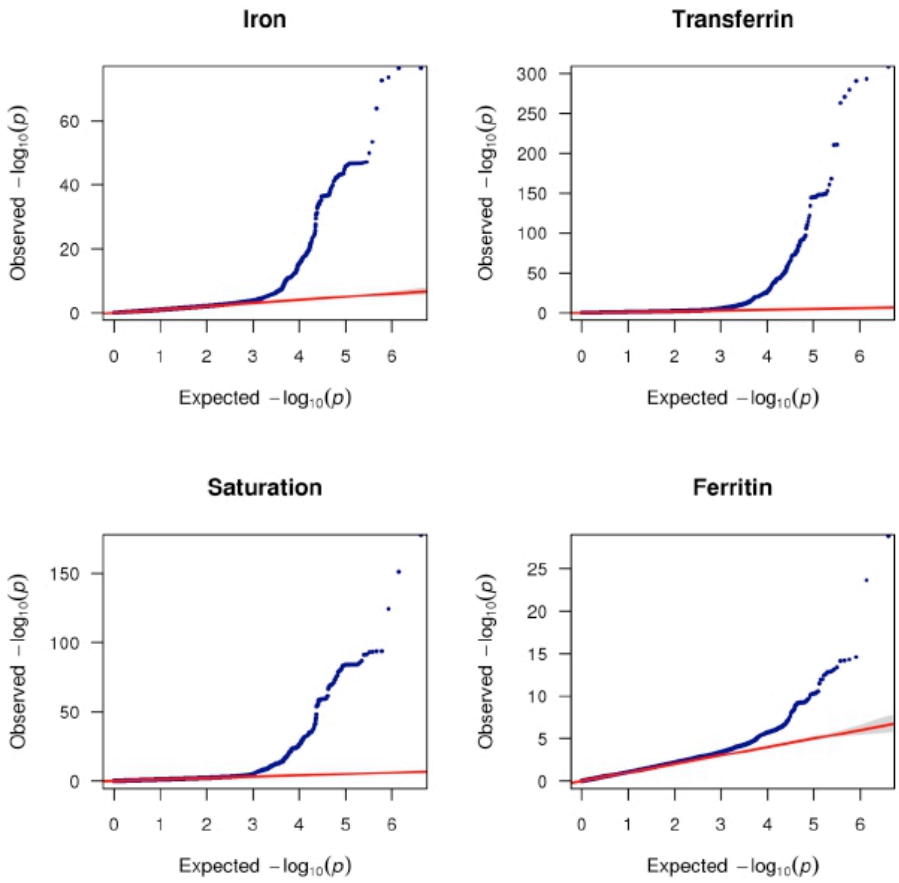
Trait°	Whole cohort					Subset 1				
	N	Beta	SE	p	R^{2*} (%)	N	Beta	SE	p	R^{2*} (%)
rs1800562 *HFE*^										
Iron	1538	0.415	0.078	1.06E-07	2.0	1128	0.457	0.090	3.71E-07	2.7
Tf	1535	-0.482	0.079	1.20E-09	3.0	1130	-0.518	0.090	1.02E-08	2.9
Tf Sat	1534	0.567	0.080	1.24E-12	3.7	1126	0.629	0.091	4.08E-12	4.7
Hb	1538	0.105	0.079	1.85E-01	-	1131	0.114	0.091	2.10E-01	-
Hct	1540	0.001	0.080	9.89E-01	-	1132	-0.016	0.090	8.63E-01	-
MCH	1529	0.308	0.080	1.21E-04	0.8	1125	0.367	0.091	5.84E-05	1.3
MCV	1531	0.179	0.080	2.63E-02	0.2	1126	0.178	0.091	5.05E-02	-
MCHC	1540	0.214	0.079	6.97E-03	0.3	1130	0.245	0.090	6.44E-03	0.4
rs855791 *TMPRSS6*$^{\$}$										
Iron	1538	-0.239	0.038	2.69E-10	2.7	1128	-0.277	0.044	2.52E-10	3.7
Tf	1535	0.000	0.038	9.92E-01	-	1130	0.004	0.044	9.35E-01	-
Tf Sat	1534	-0.222	0.038	4.52E-09	2.2	1126	-0.254	0.044	6.54E-09	3.0
Hb	1538	-0.047	0.038	2.16E-01	-	1131	-0.094	0.044	3.29E-02	0.3
Hct	1540	-0.020	0.038	6.05E-01	-	1132	-0.058	0.044	1.84E-01	-
MCH	1529	-0.185	0.038	1.35E-06	1.5	1125	-0.211	0.044	1.55E-06	1.8
MCV	1531	-0.153	0.038	7.08E-05	0.9	1126	-0.172	0.044	9.33E-05	1.1
MCHC	1540	-0.089	0.038	1.89E-02	0.3	1130	-0.080	0.043	6.44E-02	-
rs3811647 *TF*$^{\#}$										
Iron	1538	0.118	0.042	5.29E-03	0.4	1128	0.180	0.049	2.62E-04	1.2
Tf	1535	0.344	0.042	4.76E-16	5.1	1130	0.416	0.049	2.95E-17	7.0

° All the traits were corrected for covariates: sex, age, squared age and hepcidin
N: Sample size; SE: Standard Error; Tf: Transferrin; Tf Sat: Transferrin Saturation.
* R^2: Adjusted R square of the linear regression corresponding to the variance of the trait explained by each locus.
^Minor allele is A for rs1800562 *HFE* encoding C282Y (Frequency = 0 .066 in the whole cohort; Frequency = 0.068 in subset 1)
$^{\$}$Minor allele is A for rs855791 *TMPRSS6* encoding A736V. (Frequency = 0.45 in both datasets).
$^{\#}$Minor allele is A for rs3811647 *TF* (Frequency = 0.28 in both datasets).

Supplemental data of Chapter 8

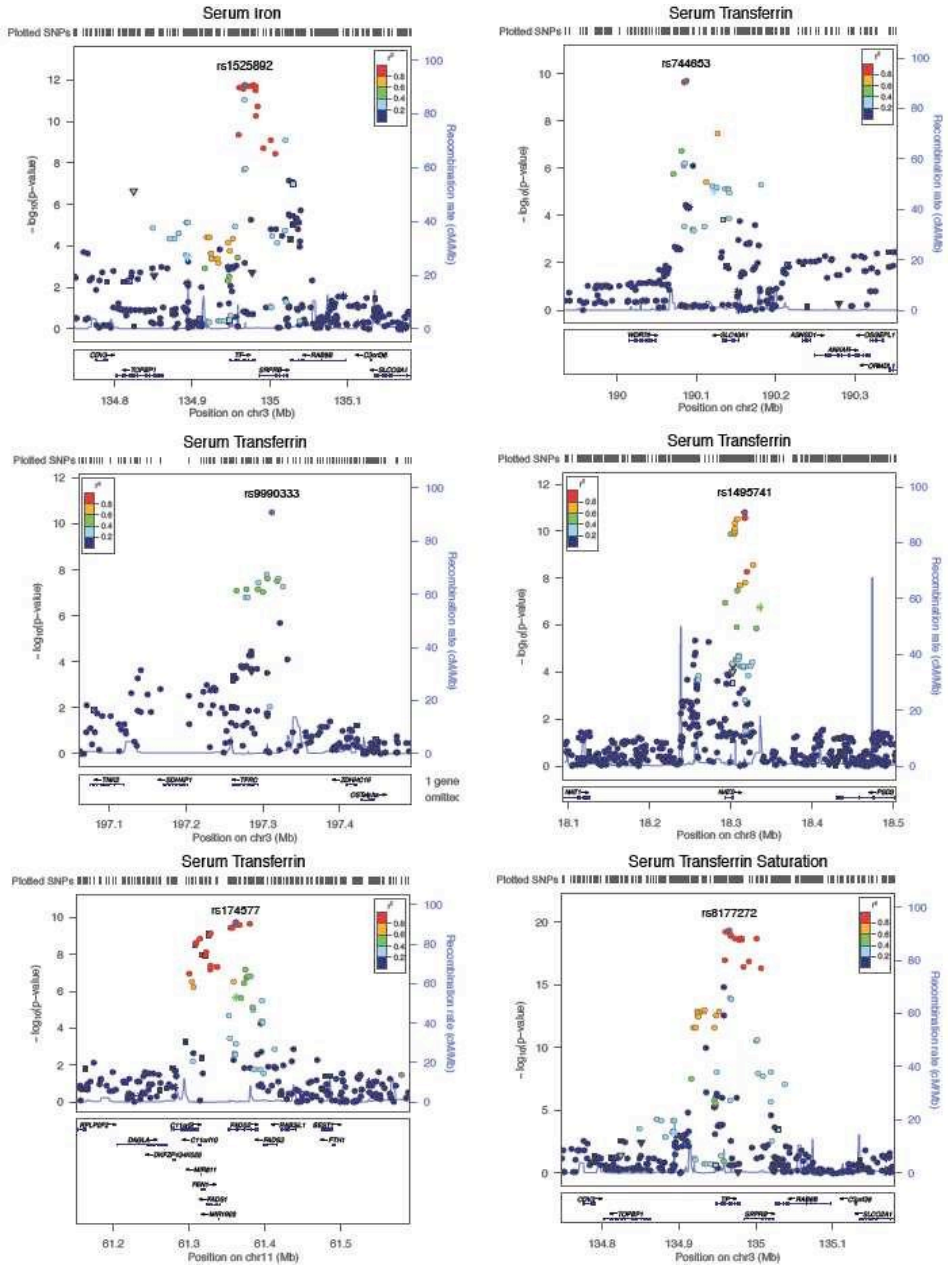
SUPPLEMENTAL TEXT, TABLES AND FIGURES.

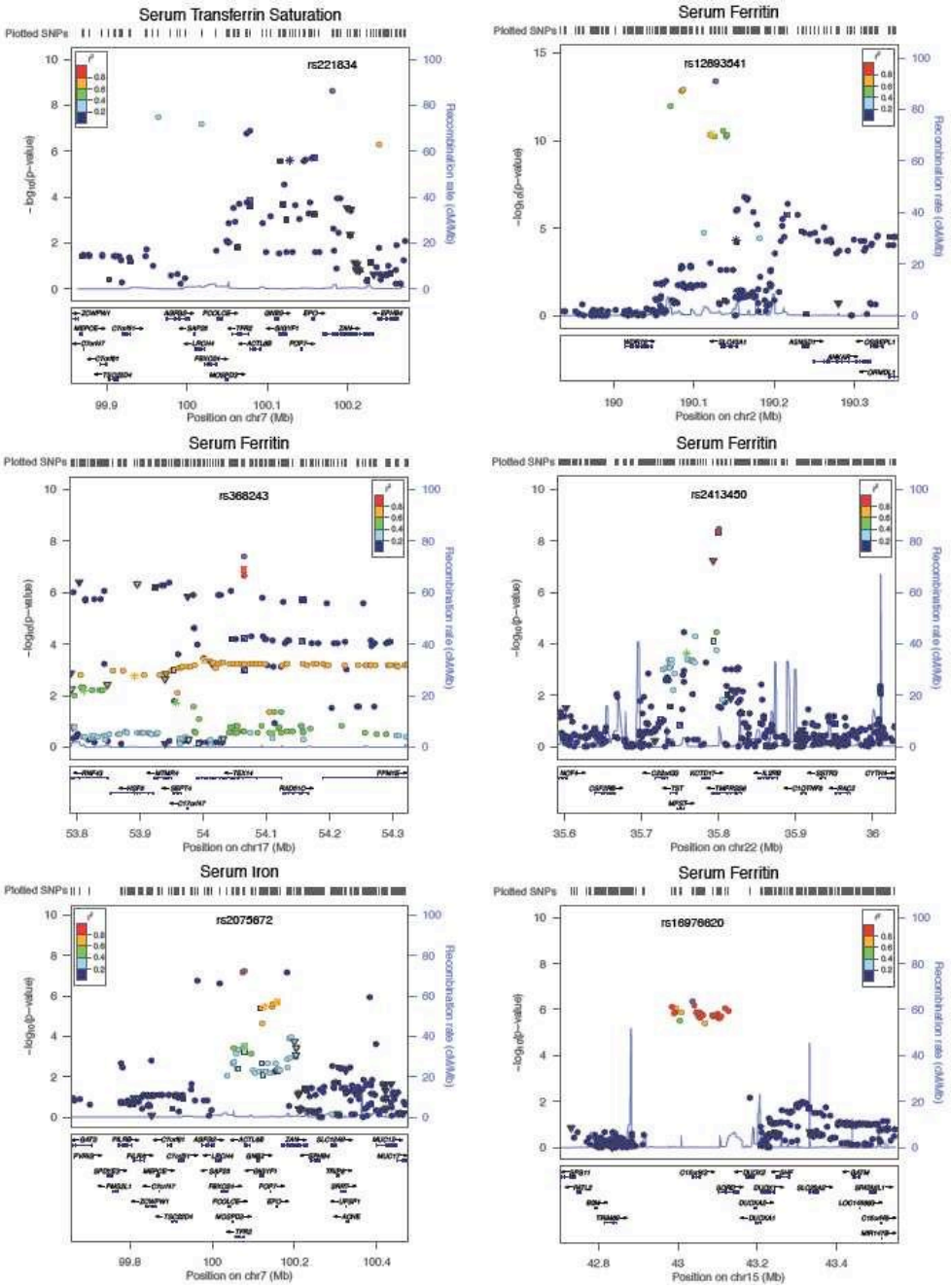
Supplementary Figure S1. Q-Q plots for iron, transferrin, saturation and ferritin in the Discovery meta-analysis. The genomic inflation factors (λ) are 1.035, 1.092, 1.051 and 1.067 for serum iron, transferrin, transferrin saturation and ferritin, respectively.



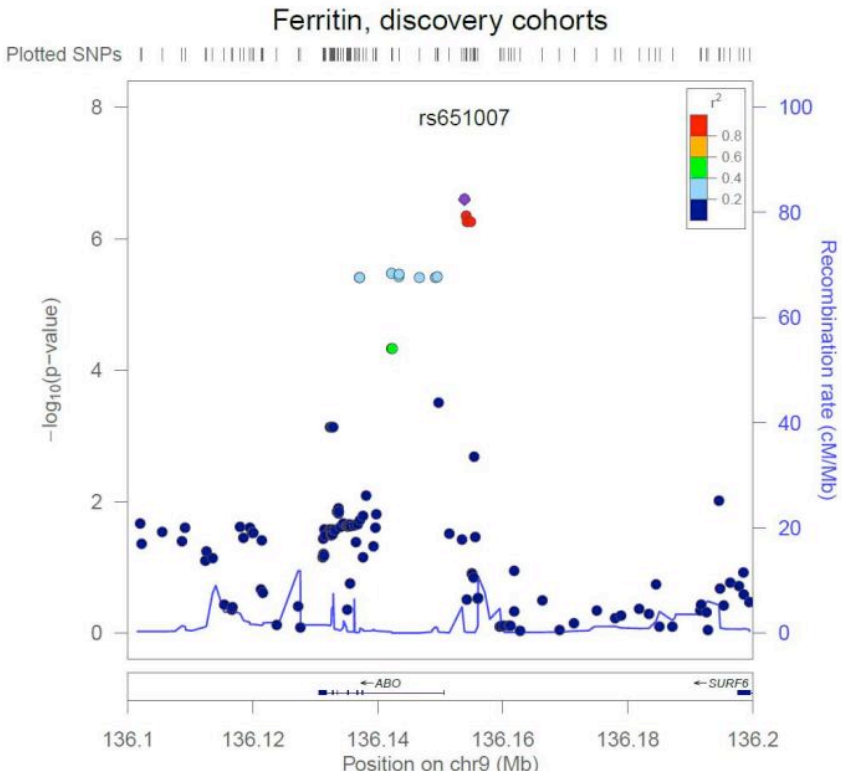
Supplementary Figure2. Regional association plots for loci with significant results in meta- analysis of data from the Discovery cohorts or the Discovery + Replication cohorts.

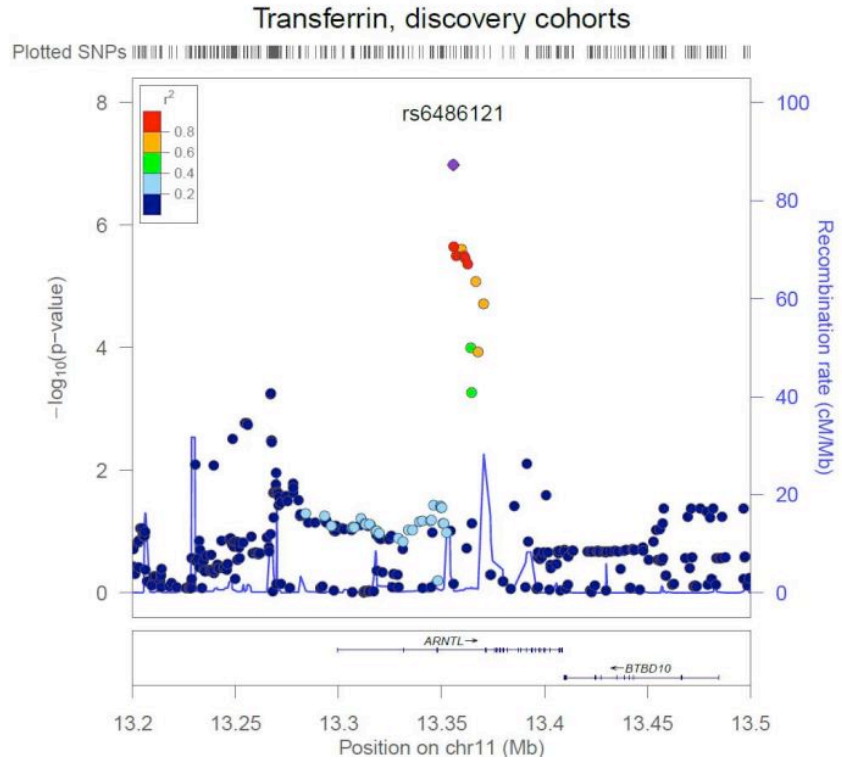
DISCOVERY



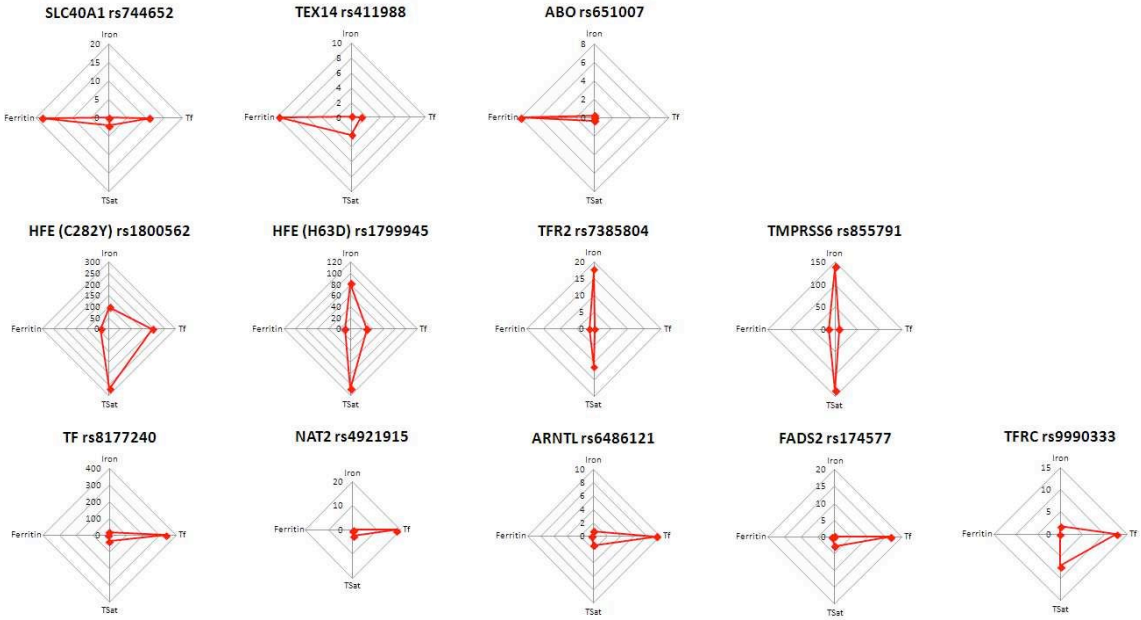

Serum Transferrin Saturation
rs221834
Position on chr7 (Mb)
Serum Ferritin
rs12693541
Position on chr2 (Mb)
Serum Ferritin
rs368243
Position on chr17 (Mb)
Serum Ferritin
rs2413450
Position on chr22 (Mb)
Serum Iron
rs2075672
Position on chr7 (Mb)
Serum Ferritin
rs16976620
Position on chr15 (Mb)
Plotted SNPs
Recombination rate (cM/Mb)

DISCOVERY + REPLICATION (data from Discovery meta-analysis only, but these loci become significant in the combined data)

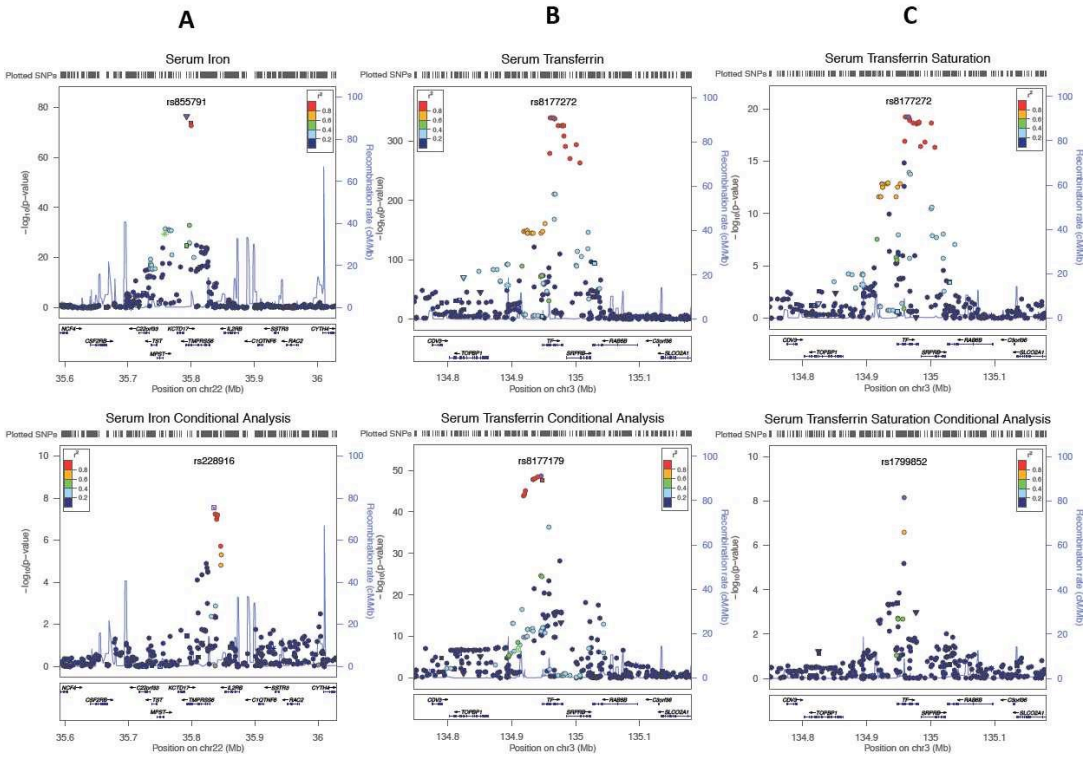




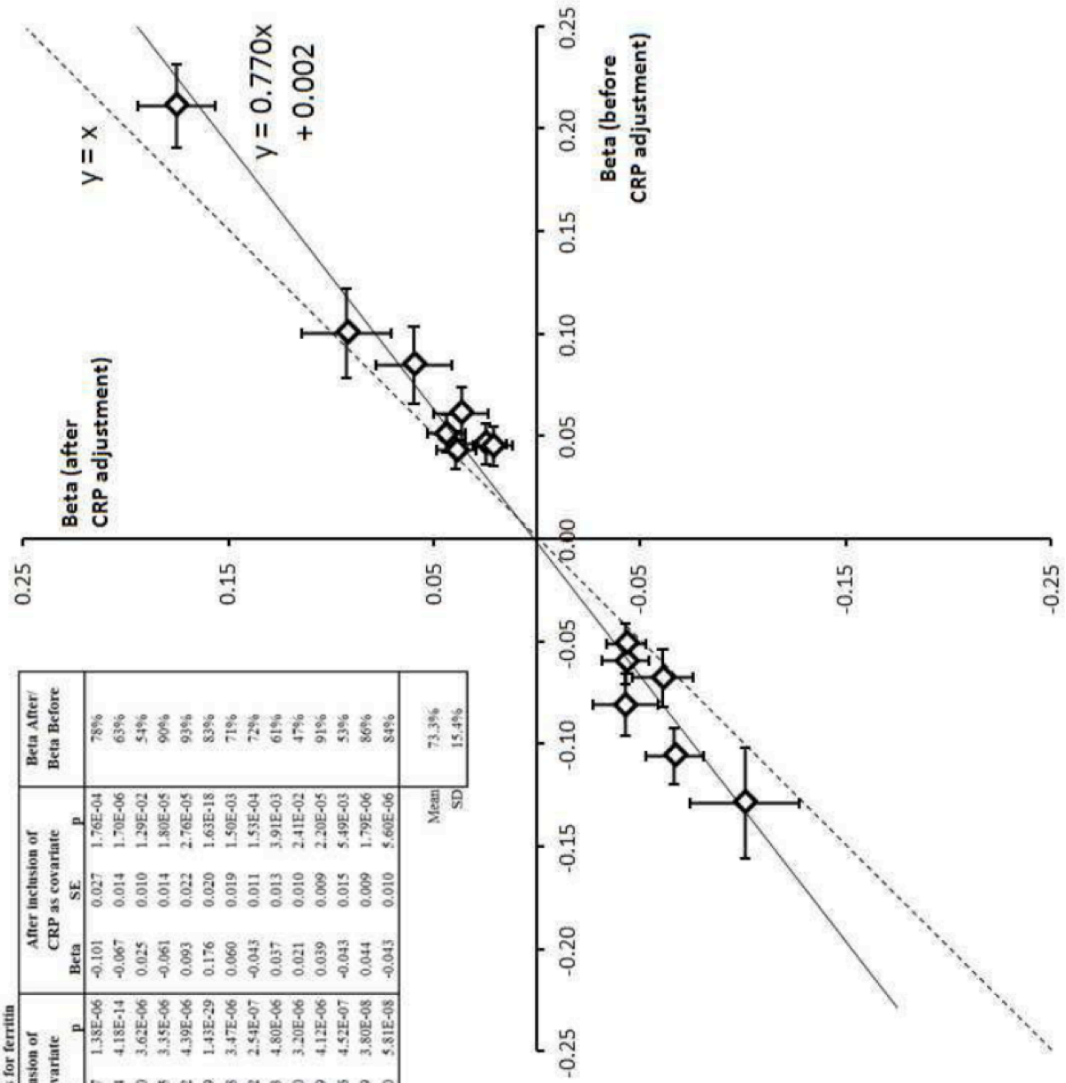
Supplementary Figure 3. Patterns of allelic effects on the four phenotypes, serumiron, transferrin, transferrin saturation and ferritin, for the most significant SNP at each locus (from the Discovery + Replication data). The top row shows loci which mainly affect ferritin, the second row shows loci which mainly affect iron and transferrin saturation, and loci in the bottom row mainly affect transferrin.



Supplementary Figure 4. Results from conditional analysis, in which original results (top panels) are compared with results obtained after including the lead SNP from the initial analysis as a covariate.



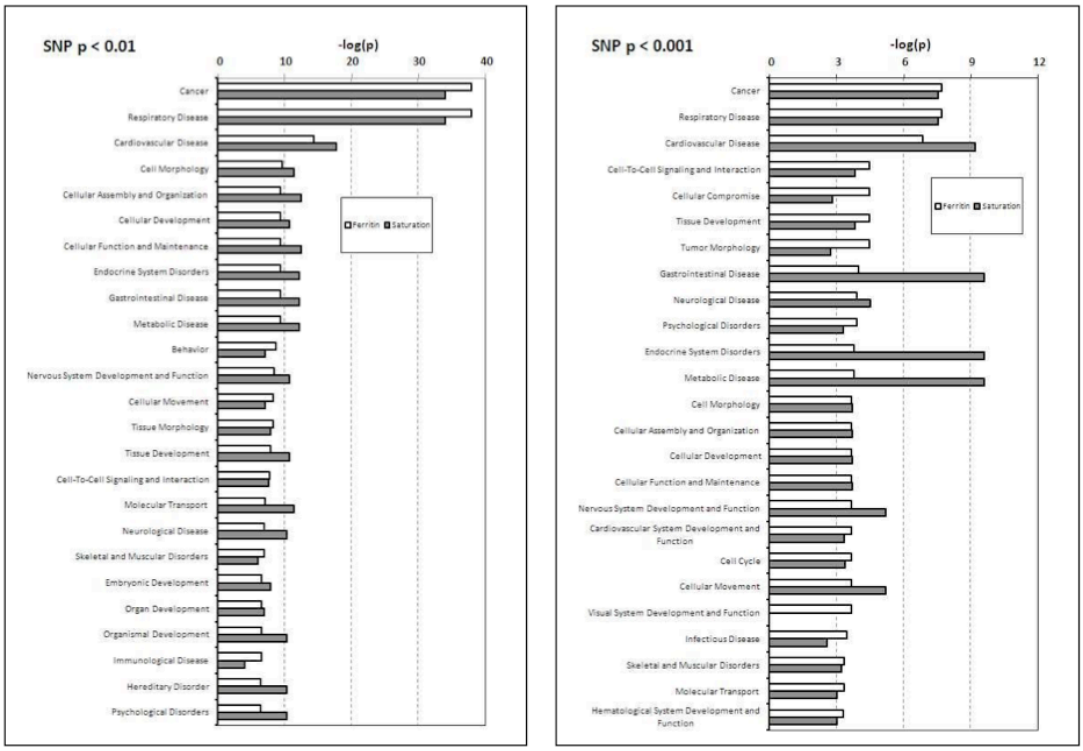
Supplementary Figure 5. Effect of adjusting for C-reactive protein (CRP) concentration on effect sizes for ferritin, showing effect sizes (beta) for the most significant SNP at loci where any SNP shows p < 5x10-6 for ferritin in the Discovery dataset. Error bars show standard errors of betas, the continuous line shows the line of best fit, the interrupted line shows equivalence between y and x.



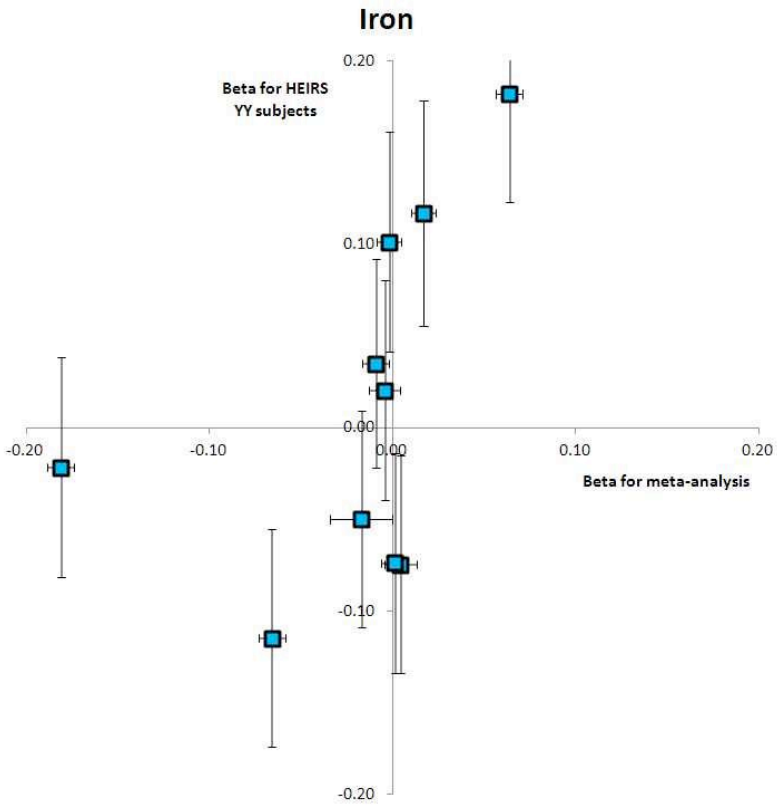
Effects of adjustment for serum CRP on standardised betas for ferritin

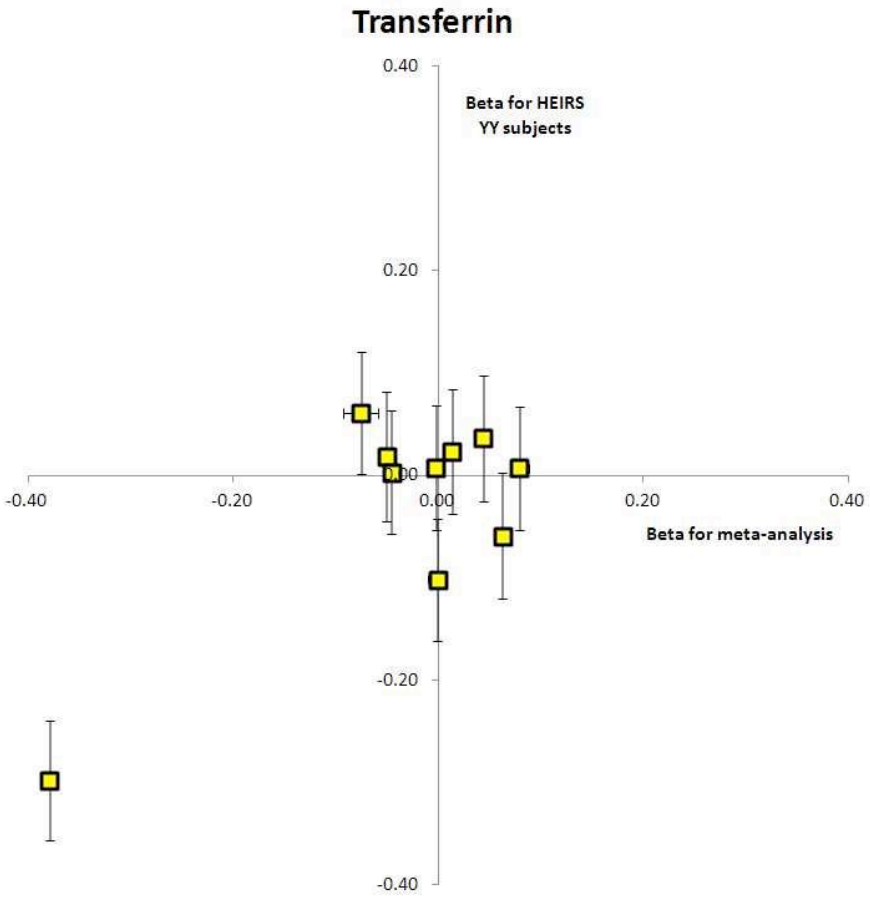
MarkerName	Chr	Gene	Before inclusion of CRP as covariate			After inclusion of CRP as covariate			Beta After/ Beta Before
			Beta	SE	p	Beta	SE	p	
rs7603193	2	*BCL11A*	-0.129	0.027	1.38E-06	-0.101	0.027	1.76E-04	78%
rs12693541	2	*SLC4A1*	-0.106	0.014	4.18E-14	-0.067	0.014	1.70E-06	63%
rs4376025	3	*CRBN-LRRN1*	0.046	0.010	3.62E-06	0.025	0.010	1.29E-02	54%
rs173780	5	*FLJ37543*	-0.068	0.015	3.35E-06	-0.061	0.014	1.80E-05	90%
rs17236666	5	*ISL1*	0.100	0.022	4.39E-06	0.093	0.022	2.76E-05	93%
rs1800562	6	*HFE*	0.211	0.019	1.43E-29	0.176	0.020	1.63E-18	83%
rs9322487	6	*RBM16-TIAM2*	0.085	0.018	3.47E-06	0.060	0.019	1.50E-03	71%
rs651007	9	*ABO*	-0.060	0.012	2.54E-07	-0.043	0.011	1.53E-04	72%
rs1752162	9	*DENND1A*	0.061	0.013	4.80E-06	0.037	0.013	3.91E-03	61%
rs7395347	11	*RAB6IP1*	0.045	0.010	3.20E-06	0.021	0.010	2.41E-02	47%
rs1050045	12	*OS9*	0.043	0.009	4.12E-06	0.039	0.009	2.20E-05	91%
rs16976620	15	*C15orf43*	-0.081	0.016	4.52E-07	-0.043	0.015	5.49E-03	53%
rs368243	17	*TEX14*	0.051	0.009	3.80E-08	0.044	0.009	1.79E-06	86%
rs855791	22	*TMPRSS6*	-0.051	0.010	5.81E-08	-0.043	0.010	5.60E-06	84%
								Mean	73.3%
								SD	15.4%

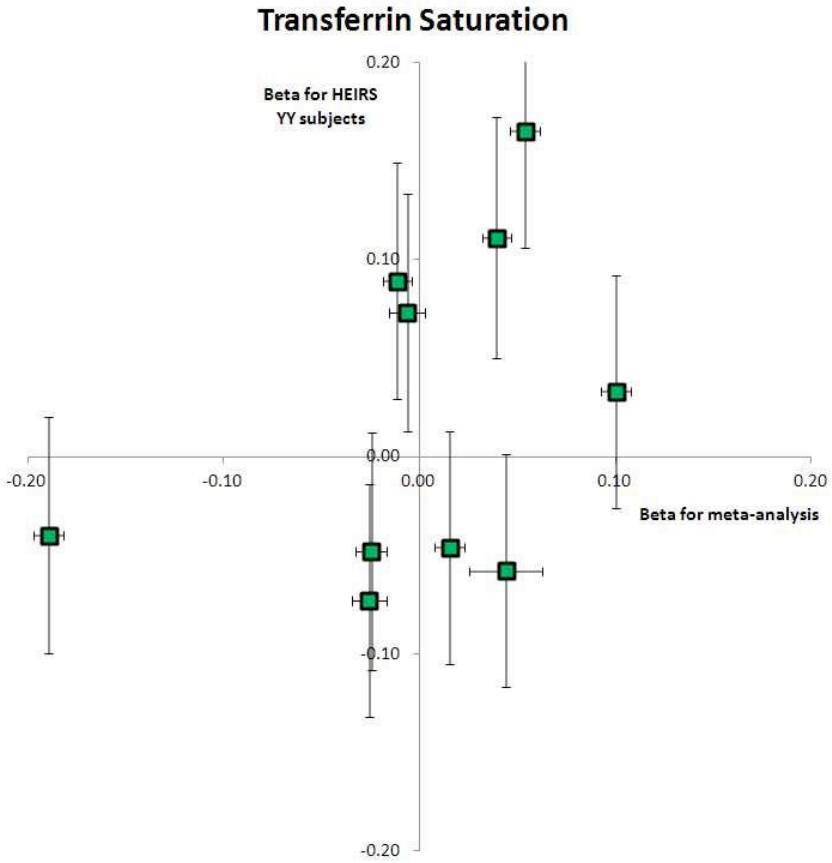
Supplementary Figure 6. Summary of disease and biological process overlap with genes identified through transferrin saturation and ferritin associations at $p < 0.01$ and $p < 0.001$, using Ingenuity Pathway Analysis.

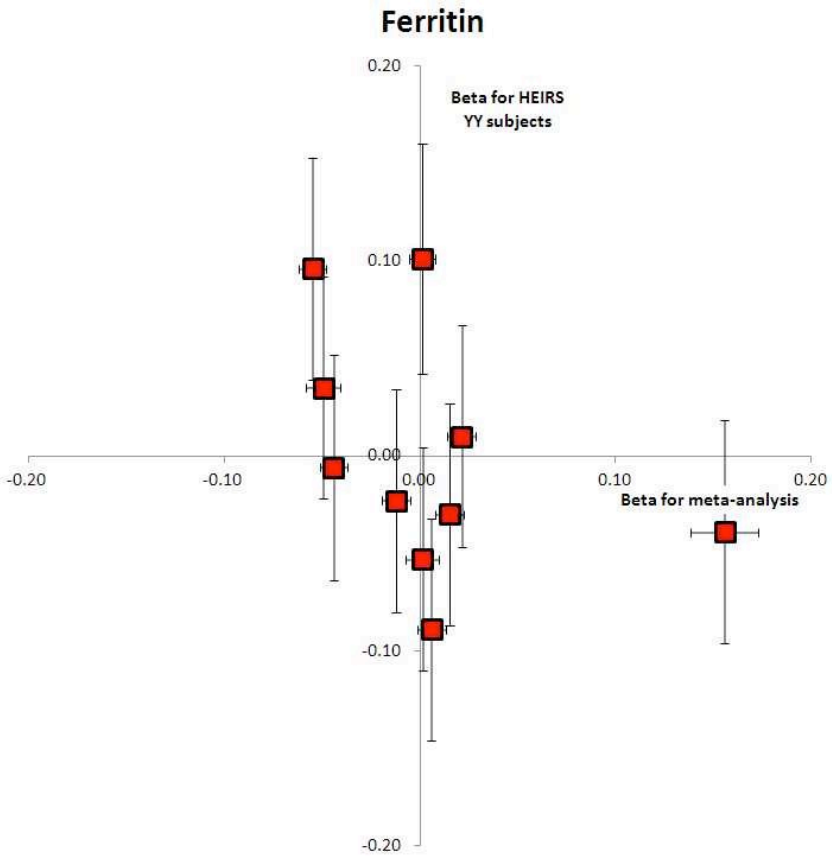


Supplementary Figure 7. Comparison of allelic effects in Discovery + Replication meta-analysis and in C282Y homozygotes from the HEIRS study. Error bars show standard errors for betas.




Transferrin
Beta for HEIRS
YY subjects
Beta for meta-analysis
0.40
0.20
0.00
-0.20
-0.40
-0.40
-0.20
0.00
0.20
0.40


Transferrin Saturation
Beta for HEIRS
YY subjects
Beta for meta-analysis
0.20
0.10
0.00
-0.10
-0.20
-0.20
-0.10
0.00
0.10
0.20



Supplementary Table 4. Initial meta-analysis; lead SNP at loci showing suggestive results ($p < 5 \times 10\text{-}6$) from meta-analysis of the Discovery datasets. Statistical tests and numbers of subjects are as described in the paper.

CHR	SNP	BP(B37)	BP (B36)	A1	A2	Freq1	Effect	StdErr	P.value	Nearby gene(s)
Iron										
2	rs12693541	190,418,690	190,126,935	t	c	0.871	-0.106	0.014	4.18E-14	*SLC40A1*
2	rs6726348	239,084,119	238,748,858	t	c	0.444	0.047	0.010	3.12E-06	*ILKAP*
3	rs7638018	133,495,461	134,978,151	a	g	0.667	-0.074	0.010	1.87E-12	*TF*
5	rs17236666	50,940,708	50,976,465	t	c	0.950	0.100	0.022	4.39E-06	*ISL1*
5	rs173780	60,903,201	60,938,958	a	g	0.135	-0.068	0.015	3.35E-06	*FLJ37543*
6	rs1800562	26,093,141	26,201,120	a	g	0.067	0.372	0.020	3.96E-77	*HFE*
6	rs4715597	56,103,037	56,210,996	c	g	0.294	0.056	0.011	4.66E-07	*COL21A1*
6	rs6920211	135,431,318	135,473,011	t	c	0.758	-0.054	0.012	3.14E-06	*HBS1L, MYB*
7	rs2075672	100,240,296	100,078,232	a	g	0.379	-0.056	0.010	5.95E-08	*TFR2*
8	rs604302	37,004,569	37,123,727	t	c	0.200	0.058	0.012	3.07E-06	*FKSG2*
9	rs1752162	126,551,037	125,590,858	t	c	0.146	0.061	0.013	4.80E-06	*DENND1A*
12	rs1050045	58,115,271	56,401,538	t	c	0.559	0.043	0.009	4.12E-06	*OS9*
15	rs16976620	45,249,892	43,037,184	a	g	0.098	-0.081	0.016	4.52E-07	*C15orf43*
15	rs7172337	61,767,743	59,555,035	t	c	0.728	-0.052	0.011	2.63E-06	*RORA*
17	rs7209063	1,892,031	1,838,781	c	g	0.487	0.048	0.010	3.61E-06	*RTN4RL1*
17	rs2007993	56,590,643	53,945,642	t	c	0.785	0.057	0.011	4.18E-07	*MTMR4*
20	rs6067410	48,973,912	48,407,319	a	t	0.456	-0.047	0.010	3.93E-06	*LOC284751*
22	rs855791	37,462,936	35,792,882	a	g	0.446	-0.187	0.010	4.31E-77	*TMPRSS6*
Transferrin										
1	rs946526	46,487,168	46,259,755	t	c	0.042	-0.122	0.026	2.98E-06	*MAST2*
2	rs11680788	33,059,096	32,912,600	t	c	0.046	-0.115	0.025	4.57E-06	*TTC27*
2	rs744653	190,378,750	190,086,995	t	c	0.854	0.092	0.014	2.00E-10	*WDR75, SLC40A1*
3	rs8177240	133,477,701	134,960,391	t	g	0.671	-0.423	0.011	< E-340	*TF*

CHR	SNP	BP(B37)	BP (B36)	A1	A2	Freq1	Effect	StdErr	P.value	Nearby gene(s)
3	rs9990333	195,827,205	197,311,602	t	c	0.460	-0.067	0.010	3.01E-11	*TFRC*
4	rs1865383	73,110,424	73,329,288	t	g	0.316	-0.052	0.011	2.17E-06	*NPFFR2, ADAMTS3*
5	rs10055024	11,149,808	11,202,808	t	c	0.386	0.051	0.010	8.98E-07	*CTNND2*
6	rs1800562	26,093,141	26,201,120	a	g	0.066	-0.550	0.021	1.26E-153	*HFE*
7	rs4291160	11,974,451	11,940,976	t	g	0.754	-0.055	0.012	3.68E-06	*TMEM106B*
8	rs1495741	18,272,881	18,317,161	a	g	0.782	0.083	0.012	1.57E-11	*NAT2*
8	rs1354342	107,200,980	107,270,156	a	g	0.051	0.126	0.024	1.28E-07	*ZFPM2, OXR1*
9	rs2165554	119,479,774	118,519,595	t	c	0.392	-0.048	0.011	4.19E-06	*ASTN2*
11	rs6486121	13,355,770	13,312,346	t	c	0.627	-0.056	0.011	1.04E-07	*ARNTL*
11	rs174577	61,604,814	61,361,390	a	c	0.333	0.068	0.011	1.90E-10	*FADS2*
12	rs12371237	29,831,076	29,722,343	a	c	0.336	-0.050	0.011	3.48E-06	*TMTC1*
12	rs2374503	106,034,829	104,558,959	c	g	0.421	-0.048	0.010	2.95E-06	*LOC387882*
19	rs12978009	17,113,634	16,974,634	a	g	0.179	-0.064	0.014	3.17E-06	*CPAMD8*
22	rs2275901	19,135,603	17,515,603	a	g	0.239	0.060	0.013	1.77E-06	*GSCL*
Saturation										
3	rs8177272	133,482,870	134,965,560	a	g	0.331	-0.097	0.011	5.52E-20	*TF*
3	rs2061336	164,591,618	166,074,312	a	g	0.913	-0.093	0.018	1.99E-07	*SI*
3	rs9990333	195,827,205	197,311,602	t	c	0.460	0.049	0.010	7.37E-07	*TFRC*
6	rs1800562	26,093,141	26,201,120	a	g	0.067	0.577	0.020	1.52E-178	*HFE*
6	rs2841000	56,077,917	56,185,876	t	c	0.701	-0.051	0.011	2.78E-06	*COL21A1*
6	rs9389269	135,427,159	135,468,852	t	c	0.727	-0.055	0.011	9.78E-07	*HBS1L, MYB*
7	rs11765024	100,125,975	99,963,911	a	g	0.897	-0.091	0.017	3.32E-08	*AGFG2 (TFR2, EPO)*
7	rs221834	100,343,175	100,181,111	c	g	0.928	-0.123	0.021	2.38E-09	*ZAN (TFR2, EPO)*
8	rs604302	37,004,569	37,123,727	t	c	0.200	0.058	0.012	3.26E-06	*FKSG2*
12	rs11046313	22,274,788	22,166,055	a	c	0.286	-0.056	0.011	6.86E-07	*CMAS, ST8SIA1*
17	rs4790859	1,897,820	1,844,570	a	g	0.474	0.048	0.010	2.29E-06	*RTN4RL1*
22	rs855791	37,462,936	35,792,882	a	g	0.446	-0.192	0.010	3.50E-80	*TMPRSS6*
Ferritin										
2	rs7603193	60,478,727	60,332,231	t	c	0.033	-0.129	0.027	1.38E-06	*BCL11A*
2	rs12693541	190,418,690	190,126,935	t	c	0.871	-0.106	0.014	4.18E-14	*SLC40A1*
3	rs4376025	3,419,984	3,394,984	t	c	0.680	0.046	0.010	3.62E-06	*CRBN, LRRN1*
5	rs17236666	50,940,708	50,976,465	t	c	0.950	0.100	0.022	4.39E-06	*ISL1*
5	rs173780	60,903,201	60,938,958	a	g	0.135	-0.068	0.015	3.35E-06	*FLJ37543*
6	rs1800562	26,093,141	26,201,120	a	g	0.068	0.211	0.019	1.43E-29	*HFE*
6	rs9322487	155,255,431	155,297,123	a	g	0.080	0.085	0.018	3.47E-06	*RBM16, TIAM2*
9	rs1752162	126,551,037	125,590,858	t	c	0.146	0.061	0.013	4.80E-06	*DENND1A*
9	rs651007	136,153,875	135,143,696	t	c	0.203	-0.060	0.012	2.54E-07	*ABO*
11	rs7395347	9,152,463	9,109,039	t	c	0.341	0.045	0.010	3.20E-06	*SCUBE2, RAB6IP1*
12	rs1050045	58,115,271	56,401,538	t	c	0.559	0.043	0.009	4.12E-06	*OS9*
15	rs16976620	45,249,892	43,037,184	a	g	0.098	-0.081	0.016	4.52E-07	*C15orf43*
17	rs368243	56,708,979	54,063,978	t	c	0.440	0.051	0.009	3.80E-08	*TEX14*
22	rs2413450	37,470,224	35,800,170	t	c	0.463	-0.056	0.010	3.57E-09	*TMPRSS6*

Supplementary Tables S2, S3, S5, S6, S7, S8, S9 and S10 are available at: http://www.nature.com/ncomms/2014/141029/ncomms5926/extref/ncomms5926-s1.pdf

SUPPLEMENTARY TEXT: COHORT INFORMATION

Discovery Cohorts:

Australia- Adult

Study participants comprised (a) adult twins, their spouses and first- degree relatives who volunteered for studies on risk factors or biomarkers for physical or psychiatric conditions; (b) people with self- reported migraine or endometriosis and unaffected relatives. These studies were approved by The Queensland Institute of Medical Research Human Research Ethics Committee and, for the studies on alcohol and nicotine genetics, also by Washington University School of Medicine Human Subjects Committee.

Benyamin et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet. 2009;41:1173-5. PMID 19820699

Painter et al. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. Nat Genet. 2011;43:51-4. PMID: 21151130

Anttila et al. Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1. Nat Genet. 2010;42:869-73. PMID: 20802479

We acknowledge funding from the Australian National Health and Medical Research Council (NHMRC grants 241944, 389875, 389891,389892, 389938, 442915, 442981, 496739 and 552485), US National Institutes of Health (NIH grants AA07535, AA10248 and AA014041) and the Australian Research Council (ARC grant DP0770096). D.R.N. and G.W.M . are supported by the NHMRC Fellowship Scheme.

Australia-Adolescent

Adolescent twins and their non-twin siblings who participated in studies on skin cancer risk factors at ages 12 and 14, and on cognition at age 16. These studies were approved by The Queensland Institute of Medical Research Human Research Ethics Committee, and both the participants and their parents or guardians gave informed consent.

Middelberg RPS, Martin NG, Whitfield JB. A longitudinal genetic study of plasma lipids in adolescent twins. Twin Research and Human Genetics 2007;10:127-135.

Powell JE, Henders AK, McRae AF, et al. The Brisbane Systems Genetics Study: genetical genomics meets complex trait genetics. PLoS One.

Financial support for aspects of the adolescent studies was provided by grants from the National Health and Medical Research Council of Australia, and the National Institute on Alcohol Abuse and Alcoholism (AA007535, AA014041).

Estonian Biobank (original cohort)

The Estonian cohort comes from the population-based biobank of the Estonian Genome Project of University of Tartu (EGCUT). The project is conducted according to the Estonian Gene Research Act and all participants have signed the broad informed consent (www.biobank.ee). In total, 52 000 individuals aged 18 years or older participated in this cohort (33% men, 67% women). The population distributions of the cohort reflect those of the Estonian population (83% Estonians, 14% Russians and 3% other). General practitioners (GP) and physicians in the hospitals randomly recruited the participants. A Computer-Assisted Personal interview was conducted during 1–2 h at doctors' offices. Data on demographics, genealogy, educational and occupational history, lifestyle and anthropometric and physiological data were assessed. These studies were approved by the Research Ethics Committee of the University of Tartu.

Website: http://www.biobank.ee/

Leitsalu L, et al. Cohort Profile: Estonian Biobank of the Estonian Genome Center, University of Tartu. Int J Epidemiol. 2014 Feb 11.

This work was supported by the Targeted Financing from the Estonian Ministry of Science and Education [SF0180142s08]; the US National Institute of Health [R01DK075787]; the Development Fund of the University of Tartu (grant SP1GVARENG); the European Regional Development Fund to the Centre of Excellence in Genomics (EXCEGEN; grant 3.2.0304.11-0312); and through FP7 grant 313010.

We acknowledge EGCUT technical personnel, especially Mr V. Soo and S. Smit. Data analyzes were carried out in part in the High Performance Computing Center of University of Tartu.

Kora (F3, F4)

The KORA study is a series of independent population-based epidemiological surveys of participants living in the region of Augsburg, Southern Germany. All survey participants are residents of German nationality identified through the registration office and were examined in 1994/95 (KORA S3) and 1999/2001 (KORA F4). In the KORA S3 and S4 studies 4,856 and 4,261 subjects have been examined implying response rates of 75% and 67%, respectively. 3,006 subjects participated in a 10-year follow-up examination of S3 in 2004/05 (KORA F3), and 3080 of S4 in 2006/2008 (KORA F4). Individuals for genotyping in KORA F3 and KORA F4 were randomly selected. The age range of the participants was 25 to 74 years of recruitment. Informed consent has been given by all participants. The study has been approved by the local ethics committee (Ethik-Kommission der Bayerische Landesärztekammer).

Holle R, Happich M, Löwel H, Wichmann HE (2005) KORA–a research platform for population based health research. Gesundheitswesen 2005 Aug;67(Suppl 1): S19–25.

Wichmann H-E, Gieger C, Illig T (2005) KORA-gen–resource for population genetics, controls and a broad spectrum of disease phenotypes. Gesundheitswesen 2005Aug ;67(Suppl 1): S26–30.

The KORA research platform (KORA, Cooperative Health Research in the Region of Augsburg) was initiated and financed by the Helmholtz Zentrum München - German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research and by the State of Bavaria. Furthermore, KORA research was supported within the Munich Center of Health Sciences (MC Health), Ludwig- Maximilians-Universität, as part of LMUinnovativ.

Val Borbera

The INGI–Val Borbera population is a collection of 1,664 genotyped samples collected in the Val Borbera Valley, a geographically isolated valley located within the Appennine Mountains in Northwest Italy1. The valley is inhabited by about 3,000 descendants from the original population, living in 7 villages along the valley and in the mountains. Participants were healthy people 18-102 years of age that had at least one grandfather living in the valley. The study plan and the informed consent form were reviewed and approved by the institutional review boards of San Raffaele Hospital in Milan.

Traglia, M. et al. Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. PLoS One 4, e7554 (2009).†

Colonna V, et al. Small effective population size and genetic homogeneity in the Val Borbera isolate. Eur J Hum Genet. 2):89-94. 2013

The research was supported by funds from Compagnia di San Paolo, Torino, Italy; Fondazione Cariplo, Italy and Ministry of Health, Ricerca Finalizzata 2008 and CCM 2010, PRIN 2009 and Telethon, Italy to DT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank the inhabitants of the VB that made this study possible, the local administrations, the Tortona and Genova archdiocese and the ASL-22, Novi Ligure (Al) for support.

We also thank Fiammetta Viganò for technical help, Corrado Masciullo and Massimiliano Cocca for building the analysis platform.

NBS (Nijmegen Biomedical Study)

The Nijmegen Biomedical Study (NBS; http://www.nijmegenbiomedischestudie.nl) is a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboud University Medical Centre, Nijmegen, The Netherlands. The study has been described before (1). Briefly, in 2002, 22,451 age and sex-stratified randomly selected adult inhabitants of Nijmegen, a city located in the eastern part of the Netherlands, received an invitation to fill out a postal questionnaire (QN) including questions about lifestyle, health status, and medical history, and to donate a blood sample for DNA isolation and biochemical studies. A total of 9350 (43%) persons filled out the QN, of which 6468 (69%) donated blood samples. A second, third and fourth questionnaire were sent out in 2005, 2008 and 2012, respectively. Approval to conduct the NBS was obtained from the Radboud University Medical Centre Institutional Review Board. All participants gave written informed consent for participation in the NBS. For this study we used the subset of 1980 NBS participants that was selected to serve as controls in GWAS (2).

1. Hoogendoorn EH, Hermus AR, de Vegt F, Ross HA, Verbeek AL, Kiemeney LA, Swinkels DW, Sweep FC, den Heijer M. Thyroid function and prevalence of anti-thyroperoxidase antibodies

in a population with borderline sufficient iodine intake: influences of age and sex. Clin Chem 2006;52:104-11.
2. Kiemeney LA, Thorlacius S, Sulem P, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. Nat Genet 2008;40:1307-12.
This work was sponsored by the Stichting Nationale Computerfaciliteiten (National Computing Facilities Foundation, NCF) for the use of supercomputer facilities, with financial support from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (Netherlands Organization for Scientific Research, NWO).
The Nijmegen Biomedical Study is a population-based survey conducted at the Department for Health Evidence, and the Department of Laboratory Medicine of the Radboud University Medical Centre. Principal investigators of the Nijmegen Biomedical Study are Lambertus A. Kiemeney, Martin den Heijer, André L.M. Verbeek, Dorine W. Swinkels and Barbara Franke.

Cambridge

The UK Blood Services (UKBS) Common Controls Panel 1 and 2 (UKBS - CC1 and UKBS - CC2) is a national collection of 3,000 DNA samples from the 12 health regions of Great Britain established in 2005 - 2006 by a partnership between NHS Blood and Transplant (NHSBT) of England, the Scottish National Blood Transfusion Service and the Welsh Blood Service. The Common Controls collection was established for use as the shared controls in the WTCCC Genome - Wide Association Studies (WGAS), and was approved by the Peterborough & Fenland Local Research Ethics Committee

Wellcome Trust Case Control Consortium. Genome - wide association study of 14, 000 cases of seven common diseases and 3,000 shared controls. Nature 447 , 661 - 78 (2007).

Research in the Ouwehand laboratory is supported by program grants from the National Institute for Health Research (NIHR) to WHO and the British Heart Foundation (to AR) under numbers RP-PG-0310-1002 and RG/09/12/28096.

Micros/EURAC

The MICROS study is part of the genomic health care program 'GenNova' and was carried out in three villages of the Val Venosta, South Tyrol (Italy), in 2001-2003. It comprised members of the populations of Stelvio, Vallelunga and Martello. A detailed description of the MICROS study is available elsewhere (Pattaro et al. 2007). Briefly, study participants were volunteers from three isolated villages located in the Italian Alps, in a German-speaking region bordering with Austria and Switzerland. Owing to geographical, historical and political reasons, the entire region experienced a prolonged period of isolation from surrounding populations. Information on the participant's health status was collected through a standardized questionnaire. Laboratory data were obtained from standard blood analyses. The study participants are connected among each other in a unique genealogy for the three villages. The study was approved by the Landesethikkomitee (ethics committee) of the autonomous province of Bolzano.

Pattaro C, Marroni F, Riegler A, Mascalzoni D, Pichler I, Volpato CB, Dal Cero U, De Grandi A, Egger C, Eisendle A, Fuchsberger C, Gögele M, Pedrotti S, Pinggera GK, Stefanov SA, Vogl FD, Wiedermann CJ, Meitinger T, Pramstaller PP. The genetic study of three population microisolates in South Tyrol (MICROS): study design and epidemiological perspectives. BMC Med Genet. 2007 Jun 5;8:29.

The 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.
For the MICROS study, we thank the primary care practitioners Raffaela Stocker, Stefan Waldner, Toni Pizzecco, Josef Plangger, Ugo Marcadent and the personnel of the Hospital of Silandro (Department of Laboratory Medicine) for their participation and collaboration in the research project.

ERF/Rotterdam

The Erasmus Rucphen Family study is part of the Genetic Research in Isolated Populations (GRIP) program. It is a cross-sectional population- based study that includes over 3000 participants descending from 22 couples who lived in the Rucphen region in the southwest Netherlands and had at least 6 children baptized in the community church between 1850 and 1900 . All living descendants of these pairs (as well as their spouses), ascertained on the basis of municipal and

baptismal records, were traced and invited to participate (n = 3000). Selection of the study participants was not based on any disease. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam approved the study and informed consent was obtained from all participants.
Aulchenko YS, Heutink P, Mackay I, et al. Linkage disequilibrium in young genetically isolated Dutch population. Eur J Hum Genet 2004;12:527-34. PMID:15054401
ERF: The genotyping for the ERF study was supported by EUROSPAN (European Special Populations Research Network) and the European Commission FP6 STRP grant (018947; LSHG-CT-2006-01947). The ERF study was further supported by grants from the Netherlands Organisation for Scientific Research, Erasmus MC, the Centre for Medical Systems Biology (CMSB) and the Netherlands Brain Foundation (HersenStichting Nederland). We are grateful to all participating individuals and their relatives, general practitioners and neurologists for their contributions and to P. Veraart for her help in genealogy, Jeannette Vergeer for the supervision of the laboratory work and P. Snijders for his help in data collection.

Busselton Health Study

Residents of the town of Busselton in the southwest of Western Australia have been involved in a series of health surveys since 1966.a The population is predominantly of European origin. In 1994/95 there was a follow-up study involving a subset of those who had attended any of the previous surveys. Cases of asthma were defined as those who reported doctor-diagnosed asthma at any survey that they attended from 1966 to 1994 (answer 'Yes' to 'Has your doctor ever told you that you had asthma?').b Controls are those who have consistently answered 'No' to 'Has your doctor ever told you that you had asthma?' at all previous surveys that they have attended from 1996 to 1994. For the GWA study, a case control sample of unrelated individuals was selected. After QC a total of 1,207 subjects were retained in the GWAS analyses. Ethical approval was obtained through the Human Research Ethics Office, University of Western Australia
Website: http://www.busseltonhealthstudy.com/
James AL, Knuiman MW, Divitini ML et al. Changes in the prevalence of asthma in adults since 1966: the Busselton Health Study. Eur Respir J 2009.
The Busselton Health Study (BHS) acknowledges the generous support for the 1994/5 follow-up study from Healthway, Western Australia and the numerous Busselton community volunteers who assisted with data collection and the study participants from the Shire of Busselton. The Busselton Health Study is supported by The Great Wine Estates of the Margaret River region of Western Australia.

Replication Cohorts:

Estonian Biobank (replication cohort)

The Estonian cohort comes from the population-based biobank of the Estonian Genome Project of University of Tartu (EGCUT). The project is conducted according to the Estonian Gene Research Act and all participants have signed the broad informed consent (www.biobank.ee). In total, 52 000 individuals aged 18 years or older participated in this cohort (33% men, 67% women). The population distributions of the cohort reflect those of the Estonian population (83% Estonians, 14% Russians and 3% other). General practitioners (GP) and physicians in the hospitals randomly recruited the participants. A Computer-Assisted Personal interview was conducted during 1–2 h at doctors' offices. Data on demographics, genealogy, educational and occupational history, lifestyle and anthropometric and physiological data were assessed. These studies were approved by the Research Ethics Committee of the University of Tartu.
Website: http://www.biobank.ee/
Leitsalu L, et al. Cohort Profile: Estonian Biobank of the Estonian Genome Center, University of Tartu. Int J Epidemiol. 2014 Feb 11.
This work was supported by the Targeted Financing from the Estonian Ministry of Science and Education [SF0180142s08]; the US National Institute of Health [R01DK075787]; the Development Fund of the University of Tartu (grant SP1GVARENG); the European Regional Development Fund to the Centre of Excellence in Genomics (EXCEGEN; grant 3.2.0304.11-0312); and through FP7 grant 313010.
We acknowledge EGCUT technical personnel, especially Mr V. Soo and S. Smit. Data analyzes were carried out in part in the High Performance Computing Center of University of Tartu.

InCHIANTI

The InCHIANTI study is a population-based epidemiological 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 previously reported[1]. Briefly, 1616 residents were selected from the population registry of Greve in Chianti (a rural area: 11,709 residents with 19.3% of the population greater than 65 years of age), and Bagno a Ripoli (Antella village near Florence; 4,704 inhabitants, with 20.3% greater than 65 years of age). The participation rate was 90% (n=1453), and the subjects ranged between 21-102 years of age. Overnight fasted blood samples were for genomic DNA extraction, and measurement of iron-related traits. Illumina Infinium HumanHap 550K SNP arrays were used for genotyping [2]. The study protocol was approved by the Italian National Institute of Research and Care of Aging Institutional Review, and Medstar Research Institute (Baltimore, MD).

1. Ferrucci, L., et al., 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, 2000. 48(12): p. 1618-25. PMID: 11129752

2. Melzer, D., et al., A genome-wide association study identifies protein quantitative trait loci (pQTLs). PLoS Genet, 2008. 4(5): p. e1000072. PMID: 18464913

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 U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336).

SardiNIA

The SardiNIA study is a longitudinal study which recruited and phenotyped 6,148 individuals, males and females, aged 14–102 y, from a cluster of four towns in the Lanusei Valley [Pilia et al Plos Genetic 2006], located in the central east coast of the Sardinia island, Italy. During physical examination of each individual, a blood sample was collected and divided into two aliquots. One aliquot was used for DNA extraction and the other to characterize several blood phenotypes. During the study, we genotyped, by common GWAS arrays (Affymtrix 10K, Affymetrix 500K and Affymetrix 6.0), 4,694 individuals selected from the whole sample to represent the largest available families, regardless of their phenotypic values. Genotyping protocol and quality checks for the genotyping arrays were described previously [Naitza et al Plos Genet 2012]. The quality controlled 731,209 autosomal markers were used to estimate genotypes for additional 1,594,772 polymorphic SNPs assessed in the CEU HapMap population (release 22) by genotype imputation. The SardiNIA study was approved by both the IRB at the National Institute on Ageing and the local Italian Ethical Committee "Azienda Unita' Sanitaria Locale (U.S.L.) N 4, Lanusei.

We thank the many individuals who generously participated in this study. We are also grateful for the important computing resources made available for imputation and analysis by the CRS4 HP Computing Cluster in Pula (Cagliari, Italy), and in particular to Lidia Leoni, Luca Carta e Michele Muggiri. This work was supported by the Intramural Research Program of the National Institute on Aging (NIA), National Institutes of Health (NIH). The SardiNIA (“Progenia”) team was supported by Contract NO1-AG-1– 2109 from the NIA.

CoLAUS

The CoLaus study is a population-based cohort study in Lausanne, Switzerland and has been described previously [Firmann M, BMC Cardiovascular Disorders, 2008, PMID 18366642]. Briefly, the baseline study was conducted between 2003 and 2006, recruiting over 6,000 subjects. The following inclusion criteria were applied: a) voluntary participation in the examination, including blood sample, b) aged 35-75 years, and c) Caucasian origin defined as having both parents and grand-parents Caucasian (determined by birth place). A follow-up visit took place from 2009-2012, hence 5 years after the baseline study, (n=5,228, 78% follow-up) and similar measurements were repeated. The Institutional Review Board of the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne and the Cantonal Ethics Committee (Commission Cantonale d’éthique de la recherche sur l’être humain) approved the study protocol for both the baseline and follow-up studies and signed informed consent was obtained from participants.

The CoLaus study was supported by research grants from GlaxoSmithKline, the Faculty of Biology and Medicine of Lausanne, Switzerland, and the Swiss National Science Foundation (grant no: 33CSCO-122661, 33CS30-139468). ZK was supported by the Leenaards Foundation and the Swiss National Science Foundation (31003A-143914).

The authors thank Peter Vollenweider, Vincent Mooser and Dawn Waterworth, Co-PIs of the CoLaus study. Special thanks to Murielle Bochud, Yolande Barreau, Mathieu Firmann, Vladimir Mayor, Anne-Lise Bastian, Binasa Ramic, Martine Moranville, Martine Baumer, Marcy Sagette, Jeanne Ecoffey and Sylvie Mermoud for data collection.

PREVEND

The PREVEND Study is a prospective, observational cohort study, focussed to assess the impact of elevated urinary albumin loss in non-diabetic subjects on future cardiovascular and renal disease. PREVEND is an acronym for Prevention of REnal and Vascular ENd- stage Disease. This study started with a population survey on the prevalence of micro-albuminuria and generation of a study cohort of the general population. The goal is to monitor this cohort for the long-term development of cardiac-, renal- and peripheral vascular end-stage disease. For that purpose the participants receive questionnaires on events and are seen every three/four years for a survey on cardiac-,renal- and peripheral vascular morbidity. 'The PREVEND study was approved by the medical ethics committee of the University Medical Center Groningen and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent. Website: http://www.prevend.org/index.php

This work was supported by the following grants: PREVEND genetics is supported by the Dutch Kidney Foundation (Grant E033), the National Institutes of Health (grant LM010098), The Netherlands Organization for Scientific Research (NWO-Groot 175.010.2007.006, NWO VENI grant 916.761.70, ZonMW 90.700.441), and the Dutch Inter University Cardiology Institute Netherlands. N. Verweij is supported by the Netherlands Heart Foundation (grant NHS2010B280).

'The PREVEND study was approved by the medical ethics committee of the University Medical Center Groningen and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

FENLAND

The Fenland study is a population based cohort in Eastern England (UK) designed to analyse gene-lifestyle interactions on intermediate quantitative traits related to obesity and type 2 diabetes risk. It combines detailed measurement of the lifestyle exposures with accurate metabolic and anthropometric phenotyping. More than 10,000 men and women born between 1950 and 1975 have been recruited since 2004 and is still ongoing. Exclusion criteria were people suffering from a psychotic illness, pregnant and lactating females, people unable to walk unaided, individuals with diagnosed diabetes or a prognosis of less than 1 year. GWAS data is currently available on 1,500 randomly selected participants. The study was approved by Cambridge Local Research Ethics Committee (NHS).

De Lucia Rolfe E, Am J Clin Nutr, 2010, PMID 21248185 The Fenland Study is funded by the Medical Research Council (MC_UU_12015/1). Clara Podmore is funded by the Wellcome Trust (097451/Z/11/Z).

We are grateful to all the volunteers for their time and help, and to the General Practitioners and practice staff for assistance with recruitment. We thank the Fenland Study Investigators, Fenland Study Co- ordination team and the Epidemiology Field, Data and Laboratory teams. Biochemical assays were performed by the National Institute for Health Research, Cambridge Biomedical Research Centre, Core Biochemistry Assay Laboratory, and the Cambridge University Hospitals NHS Foundation Trust, Department of Clinical Biochemistry.

INTERACT

The InterAct study is a case-cohort study of incident cases of type 2 diabetes (T2D) from eight of the ten countries involved in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohorts [Langenberg C, Diabetologia 2011 PMID 21717116]. In brief, 12,403 verfied incident cases of T2D occured between 1991 and 2007 among the participants eligible for inclusion in InterAct, and a centre-stratified subcohort of 16,154 individuals was defined for comparative analysis. As part of EPIC, standardised information had been collected on participants, including information on lifestyles exposures, diet, physical activity, standard anthropometric data and biomarker measurements on stored blood samples. The study was approved by the Internal Review Board of the International Agency for Research on Cancer, in addition to the local ethics committees in the participating countries.

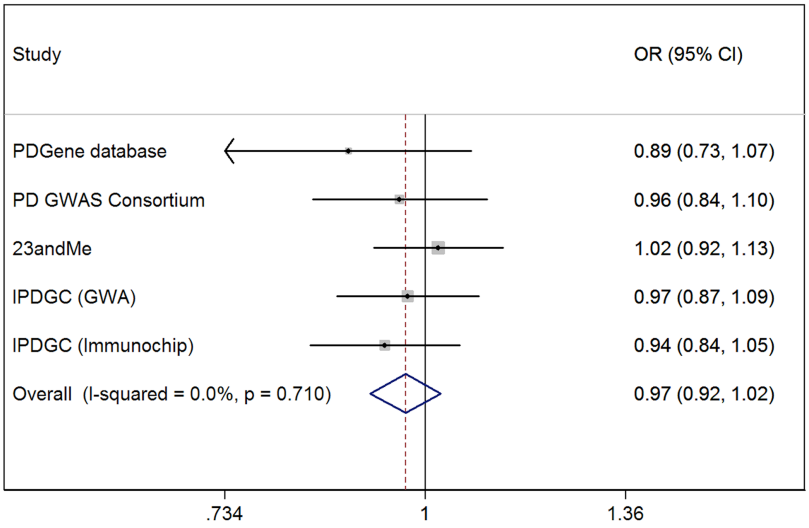
InterAct was funded by the EU Integrated Project LSHM-CT-2006- 037197.

We thank all EPIC participants and staff for their contribution to the study.

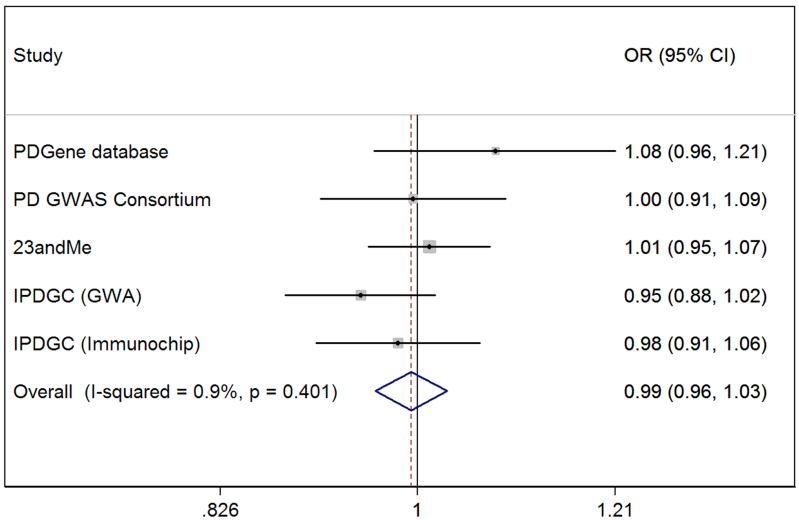
Supplemental data of Chapter 9

SUPPLEMENTAL TEXT, TABLES AND FIGURES.

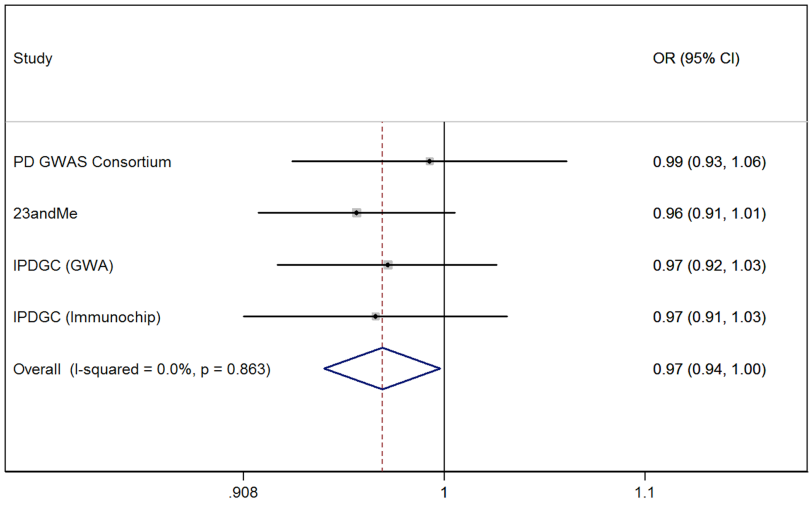
Supplementary Figure S1. Forest plot of the meta-analysis of the studies included for the effect of HFE rs1800562 on PD risk. The boxes indicate the genetic (additive) effects of individual studies, with the size of the box being inversely proportional to the variance and horizontal lines indicating 95% confidence intervals. The diamond indicates the pooled effect estimate, obtained using inverse-variance weighted fixed-effect meta-analysis, and its 95% confidence interval. The full vertical line shows the value for no effect, as opposed to the dashed line indicating the estimated pooled effect.



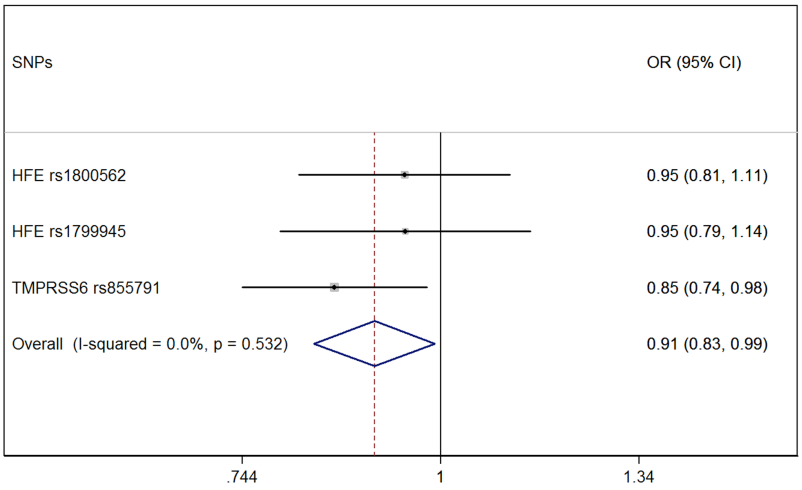
Supplementary Figure S2. Forest plot of the meta-analysis of the studies included for the effect of HFE rs1799945 on PD risk. The boxes indicate the genetic (additive) effects of individual studies, with the size of the box being inversely proportional to the variance and horizontal lines indicating 95% confidence intervals. The diamond indicates the pooled effect estimate, obtained using inverse-variance weighted fixed-effect meta-analysis, and its 95% confidence interval. The full vertical line shows the value for no effect, as opposed to the dashed line indicating the estimated pooled effect.



Supplementary Figure S3. Forest plot of the meta-analysis of the studies included for the effect of TMPRSS6 rs855791 on PD risk. The boxes indicate the genetic (additive) effects of individual studies, with the size of the box being inversely proportional to the variance and horizontal lines indicating 95% confidence intervals. The diamond indicates the pooled effect estimate, obtained using inverse-variance weighted fixed-effect meta-analysis, and its 95% confidence interval. The full vertical line shows the value for no effect, as opposed to the dashed line indicating the estimated pooled effect.



Supplementary Figure S4. Sensitivity analysis: Forest plot of the mendelian randomization estimates after exclusion of nine studies from the PDGene dataset that had not adjusted for population stratification (see Table S2).



Supplementary Table S1. Characteristics and sample size of the individual studies included for the gene–iron association. In all studies, the analyses were adjusted for age and sex, as well as for the first five MDS (multidimensional scaling) or principal components to control for population stratification.

Data source	N. studies	Country	Type of study	Study design	Sample size[1]
Genetics of Iron Status (GIS) Consortium[2]	10		GWA	M-A of two family-based and eight population-based studies	22,444
Australia-Adult	1	Australia	GWA	Family based study	9,148
Australia-Adolescent	1	Australia	GWA	Family based study	2,544
Estonia	1	Estonia	GWA	Population-based study	893
KORA	1	Germany	GWA	Population-based study	1,809
Milano	1	Italy	GWA	Population-based study	1,659
Nijmegen	1	The Netherlands	GWA	Population-based study	1,791
MICROS	1	Italy	GWA	Population-based study	1,218
ERF/Rotterdam	1	The Netherlands	GWA	Population-based study	871
KORA F3	1	Germany	GWA	Population-based study	1,634
BHS-WA	1	Australia	GWA	Population-based study	877

[1] The original sample size was 22,444, but genotype and phenotype data were available only for 21,567 (see Table S3).
[2] Personal communication B. Benyamin.

Supplementary Table 2. Characteristics and sample size of the individual studies included for the gene–PD association.

Data source	N. studies	Country	Type of study	Study design	Sample size	Covariates
PDGene database	9		candidate gene studies	M-A	2,384 cases; 6,908 controls	no covariates
Greco V et al. 2011 [12]		Italy	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	181 cases; 180 controls	no covariates
Halling J et al. 2008 [13]		Faroe Islands	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	79 cases; 154 controls	no covariates
Guerreiro RJ et al. 2006 [14]		Portugal	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	132 cases; 115 controls	no covariates
Dekker MC et al. 2003 [15]		The Netherlands	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	197 cases; 2,914 controls	no covariates
Borie C et al. 2002 [16]		France	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	216 cases; 193 controls	no covariates
Aamodt AH et al. 2007 [17]		Norway	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	388 cases; 505 controls	no covariates
Biasiotto G et al. 2008 [18]		Italy	candidate gene study (*HFE* rs1800562 and *HFE* rs1799945)	case / control	475 cases; 2,100 controls	no covariates
Buchanan DD et al. 2002 [19]		Australia	candidate gene study (*HFE* rs1800562)	case / control	438 cases; 485 controls	no covariates
Akbas N et al. 2006 [20]		Germany	candidate gene study (*HFE* rs1799945)	case / control	278 cases; 262 controls	no covariates
PD GWAS Consortium [6]	5		GWA	M-A	4,238 cases; 4,239 controls	sex, (age), PC
PROGENI/GenePD [3]		USA, Germany, Italy, UK, Canada, Australia	GWA	case / control	840 cases; 862 controls	sex, age, PC
NIA Phase I [1]		USA	GWA	case / control	245 cases; 256 controls	sex, PC
NIA Phase II [2]		USA	GWA	case / control	618 cases; 520 controls	sex, age, PC
HIHG [4]		USA	GWA	case / control	579 cases; 619 controls	sex, PC
NGRC [5]		USA	GWA	case / control	1,956 cases; 1,982 controls	sex, PC
23andMe[1] [9]	1	Europe, USA	GWA	case / control	4,127 cases; 62,037 controls	sex, age, five PC
IPDGC[2] [10,11]	9		GWA / Immunochip genotyping	M-A	10,060 cases; 15,708 controls	two PC
United Kingdom (stage I)		United Kingdom	GWA	case / control	1,705 cases; 5,200 controls	two PC
German (stage I)		Germany	GWA	case / control	742 cases; 944 controls	two PC
French (stage I)		France	GWA	case / control	1,039 cases; 1,984 controls	two PC
Dutch (stage II)		The Netherlands	GWA	case / control	772 cases; 2,024 controls	two PC
USA (stage II)		USA	Immunochip genotyping	case / control	2,807 cases; 2,215 controls	two PC
United Kingdom (stage II)		United Kingdom	Immunochip genotyping	case / control	1,271 cases; 1,864 controls	two PC
Dutch (stage II)		The Netherlands	Immunochip genotyping	case / control	304 cases; 402 controls	two PC
French (stage II)		France	Immunochip genotyping	case / control	267 cases; 363 controls	two PC
German (stage II)		Germany	Immunochip genotyping	case / control	1,153 cases; 712 controls	two PC

PC: principal components
1 23andMe: slightly expanded version of the cohort used in [9].
2 IPDGC (International Parkinson's Disease Genomics Consortium): USA-NIA and USA-dbGAP studies were not included in our analysis due to overlap with PD GWAS Consortium; the Icelandic dataset was not available for analysis.

Supplementary Figure S3. Gene–iron association: GIS-consortium meta-analysis. The effect size for the genetic effects on iron levels is expressed as number of SDs from the mean (Z-scores).

SNP	Chr.	Gene	Ref. allele / other	Frequency ref. allele	Tot. sample size	Beta (95%CI)	p-value	% Var.
rs1800562	6	*HFE*	A / G	0.02	21,567	0.37 (0.33-0.41)	$3.96x10^{-77}$	1.74
rs1799945	6	*HFE*	G / C	0.08	21,567	0.19 (0.17-0.21)	$1.65x10^{-42}$	0.92
rs855791	22	*TMPRSS6*	G / A	0.6	21,567	0.19 (0.17-0.21)	$4.31x10^{-77}$	1.72

Chr., chromosome; SE, standard error; ref. allele, reference allele.
% Var., percentage variance explained.
Frequency ref. allele from 1000 Genomes project.

Supplementary Table S4. Gene–PD association: meta-analysis of all available candidate gene and GWA studies

SNP	Chr.	Gene	Ref. allele / other	Frequency ref. allele	Tot. cases/ tot. controls	OR (95%CI)	p-value
rs1800562	6	*HFE*	A / G	0.02	20,531 / 88,630	0.97 (0.92-1.02)	0.281
rs1799945	6	*HFE*	G / C	0.08	20,371 / 88,407	0.99 (0.96-1.03)	0.715
rs855791	22	*TMPRSS6*	G / A	0.6	18,425 / 81,984	0.97 (0.94-1.00)	0.034

Chr., chromosome; SE, standard error; ref. allele, reference allele.
Frequency ref. allele from 1000 Genomes project.

Supplementary Text S1. Detailed description of the studies included in the three GWA investigations of PD risk.

PD GWAS Consortium

For this dataset (4,238 cases and 4,239 controls), two publicly available and three additional GWA studies were meta-analyzed. All studies employed standard UK Brain Bank criteria for the diagnosis of PD, with a modification to allow cases with a family history of PD to be included. PD cases with a reported age of onset below 18 years of age were removed (n=17). When data were available, any PD cases known to carry a causative mutation, either two *Parkin* mutations or a single *LRRK2* mutation, were excluded from the analysis (n=57).

PROGENI/GenePD

PD cases were selected from the PROGENI and GenePD studies of familial PD. Both studies ascertained multiplex PD families consisting of at least a sibling pair, both of whom were reported to be affected with PD. Control samples were obtained from the NINDS Human Genetics Resource Center at the Coriell Institute, Coriell Cell Repositories (Camden, NJ).

NIA Phase I

PD samples were derived from the NINDS Neurogenetics repository hosted by the Coriell Institute for Medical research (NJ, USA). For the PD cohort, blood was obtained from unique and unrelated white individuals with idiopathic PD. Both those with and without a reported family history of PD were included. For the control population, blood samples were drawn from neurologically normal, unrelated, white individuals at many different sites within the USA.

NIA Phase II

PD patients were derived from the NINDS Neurogenetics Repository at the Coriell Institute for Medical research (NJ, USA). In addition, 75 PD cases collected by a movement disorders specialist in the Laboratory of Neurogenetics were included. All patients were Caucasian individuals with idiopathic PD from the USA.

MIHG

Samples in the MIHG GWAS include individuals with PD collected by one of 13 ascertainment centers in the PD Genetics Collaboration or by the Morris K. Udall Parkinson Disease Center of Excellence ascertainment core. These participants were recruited by participating movement disorder and neurology clinics, referrals, and advertisements. Unaffected spouse and friend controls were recruited when available and willing to participate.

NGRC

PD patients and control subjects were recruited from eight NGRC-affiliated neurology clinics in Oregon, Washington, Georgia and New York. Controls were community volunteers and patient spouses.

23andMe

PD patients of the 23andMe study were recruited through a targeted email campaign together with the Michael J. Fox Foundation, the Parkinson's Institute and Clinical Center, and many other PD patient groups and clinics. Patients who stated in an online screening questionnaire that they had been diagnosed with PD were offered the 23andMe Personal Genome Service. Controls were drawn from the customer database of the 23andMe company. Individuals included in the PD GWA study were selected for being of primarily European ancestry, and overlapping samples with publically available PD studies from dbGAP were removed. The dataset available for our study included 4,127 cases and 62,037 controls from this growing study.

International Parkinson's Disease Genomics Consortium, IPDGC

From IPDGC, we included four GWA studies (United Kingdom, German, French, and Dutch datasets) with a total of 4,258 cases and 10,152 controls in our meta-analysis. In addition, five studies genotyped with a custom genotyping array (Immunochip Illumina iSelect array) with genotypes for all three genetic variants were available for our study. These studies consisted of a total of 5,802 cases and 5,556 control samples from USA, United Kingdom, Netherlands, Germany, and France. Details of the studies included are reported below and summarized in Table S2.

United Kingdom (UK) dataset

For the UK dataset sample recruitment mostly targeted sporadic cases without familial history of PD. Half of the case collection was tested for the highly penetrant G0219S variant in the *LRRK2* gene and carriers were excluded from the GWA scan. The control set is a shared resource of UK samples (1958 British Birth Cohort and blood donors recruited by the National Blood Services) genotyped by the Wellcome Trust Case Control Consortium.

German dataset

The German dataset was collected by movement disorder specialists of the Universities of Munich and Tübingen in Southern Germany. The control dataset was derived from the population based studies KORA and Popgen.

French dataset

The patients for the French dataset were recruited through the French network for the study of Parkinson's disease Genetics (GPD). The patients were enriched for cases with a positive family history of PD. The controls were derived from the French Three-City (3C) cohort, a population-based, prospective study of relationship between vascular factors and dementia.

Dutch dataset

The PD patients were recruited from four different centers within the Netherlands (Scales for Outcomes in Parkinson's disease, SCOPA, http://www.scopa-propark.eu; the Academic Medical Center Amsterdam, AMC, http://www.amc.uva.nl; the Parkinson Centrum Nijmegen, ParC, http://www.umcn.nl; and the VU University medical centre, VUmc, http://www.vumc.nl). Genotyping data from control participants from the Rotterdam study III (ERGO Young) were used as control population.

Studies genotyped with Immunochip

The US dataset consisted of samples collected in the Parkinson's, Genes and Environment (PAGE) and PostCept Studies, as well as additional samples from the Washington University of Saint Louis and the Coriell Repository. The UK dataset consisted of samples contributed by the University College London, Cardiff University and Wellcome Trust population control samples. In addition, Dutch, German, and French case-control samples were available.

REFERENCES

1. Fung HC, Scholz S, Matarin M, Simon-Sanchez J, Hernandez D, et al. (2006) Genome-wide genotyping in Parkinson's disease and neurologically normal controls: First stage analysis and public release of data. Lancet Neurology 5: 911-916.

2. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, et al. (2009) Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nature Genetics 41: 1308-1312.

3. Pankratz N, Wilk JB, Latourelle JC, DeStefano AL, Halter C, et al. (2009) Genomewide association study for susceptibility genes contributing to familial Parkinson disease. Human Genetics 124: 593-605.

4. Edwards TL, Scott WK, Almonte C, Burt A, Powell EH, et al. (2010) Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Annals of Human Genetics 74: 97-109.

5. Hamza TH, Zabetian CP, Tenesa A, Laederach A, Montimurro J, et al. (2010) Common genetic variation in the HLA region is associated with late-onset sporadic Parkinson's disease. Nature Genetics 42: 781-785.

6. Pankratz N, Beecham GW, DeStefano AL, Dawson TM, Doheny KF, et al. (2012) Meta-analysis of Parkinson's disease: Identification of a novel locus, RIT2. Annals of Neurology 71: 370-384.

7. Hughes AJ, Daniel SE, Kilford L, Lees AJ. (1992) Accuracy of clinical diagnosis of idiopathic parkinson's disease: A clinico-pathological study of 100 cases. Journal of Neurology, Neurosurgery, and Psychiatry 55: 181-184.

8. Scott WK, Nance MA, Watts RL, Hubble JP, Koller WC, et al. (2001) Complete genomic screen in Parkinson disease: Evidence for multiple genes. JAMA: The Journal of the American Medical Association 286: 2239-2244.

9. Do CB, Tung JY, Dorfman E, Kiefer AK, Drabant EM, et al. (2011) Web-based genome-wide association study identifies two novel loci and a substantial genetic component for Parkinson's disease. PLoS Genetics 7: e1002141.

10. International Parkinson Disease Genomics Consortium, Nalls MA, Plagnol V, Hernandez DG, Sharma M, et al. (2011) Imputation of sequence variants for identification of genetic risks for Parkinson's disease: A meta-analysis of genome-wide association studies. Lancet 377: 641-649.
11. International Parkinson's Disease Genomics Consortium (IPDGC), Wellcome Trust Case Control Consortium 2 (WTCCC2). (2011) A two-stage meta-analysis identifies several new loci for Parkinson's disease. PLoS Genetics 7: e1002142.
12. Greco V, De Marco EV, Rocca FE, Annesi F, Civitelli D, et al. (2011) Association study between four polymorphisms in the HFE, TF and TFR genes and Parkinson's disease in southern Italy. Neurological Sciences : Official Journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology 32: 525-527.
13. Halling J, Petersen MS, Grandjean P, Weihe P, Brosen K. (2008) Genetic predisposition to parkinson's disease: CYP2D6 and HFE in the Faroe Islands. Pharmacogenetics and Genomics 18: 209-212.
14. Guerreiro RJ, Bras JM, Santana I, Januario C, Santiago B, et al. (2006) Association of HFE common mutations with Parkinson's disease, Alzheimer's disease and mild cognitive impairment in a portuguese cohort. BMC Neurology 6: 24.
15. Dekker MC, Giesbergen PC, Njajou OT, van Swieten JC, Hofman A, et al. (2003) Mutations in the hemochromatosis gene (HFE), Parkinson's disease and parkinsonism. Neuroscience Letters 348: 117-119.
16. Borie C, Gasparini F, Verpillat P, Bonnet AM, Agid Y, et al. (2002) Association study between iron-related genes polymorphisms and Parkinson's disease. Journal of Neurology 249: 801-804.
17. Aamodt AH, Stovner LJ, Thorstensen K, Lydersen S, White LR, et al. (2007) Prevalence of haemochromatosis gene mutations in Parkinson's disease. Journal of Neurology, Neurosurgery, and Psychiatry 78: 315-317.
18. Biasiotto G, Goldwurm S, Finazzi D, Tunesi S, Zecchinelli A, et al. (2008) HFE gene mutations in a population of Italian Parkinson's disease patients. Parkinsonism & Related Disorders 14: 426-430.
19. Buchanan DD, Silburn PA, Chalk JB, Le Couteur DG, Mellick GD. (2002) The Cys282Tyr polymorphism in the HFE gene in Australian Parkinson's disease patients. Neuroscience Letters 327: 91-94.
20. Akbas N, Hochstrasser H, Deplazes J, Tomiuk J, Bauer P, et al. (2006) Screening for mutations of the HFE gene in Parkinson's disease patients with hyperechogenicity of the substantia nigra. Neuroscience Letters 407: 16-19.

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A mio papà.

Training and international meetings

INTERNATIONAL INTERNSHIP

Project:'ESGI: Quality check of next-generation sequencing data on a subset of Val Borbera individuals'
Welcome Trust Sanger Institute Hinxton,UK – June 2012-July 2012

COURSES

HarvardX: 'Data Analysis for Genomics'
Verified certificate online course edX June 2014

PARTECIPATION IN INTERNATIONAL MEETINGS

M. Traglia, M. Cocca, J. Huang,Y. Memari, C. Sala, C. Masciullo, C. Barbieri, P. D'Adamo, P. Gasparini, N. Soranzo, D. Toniolo
Several loci enriched in lower frequency variants are associated to risk factor for metabolism and cardiovascular diseases in 4,000 Italian isolated individuals
ESHG - European Society of Human Genetics conference 2014
Milan– May 2014 - poster

C. Barbieri, **M. Traglia**, V. Vanni, T. Nutile, S. Ulivi, L. Portas, C. Masciullo, C. Sala, M. Cocca, M. Ciullo, P. Gasparini, M. Pirastu, A. La Marca, P. Panina, E. Papaleo, D. Toniolo Anti Müllerian Hormone (AMH) association analysis on about 1,000 caucasian women highlights 2 novel suggestive loci for fertility
ESHG - European Society of Human Genetics conference 2014
Milan– May 2014 - poster

J. O'Connell, O. Delaneau, N. Pirastu, S. Ulivi, M. Cocca, **M. Traglia**, J. Huang, J. E. Huffman, I. Rudan, R. McQuillan, R. M. Fraser, H. Campbell, O. Polasek, C. Hayward, A. F. Wright, V. Vitart, P. Navarro, J. F. Zagury, J. F. Wilson, D. Toniolo, P. Gasparani, N. Soranzo, J. Marchini
Haplotype phasing across the full spectrum of relatedness.
ASHG – American Society of Human Genetics conference 2013
Boston – October 2013 – selected for oral presentation

M. Cocca, **M. Traglia**, G. Girotto, C. Sala, Y. Memari, K. Walter, C. Masciullo, C. Barbieri, P. D'Adamo, P. Gasparini, N. Soranzo, D. Toniolo,
Next-generation sequencing approach allowed to draw a whole-genome reference panel enriched in Italian lower-frequency variants.
ESHG - European Society of Human Genetics conference 2013
Paris– May 2013 - poster

M. Traglia, M. Cocca, C. Sala, C. Masciullo , C. Barbieri , Y. Memari , N. Soranzo , D. Toniolo
Whole-genome sequencing of Val Borbera cohort highlights associated rare variants with several complex traits.
ESHG - European Society of Human Genetics conference 2013
Paris – May 2013 - poster

M. Traglia, D. Girelli, G. Biino, N. Campostrini, M. Corbella, C. Sala, C. Masciullo, F. Viganò, I. Buetti, G. Pistis, M. Cocca, C. Camaschella, D. Toniolo
Serum Hepcidin levels and association studies of TMPRSS6 and HFE variants provide further insights into regulation of iron homeostasis.
ESHG - European Society of Human Genetics conference 2011
Amsterdam – May 2011 - poster

A. Nai, A. Pagani, L. Silvestri, N. Campostrini, D. Girelli, **M. Traglia**, D Toniolo, C Camaschella
The TMPRSS6 rs855791 common variant modulates hepcidin promoter in vitro and is associated with low serum hepcidin/ferritin ratio
4th Meeting of the international BioIron Society 2011
Vancouver – May 2011 – selected for oral presentation

G. Pistis, C. Sala, **M. Traglia**, T. Corre, C. Masciullo, I. Buetti, M. Cocca, N. Pirastu, N. Soranzo, P. Gasparini, S. K. Ganesh and D. Toniolo
New loci associated with blood cell traits in the isolated population of Val Borbera
ESHG - European Society of Human Genetics conference 2010
Gothenburg - June 2010 - poster

M. Traglia, C. Sala, C. Masciullo, V. Cverhova, F. Lori, I. Buetti, G. Pistis, S. Bione, C. Camaschella, E. Petretto and D. Toniolo
Characterization of phenotypic traits in Val Borbera, a large genetic isolate in Northern Italy.
4th International Meeting on genetics of complex diseases and isolated populations
Trieste - June 2009 - poster

F. Lori, G. Pistis, C. Sala, C. Masciullo, **M. Traglia**, I. Buetti, O. Semino, A. Torroni, C. Nici, V. Battaglia, S. Fornarino, S. Bione, E. Petretto and D. Toniolo.
Descriptive analysis of Val Borbera population structure: mtDNA, Y chromosome polymorphism and linkage disequilibrium.
4th International Meeting on genetics of complex diseases and isolated populations
Trieste - June 2009 – selected for oral presentation

LIST OF PUBLICATIONS

2014

1. Choi SH, Ruggiero D, Sorice R, Song C, Nutile T, Vernon Smith A, Concas MP, **Traglia M**, Barbieri C et al (2014) *'Six novel loci associated with circulating VEGF levels identified by a meta-analysis of genome-wide association studies'* [in preparation]

2. Taylor PN*, UK10K core analysts* Porcu E*, Chew S*, Campbell PJ*, **Traglia M**, Brown SJ, Mullin BH, Min J, Walter K, Memari Y, Huang J, Beilby JP, Charoen P, Danecek P, Dudbridge F, Forgetta V, Greenwood C, Grundberg E, Johnson AD, Hui J, Lim EM, McCarthy S, Muddyman D, Panicker V, Perry J, Schlessinger D, Abecasis G, Cucca F , Sanna S, Surdulescu GL, Woltersdorf W, Zeggini E, Zheng H, Toniolo D, Dayan CM, Naitza S, Walsh JP, Spector TD, Davey-Smith G, Durbin R, Richards JB, Soranzo N, Timpson NJ*, Wilson SG*, and the UK10K Consortium (2014) *'Whole genome sequence based analysis of thyroid function*' [in preparation]

3. Benyamin B, Esko T, Ried JS, Radhakrishnan A, Vermeulen SH, **Traglia M**, Gögele M, Anderson D, Broer L, Podmore C, Luan J, Kutalik Z, Sanna S, van der Meer P, Tanaka T, Wang F, Westra H-J, Franke L, Mihailov E, Milani L, Häldin J, Winkelmann J, Meitinger T, Thiery J, Peters A, Waldenberger M, Rendon A, Jolley J, Sambrook J, Kiemeney LA, Sweep FC, Sala CF, Schwienbacher C, Pichler I, Hui J, Demirkan A, Isaacs A, Amin N, Steri M, Waeber G, Verweij N, Powell JE, Nyholt DR, Heath AC, Madden PAF, Visscher PM, Wright MJ, Montgomery GW, Martin NG, Hernandez D, Bandinelli S, van der Harst P, Uda M, Vollenweider P, Scott RA, Langenberg C, Wareham NJ, InterAct Consortium, van Duijn C, Beilby J, Pramstaller PP, Hicks AA, Ouwehand WH, Oexle K, Gieger C, Metspalu A, Camaschella C, Toniolo D, Swinkels DW, Whitfield JB (2014) '*Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis'* Nature Communication. 2014 Oct 29;5:4926 doi: 10.1038/ncomms5926

4. O'Connell J, Gurdasani D, Delaneau O, Pirastu N, Ulivi S, Cocca M, **Traglia M**, Huang J, Huffman JE, Rudan I, McQuillan R, Fraser RM, Campbell H, Polasek O, Asiki G, Ekoru K, Hayward C, Wright AF8, Vitart V8, Navarro P, Zagury JF, Wilson JF, Toniolo D, Gasparini P, Soranzo N, Sandhu MS, Marchini J (2014) *'A General Approach for Haplotype Phasing across the Full Spectrum of Relatedness*' PLoS Genet. 2014 Apr 17;10(4):e1004234. doi: 10.1371/journal.pgen.1004234. eCollection 2014

5. Olden M, Corre T, Hayward C, Toniolo D, Ulivi S, Gasparini P, Pistis G, Hwang SJ, Bergmann S, Campbell H, Cocca M, Gandin I, Girotto G, Glaudemans B, Hastie ND, Loffing J, Polasek O, Rampoldi L, Rudan I, Sala C, **Traglia M**, Vollenweider P, Vuckovic D, Youhanna S, Weber J, Wright AF, Kutalik Z, Bochud M, Fox CS, Devuyst O. (2014) *'Common Variants in UMOD Associate with Urinary Uromodulin Levels: A Meta-Analysis*'. J Am Soc Nephrol. 2014 Mar 27. doi: 10.1681/ASN.2013070781 [Epub ahead of print]

6. Pirastu N, Kooyman M, **Traglia M**, Robino A, Willems SM, Pistis G, d'Adamo P, Amin N, d'Eustacchio A, Navarini L, Sala C, Karssen LC, van Duijn C, Toniolo D, Gasparini P (2014) *'Association Analysis of Bitter Receptor Genes in Five Isolated Populations Identifies a Significant Correlation between TAS2R43 Variants and Coffee Liking*' PLoS One. 2014 Mar 19;9(3):e92065. doi: 10.1371/journal.pone.0092065. eCollection 2014

7. Medici M, Porcu E, Pistis G, Teumer A, Brown SJ, Jensen RA, Rawal R, Roef GL, Plantinga TS, Vermeulen SH, Lahti J, Simmonds MJ, Husemoen LL, Freathy RM, Shields BM, Pietzner D, Nagy R, Broer L, Chaker L, Korevaar TI, Plia MG, Sala C, Völker U, Richards JB, Sweep FC, Gieger C, Corre T, Kajantie E, Thuesen B, Taes YE, Visser WE, Hattersley AT, Kratzsch J, Hamilton A, Li W, Homuth G, Lobina M, Mariotti S, Soranzo N, Cocca M, Nauck M, Spielhagen C, Ross A, Arnold A, van de Bunt M, Liyanarachchi S, Heier M, Grabe HJ, Masciullo C, Galesloot TE, Lim EM, Reischl E, Leedman PJ, Lai S, Delitala A, Bremner AP, Philips DI, Beilby JP, Mulas A, Vocale M, Abecasis G, Forsen T, James A, Widen E, Hui J, Prokisch H, Rietzschel EE, Palotie A,

Feddema P, Fletcher SJ, Schramm K, Rotter JI, Kluttig A, Radke D, **Traglia M**, Surdulescu GL, He H, Franklyn JA, Tiller D, Vaidya B, de Meyer T, Jørgensen T, Eriksson JG, O'Leary PC, Wichmann E, Hermus AR, Psaty BM, Ittermann T, Hofman A, Bosi E, Schlessinger D, Wallaschofski H, Pirastu N, Aulchenko YS, de la Chapelle A, Netea-Maier RT, Gough SC, Meyer Zu Schwabedissen H, Frayling TM, Kaufman JM, Linneberg A, Räikkönen K, Smit JW, Kiemeney LA, Rivadeneira F, Uitterlinden AG, Walsh JP, Meisinger C, den Heijer M, Visser TJ, Spector TD, Wilson SG, Völzke H, Cappola A, Toniolo D, Sanna S, Naitza S, Peeters RP. (2014) *'Identification of novel genetic Loci associated with thyroid peroxidase antibodies and clinical thyroid disease*' PLoS Genet. 2014 Feb 27;10(2):e1004123. doi: 10.1371/journal.pgen.1004123. eCollection 2014

2013

8. Pistis G, Okonkwo SU, **Traglia M**, Sala C, Shin SY, Masciullo C, Buetti I, Massacane R, Mangino M, Thein SL, Spector TD, Ganesh S, Pirastu N, Gasparini P, Soranzo N, Camaschella C, Hart D, Green MR, Toniolo D. (2013) *'Genome Wide Association Analysis of a Founder Population Identified TAF3 as a Gene for MCHC in Humans*.' PLoS One. 2013 Jul 31;8(7):e69206. doi: 10.1371/journal.pone.0069206. Print 2013

9. Pichler I, Del Greco M F, Gögele M, Lill CM, Bertram L, Do CB, Eriksson N, Foroud T, Myers RH; PD GWAS Consortium, Nalls M, Keller MF; International Parkinson's Disease Genomics Consortium; Wellcome Trust Case Control Consortium 2, Benyamin B, Whitfield JB; Genetics of Iron Status Consortium, Pramstaller PP, Hicks AA, Thompson JR, Minelli C. (2013) *'Serum iron levels and the risk of Parkinson disease: a mendelian randomization study*.' PLoS Med. 2013;10(6):e1001462. doi: 10.1371/journal.pmed.1001462. Epub 2013 Jun 4

10. Paul DS, Albers CA, Rendon A, Voss K, Stephens J; HaemGen Consortium, van der Harst P, Chambers JC, Soranzo N, Ouwehand WH, Deloukas P., HaemGen consortium (2013) '*Maps of open chromatin highlight cell type-restricted patterns of regulatory sequence variation at hematological trait loci'* Genome Res. 2013 Jul;23(7):1130-41. doi: 10.1101/gr.155127.113. Epub 2013 Apr 9

11. Pelusi S, Girelli D, Rametta R, Campostrini N, Alfieri C, **Traglia M**, Dongiovanni P, Como G, Toniolo D, Camaschella C, Messa P, Fargion S, Valenti L (2013) '*The A736V TMPRSS6 polymorphism influences hepcidin and iron metabolism in chronic hemodialysis patients: TMPRSS6 and hepcidin in hemodialysis*.' BMC Nephrol. 2013 Feb 22;14:48. doi: 10.1186/1471-2369-14-48

12. Biino G, Santimone I, Minelli C, Sorice R, Frongia B, **Traglia M**, Ulivi S, Di Castelnuovo A, Gögele M, Nutile T, Francavilla M, Sala C, Pirastu N, Cerletti C, Iacoviello L, Gasparini P, Toniolo D, Ciullo M, Pramstaller P, Pirastu M, di Gaetano G, L Balduini CL (2013) '*Age- and sex- related variations in platelet count in Italy: a proposal of reference ranges based on 40987 subject's data*' PLoS One. 2013;8(1):e54289. doi: 10.1371/journal.pone.0054289. Epub 2013 Jan 31

13. Martinelli N, **Traglia M**, Campostrini N, Biino G, Corbella M, Sala C, Busti F, Masciullo C, Manna D, Previtali S, Castagna A, Pistis G, Olivieri O, Toniolo D, Camaschella C, Girelli D. (2013) '*Increased Serum Hepcidin Levels in Subjects with the Metabolic Syndrome: A Population Study*.' PLoS One. 2013 Jun 26;8(6). doi: 10.1371/annotation/233a5ac3-8118-4bdc-a139-950223506864. Print 2013

14. Perry JR, Corre T, Esko T, Chasman DI, Fischer K, Franceschini N, He C, Kutalik Z, Mangino M, Rose LM, Vernon Smith A, Stolk L, Sulem P, Weedon MN, Zhuang WV, Arnold A, Ashworth A, Bergmann S, Buring JE, Burri A, Chen C, Cornelis MC, Couper DJ, Goodarzi MO, Gudnason V, Harris T, Hofman A, Jones M, Kraft P, Launer L, Laven JS, Li G, McKnight B, Masciullo C, Milani L, Orr N, Psaty BM; ReproGen Consortium, Ridker PM, Rivadeneira F, Sala C, Salumets A, Schoemaker M, **Traglia M**, Waeber G, Chanock SJ, Demerath EW, Garcia M, Hankinson SE, Hu FB, Hunter DJ, Lunetta KL, Metspalu A, Montgomery GW, Murabito JM, Newman AB, Ong KK, Spector TD, Stefansson K, Swerdlow AJ, Thorsteinsdottir U, Van Dam RM, Uitterlinden AG, Visser JA, Vollenweider P, Toniolo D, Murray A. (2013) '*A genome-wide association study of early menopause and the combined impact of identified variant's*. Hum Mol Genet. 2013 Apr 1;22(7):1465-72. doi: 10.1093/hmg/dds551. Epub 2013 Jan 9

2012

15. Esko T, Mezzavilla M, Nelis M, Borel C, Debniak T, Jakkula E, Julia A, Karachanak S, Khrunin A, Kisfali P, Krulisova V, Aušrelé Kučinskiené Z, Rehnström K, **Traglia M**, Nikitina-Zake L, Zimprich F, Antonarakis SE, Estivill X, Glavač D, Gut I, Klovins J, Krawczak M, Kučinskas V, Lathrop M, Macek M, Marsal S, Meitinger T, Melegh B, Limborska S, Lubinski J, Paolotie A, Schreiber S, Toncheva D, Toniolo D, Wichmann HE, Zimprich A, Metspalu M, Gasparini P, Metspalu A, D'Adamo P (2012) *'Genetic characterization of northeastern Italian population isolates in the context of broader European genetic diversity*' Eur J Hum Genet. 2013 Jun;21(6):659-65. doi: 10.1038/ejhg.2012.229. Epub 2012 Dec 19

16. Campostrini N , **Traglia M** , Martinelli N, Corbella M , Cocca M, Manna D, Castagna A, Masciullo C, Silvestri L, Olivieri O, Toniolo , Camaschella C, Girelli D:(2012) '*Serum levels of the hepcidin-20 isoform in a large general population: The Val Borbera study*' J Proteomics. 2012 Aug 21. doi: 10.1016/j.jprot.2012.08.006

17. Boraska V, Jerončić A, Colonna V, Southam L, Nyholt DR, Rayner NW, Perry JR, Toniolo D, Albrecht E, Ang W, Bandinelli S, Barbalic M, Barroso I, Beckmann JS, Biffar R, Boomsma D, Campbell H, Corre T, Erdmann J, Esko T, Fischer K, Franceschini N, Frayling TM, Girotto G, Gonzalez JR, Harris TB, Heath AC, Heid IM, Hoffmann W, Hofman A, Horikoshi M, Zhao JH, Jackson AU, Hottenga JJ, Jula A, Kähönen M, Khaw KT, Kiemeney LA, Klopp N, Kutalik Z, Lagou V, Launer LJ, Lehtimäki T, Lemire M, Lokki ML, Loley C, Luan J, Mangino M, Mateo Leach I, Medland SE, Mihailov E, Montgomery GW, Navis G, Newnham J, Nieminen MS, Palotie A, Panoutsopoulou K, Peters A, Pirastu N, Polasek O, Rehnström K, Ripatti S, Ritchie GR, Rivadeneira F, Robino A, Samani NJ, Shin SY, Sinisalo J, Smit JH, Soranzo N, Stolk L, Swinkels DW, Tanaka T, Teumer A, Tönjes A, **Traglia M**, Tuomilehto J, Valsesia A, van Gilst WH, van Meurs JB, Smith AV, Viikari J, Vink JM, Waeber G, Warrington NM, Widen E, Willemsen G, Wright AF, Zanke BW, Zgaga L; Wellcome Trust Case Control Consortium, Boehnke M, d'Adamo AP, de Geus E, Demerath EW, den Heijer M, Eriksson JG, Ferrucci L, Gieger C, Gudnason V, Hayward C, Hengstenberg C, Hudson TJ, Järvelin MR, Kogevinas M, Loos RJ, Martin NG, Metspalu A, Pennell CE, Penninx BW, Perola M, Raitakari O, Salomaa V, Schreiber S, Schunkert H, Spector TD, Stumvoll M, Uitterlinden AG, Ulivi S, van der Harst P, Vollenweider P, Völzke H, Wareham NJ, Wichmann HE, Wilson JF, Rudan I, Xue Y, Zeggini E.(2012) '*Genome-wide Meta-analysis of common variant differences between men and women*' Hum. Mol. Genet. first published online July 27, 2012 doi:10.1093/hmg/dds304

18. van der Harst P, Zhang W, Mateo Leach I, Rendon A, Verweij N, Sehmi J, Paul DS, Elling U, Allayee H, Li X, Radhakrishnan A, Tan ST, Voss K, Weichenberger CX, Albers CA, Al-Hussani A, Asselbergs FW, Ciullo M, Danjou F, Dina C, Esko T, Evans DM, Franke L, Gögele M, Hartiala J, Hersch M, Holm H, Hottenga JJ, Kanoni S, Kleber ME, Lagou V, Langenberg C, Lopez LM, Lyytikäinen LP, Melander O, Murgia F, Nolte IM, O'Reilly PF, Padmanabhan S, Parsa A, Pirastu N, Porcu E, Portas L, Prokopenko I, Ried JS, Shin SY, Tang CS, Teumer A, **Traglia M**, Ulivi S, Westra HJ, Yang J, Zhao JH, Anni F, Abdellaoui A, Attwood A, Balkau B, Bandinelli S, Bastardot F, Benyamin B, Boehm BO, Cookson WO, Das D, de Bakker PI, de Boer RA, de Geus EJ, de Moor MH, Dimitriou M, Domingues FS, Döring A, Engström G, Eyjolfsson GI, Ferrucci L, Fischer K, Galanello R, Garner SF, Genser B, Gibson QD, Girotto G, Gudbjartsson DF, Harris SE, Hartikainen AL, Hastie CE, Hedblad B, Illig T, Jolley J, Kähönen M, Kema IP, Kemp JP, Liang L, Lloyd-Jones H, Loos RJ, Meacham S, Medland SE, Meisinger C, Memari Y, Mihailov E, Miller K, Moffatt MF, Nauck M, Novatchkova M, Nutile T, Olafsson I, Onundarson PT, Parracciani D, Penninx BW, Perseu L, Piga A, Pistis G, Pouta A, Puc U, Raitakari O, Ring SM, Robino A, Ruggiero D, Ruokonen A, Saint-Pierre A, Sala C, Salumets A, Sambrook J, Schepers H, Schmidt CO, Silljé HH, Sladek R, Smit JH, Starr JM, Stephens J, Sulem P, Tanaka T, Thorsteinsdottir U, Tragante V, van Gilst WH, van Pelt LJ, van Veldhuisen DJ, Völker U, Whitfield JB, Willemsen G, Winkelmann BR, Wirnsberger G, Algra A, Cucca F, d'Adamo AP, Danesh J, Deary IJ, Dominiczak AF, Elliott P, Fortina P, Froguel P, Gasparini P, Greinacher A, Hazen SL, Jarvelin MR, Khaw KT, Lehtimäki T, Maerz W, Martin NG, Metspalu A, Mitchell BD, Montgomery GW, Moore C, Navis G, Pirastu M, Pramstaller PP, Ramirez-Solis R, Schadt E, Scott J, Shuldiner AR, Smith GD, Smith JG, Snieder H, Sorice R, Spector TD, Stefansson K, Stumvoll M, Tang WH, Toniolo D, Tönjes A, Visscher PM, Vollenweider P, Wareham NJ, Wolffenbuttel BH, Boomsma DI, Beckmann JS, Dedoussis GV, Deloukas P, Ferreira MA, Sanna S, Uda M, Hicks AA, Penninger JM, Gieger C, Kooner JS, Ouwehand WH, Soranzo N, Chambers JC (2012) '*Seventy-five genetic loci influencing the human*

red blood cell' Nature. 2012 Dec 20;492(7429):369-75. doi: 10.1038/nature11677. Epub 2012 Dec 5

2011

19. Nai A, Pagani A, Silvestri L, Campostrini N, Girelli D, **Traglia M**, Toniolo D, Camaschella C (2011) '*TMPRSS6 rs855791 modulates hepcidin transcription in vitro and levels of serum hepcidin according to iron status in normal individuals*'. Blood 2011 doi:10.1182/blood-2011-06-364034

20. **Traglia M**, Girelli D, Biino G, Campostrini N, Corbella M, Sala C, Masciullo C, Viganò F, Buetti I, Pistis G, Cocca M, Camaschella C, Toniolo D (2011) '*The association of HFE and TMPRSS6 genetic variants to iron and erythrocyte parameters is only in part dependent from serum hepcidin' levels*. J Med Genet 2011;48:629e634. doi:10.1136/jmedgenet-2011-100061

21. Masciullo C, Milani G, Sala C, Bellazzi R, Buetti I, Pistis,G **Traglia M**, Toniolo D and Larizza C (2011) *'Computer-based genealogy reconstruction in founder populations*' J Biomed Inform, doi:10.1016/j.jbi.2011.08.004

2010

22. La Marca A, Sighinolfi G, **Traglia M**, Argento C, Sala C, Masciullo C, Volpe A,Toniolo D (2009) '*Normal serum levels of anti-Mullerian hormone in women with regular menstrual cycles'*. Reprod Biomed Online. 2010 Oct;21(4):463-9

2009

23. **Traglia M**, Sala C, Masciullo C, Cverhova V, Lori F, Pistis G,Bione S, Gasperini P, Ulivi S, Ciullo M, Nutile T, Bosi E, Sirtori M, Mignogna G, Rubinacci A, Buetti I, Camaschella C, Petretto E, Toniolo D. (2009) '*Heritability and Demographic Analyses in the Large Isolated Population of Val Borbera Suggest Advantages in Mapping Complex Traits Genes*'. PLoS ONE 4(10): e7554. doi:10.1371/journal.pone.0007554.