TESSEL GALESLOOT

Hepcidin: population-based studies into genetic determinants and effects on atherosclerosis

Stellingen

behorende bij het proefschrift

Hepcidin: population-based studies into genetic determinants and effects on atherosclerosis

Van Tessel Galesloot

1 In onderzoek naar de rol van hepcidine in gezondheid en ziekte dient ook de rol van de ratio hepcidine/ferritine te worden geëvalueerd. [dit proefschrift]

2 In de algemene Kaukasische populatie komen geen single nucleotide polymorfismen voor die meer dan 1% van de variatie in de concentratie van serum hepcidine verklaren. [dit proefschrift]

3 De verdeling van ijzer over het lichaam en niet de absolute hoeveelheid ijzer in het lichaam lijkt een causale rol te spelen in de ontwikkeling van atherosclerose. [dit proefschrift]

4 Multivariate genoomwijde associatieanalyse moet standaard worden toegepast bij genoomwijde associatieanalyse van meerdere (gecorreleerde) traits om de power voor het identificeren van genetische determinanten te vergroten. [dit proefschrift]

5 De resultaten van humane populatiestudies dienen in sterkere mate geïntegreerd te worden in het design en de interpretatie van resultaten van cel- en dierstudies.

6 Succes in de wetenschap is in te grote mate afhankelijk van de uitkomsten van wetenschappelijk onderzoek.

7 Om meer grip te krijgen op negatieve bevindingen zouden alle wetenschappelijke studies, en dus niet alleen trials, bij aanvang standaard geregistreerd moeten worden.

8 De uitspraak 'de ziel gaat te paard' (Harry Mulisch) is wetenschappelijk gezien niet verdedigbaar, maar als metafoor een waarheid als een koe.

9 Life is like riding a bicycle. To keep your balance you must keep moving. [Albert Einstein]

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The work presented in this thesis was carried out within the Radboud Institute for Health Sciences.

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CHAPTER 1

General introduction

Hepcidin

In the beginning of the 21st century, a 25-amino acid peptide was discovered and named **hepcidin**, because of its high expression in the liver and antimicrobial activity [1,2]. Hepcidin, encoded by the hepcidin antimicrobial peptide gene (*HAMP*), is predominantly produced by hepatocytes, but expression at much lower levels has also been demonstrated for other cell types, *e.g.* cardiomyocytes and kidney tubule [3,4]. Serum hepcidin circulates in the bloodstream, partly bound to α2-macroglobulin. Reports are inconsistent about the exact percentage of hepcidin that is freely circulating [5,6]. Besides the 25-amino acid peptide, three smaller hepcidin isoforms of 24, 22 and 20 amino acids exist, but these are only present in serum in patients with diseases that are associated with increased hepcidin-25 concentrations [7,8]. Hepcidin has been reported to leave the body via excretion by the kidneys [9,10].

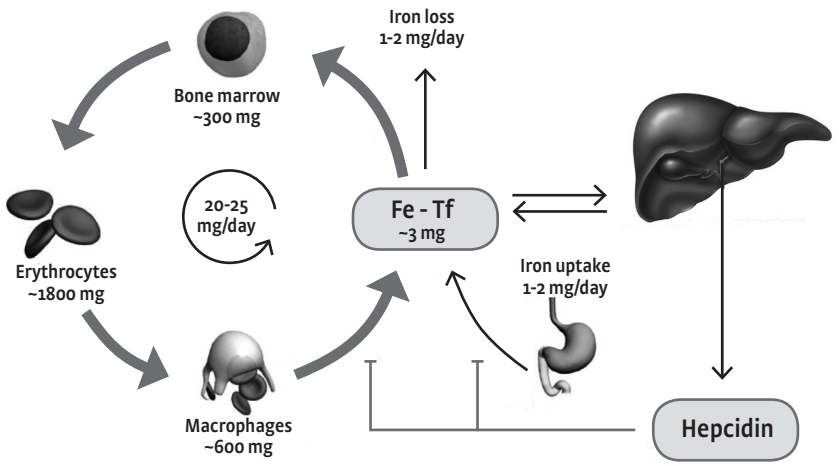
Hepcidin is the key regulator of systemic iron homeostasis

Iron (Fe) is an essential trace element for all living organisms, but free iron is highly toxic because of its redox activity that catalyzes the formation of reactive oxygen species. For this reason, the systemic iron concentration is tightly controlled with hepcidin as key regulator. The hepcidin receptor is ferroportin, the major cellular iron exporter in the membrane of macrophages, hepatocytes and the basolateral site of enterocytes [11,12]. By binding to ferroportin and inducing its internalization and degradation [13], hepcidin regulates the release of iron from these cells into the bloodstream: from duodenal enterocytes which absorb dietary iron, from macrophages which are involved in recycling of iron from senescent erythrocytes, and from hepatocytes involved in iron storage (Figure 1.1). Thus, hepcidin affects body iron distribution, with increased serum hepcidin concentration leading to a decreased flow of iron into the bloodstream and an increased amount of iron trapped inside the iron-exporting cells, predominantly reticulo-endothelial macrophages [14].

Several other parameters that reflect iron status and homeostasis of the human body are used in (clinical) practice. In the blood, iron is bound to **transferrin**, the iron transport protein. Transferrin can either be directly measured by immunochemical methods or indirectly

by the assessment of the **total iron-binding capacity (TIBC)**, a parameter that indicates the capacity of the blood to bind iron and thus reflects the transferrin concentration. **Transferrin saturation (TS)** is the ratio of serum iron divided by TIBC times 100% and reflects the percentage of the iron loading places of transferrin that are occupied by iron. Intracellular iron is stored in the cytoplasmic protein complex **ferritin**, from which iron is released in the circulation during periods of high iron demand. An iron-poor form of ferritin is released in the bloodstream, primarily by macrophages [15]. The measurement of circulating ferritin is used to quantify body iron stores, since in most circumstances its concentration correlates with liver iron concentrations as quantified by magnetic resonance imaging [16,17].

FIGURE 1.1 Systemic iron homeostasis: iron uptake and recycling.



The largest flux of body iron is the recycling of iron from senescent erythrocytes by macrophages and its subsequent incorporation in new erythroid precursors in the bone marrow. Only ±1-2 mg of iron is absorbed from the diet and lost from the body every day. Hepcidin regulates the systemic iron concentration (Fe-Tf) by inhibiting iron export from duodenal enterocytes and reticulo-endothelial macrophages. It is predominantly produced by hepatocytes. Figure adapted from Swinkels *et al*., 2006 [18].

Little is known about determinants of serum hepcidin in healthy human populations

Most of the current knowledge about hepcidin regulation is based on animal models and *in vitro* research. There have been studies on serum hepcidin concentrations in humans, but these are mostly based on patient series. Data on serum hepcidin concentrations in healthy individuals are scarce.

Evidence from animal and *in vitro* studies

The major factor in hepcidin regulation is iron status: when iron is abundant, hepcidin production is increased, and vice versa (Figure 1.2). Iron status regulates hepcidin expression via *circulating iron* and *iron stores*.

The signal of *circulating iron* is suggested to be provided by iron-loaded transferrin that binds to transferrin receptor 1 and 2 (TfR1 and TfR2) on hepatocytes. The hemochromatosis protein Hfe appears to modulate this signal by acting as a bimodal switch between TfR1 and TfR2 [19]. It is hypothesized that high concentrations of Tf-Fe_2 displace Hfe from TfR1 to promote its interaction with TfR2. The Hfe-TfR2 complex then activates the transcription of *HAMP* via extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and bone morphogenetic protein (BMP)/son of mothers against decapentaplegic (SMAD) signaling [20-22].

Intracellular iron stores have been found to increase hepcidin expression via bone morphogenetic proteins (BMP), particularly BMP-6, which bind to BMP receptors and activate SMAD signaling [23-25]. Hemojuvelin (HJV), a BMP co-receptor, appears to enhance this signaling [26]. HJV is cleaved by matriptase-2 (MT-2), a transmembrane serine protease encoded by the transmembrane serine protease 6 gene (*TMPRSS6*) in case of low iron status, leading to decreased BMP signaling [27].

Animal models have revealed that mice with a full knockout for positive regulators of hepcidin transcription, such as *HFE* and *TfR2*, present with decreased hepcidin expression leading to iron overload [28,29], whereas mice knockout for *TMPRSS6* have increased hepcidin expression leading to anemia [30].

Besides iron status, signals reflecting erythropoietic activity, hypoxia and inflammation regulate hepcidin expression (Figure 1.2) [8,20-22,31-33]. Hepcidin expression is decreased under conditions of increased erythropoiesis, due to the high iron demand of this process [34], and during hypoxia, whereas inflammation leads to increased hepcidin expression [35]. Finally, hepcidin mRNA levels were reported to be influenced by various other factors, *e.g.* testosterone [36,37], estrogen [38,39], and growth factors [40], but it is not precisely known whether these factors affect hepcidin expression directly or via signals of iron status, inflammation, hypoxia and/or erythropoiesis (Figure 1.2).

Evidence from studies in patients

There is some evidence about regulation of serum hepcidin in humans based on studies in patients with inherited and acquired disorders associated with either a hepcidin deficiency or a hepcidin excess [8]. The first group includes most forms of hereditary hemochromatosis (HH), which are characterized by body iron overload, ultimately resulting in iron-mediated organ injury like liver cirrhosis [8,18]. The most common form of HH is caused by homozygosity

for the single nucleotide polymorphism (SNP) rs1800562 (p.Cys282Tyr) in *HFE*, but defects in other genes that positively regulate hepcidin are also known to cause HH, namely *TfR2*, *HJV*, and, but rarely, *HAMP* itself [8,18,41,42]. Also, hepcidin synthesis is decreased in iron-loading anemias, including β-thalassemia intermedia and major and congenital dyserythropoietic anemias, due to overactivity of the diseased erythron despite the presence of iron loading [33,43,44]. Furthermore, hepcidin concentrations are relatively low for the level of iron load in acquired liver diseases such as excessive alcohol consumption and nonalcoholic fatty liver disease, and in patients with iron-deficiency anemia or low ferritin concentrations without anemia [8].

Hepcidin concentrations are increased relative to TS in patients with iron-refractory iron deficiency anemia (IRIDA), an inherited form of iron deficiency anemia that is resistant to oral iron therapy and only partially responds to parenteral iron administration, which results from defects in the *TMPRSS6* gene [45]. Furthermore, hepcidin is increased in infectious and inflammatory diseases, and in renal diseases due to impaired renal clearance and associated (low-grade) inflammation [8,46].

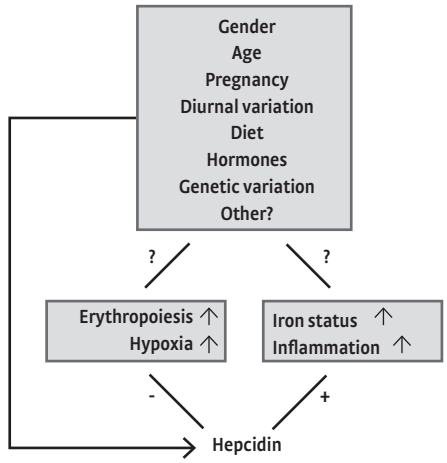
Evidence from studies in healthy volunteers

Evidence about hepcidin regulation in healthy individuals is scarce and mostly based on studies that investigated serum hepcidin levels in relatively small groups, ranging from 23 to 114 individuals [9,10,47-49]. Data from these studies revealed that serum hepcidin concentrations vary substantially between healthy persons, and that gender is a major determinant of serum hepcidin at population level, with higher hepcidin levels in men than in women [9,10,47-49]. The largest study showed median (P5-P95) serum hepcidin concentrations of 112 (29-254) and 65 (17-286) ng/mL in men and women, respectively [9]. Hepcidin concentration was shown to increase during a working day [9,47,48,50], which was recently described to be mediated by an innate diurnal rhythm rather than dietary iron [51]. The influence of age was investigated by only one of the studies on healthy subjects, which showed a trend of increasing hepcidin with age for both genders. However, this trend was non-significant and the authors called for further confirmation [9]. A high correlation between serum hepcidin and serum ferritin has been reported by several studies on healthy volunteers [9,10,49,52]. In addition, oral iron dosing was shown to increase serum hepcidin levels [9,53-56]. These findings confirm the evidence from animal and *in vitro* studies on the role of iron stores and circulating iron in hepcidin regulation (Figure 1.2).

Studies of hepcidin levels in healthy pregnant women revealed a decreased serum hepcidin concentration during pregnancy [57,58]. A recent longitudinal study reported that hepcidin levels decrease gradually during the course of pregnancy and are undetectable (≤0.5 nmoles/L) in nearly all women in their third trimester [57]. This is probably a result of maternal iron deficiency caused by an increased iron demand due to the growing fetus and placenta, and an increased iron need for erythropoiesis to maintain hemoglobin levels (Figure 1.2).

Genetic variation influencing serum hepcidin concentration in the general population has been unexplored until now, but (common) variants in the genes *HFE* and *TMPRSS6* are likely candidates.

FIGURE 1.2 Regulation of serum hepcidin.



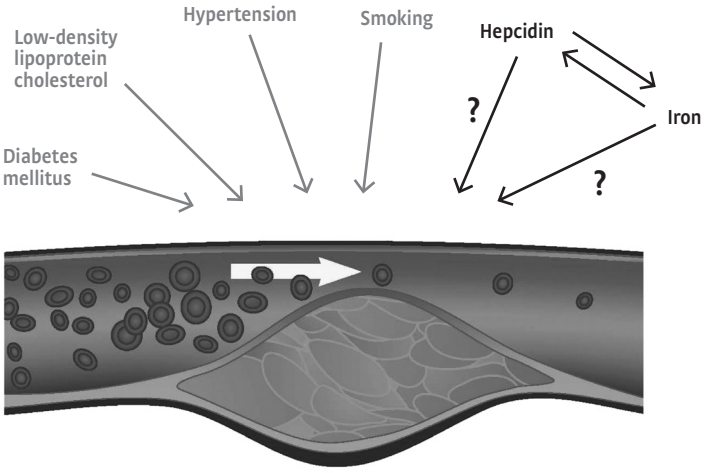
Increased erythropoiesis and hypoxia result in decreased serum hepcidin levels, whereas elevated levels of circulating iron and increased body iron status and inflammation increase serum hepcidin. Other factors might either influence hepcidin levels via these or other (unknown) processes.

Iron is hypothesized to play a role in the development of atherosclerosis: what about hepcidin?

Iron has been linked to multiple diseases, including neurodegenerative disorders [59], type II diabetes [60], and atherosclerosis. Atherosclerosis is the gradual process of accumulation of fatty substances, cholesterol, cellular waste products, calcium and fibrin in the inner lining of an artery, leading to a thickening of the arterial wall, a so called 'plaque' [61,62]. These plaques can rupture or erode, subsequently leading to thrombosis and cardiovascular diseases (CVD) such as myocardial infarcts and cerebrovascular accidents [61]. In 1981, Sullivan proposed the 'iron hypothesis', which was based on the observation that men have a higher risk of heart disease compared to premenopausal women, but not compared to postmenopausal women [63]. He suggested that iron deficiency plays a protective role against heart disease and that women are relatively protected because of loss of iron with menstruation. Iron, stored in macrophages that reside in the vessel wall, can catalyze the formation of reactive oxygen species. These oxidize low-density lipoprotein cholesterol, resulting in transformation of the macrophages into foam cells and ultimately atherosclerosis [64,65]. Until now, however, epidemiological studies on iron and atherosclerosis or the harder endpoint CVD showed inconsistent results [66-73]. In addition, the fact that HH patients with iron overload do not show a higher incidence of atherosclerosis provides evidence against the 'iron

hypothesis' [74]. Recently, Sullivan extended the 'iron hypothesis' and proposed that hepcidin plays a role in the development of atherosclerosis via promotion of iron deposition in macrophages, thereby increasing their atherogenic potential (Figure 1.3) [75]. This hypothesis has been confirmed by *in vitro* studies [76,77] and studies in patients on hemodialysis [78,79], but has never been tested in the general population. Indeed, if hepcidin proves to be causally related to atherosclerosis, it might contribute to CVD risk prediction and offer a new therapeutic target for atherosclerosis and CVD.

FIGURE 1.3 Risk factors of atherosclerosis.



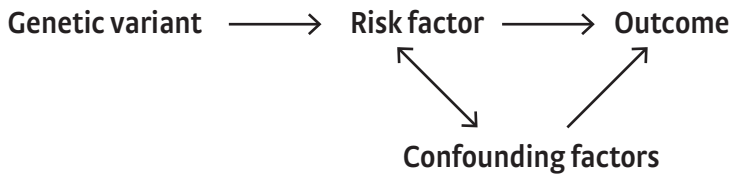
Established risk factors include diabetes mellitus, low-density lipoprotein cholesterol, hypertension and smoking. Iron and hepcidin are hypothesized to play a role in the development of atherosclerosis.

Additional insight into hepcidin genetics will help to decipher regulation of hepcidin and its role in disease

Both iron and hepcidin levels are viewed as multifactorial (or compex) traits that are influenced by many environmental and genetic factors and their interactions. There is a growing body of evidence on the genetic variants that affect iron homeostasis in the general population. This knowledge has been acquired by application of genome-wide association studies (GWAS), in which the entire genome is scanned in a hypothesis-free manner using SNP-arrays to identify common genetic variation associated with a trait of interest. GWAS have been performed for serum iron, transferrin, TS and ferritin, and revealed associations with variants in *e.g. HFE*, *TMPRSS6*, the transferrin gene (*TF*), and the transferrin receptor 2 gene (*TfR2*) [80-84]. On the contrary, relatively little is known about genetic determinants of hepcidin variation in the general population. No GWAS for serum hepcidin has been performed yet, and there are no studies that investigated associations between serum hepcidin and

common variants in plausible candidate genes such as *HFE* and *TMPRSS6*. Knowledge on the genetic background of hepcidin will increase insight into (potentially modifiable) molecular processes that regulate hepcidin production in the general population. Furthermore, it will increase insight into its relation with disease phenotypes via enabling so-called Mendelian randomization (MR) studies. In this approach, genetic variants are used as proxies for a risk factor of interest to test for a causal effect of this risk factor on an outcome of interest (Figure 1.4)[85]. It allows for evaluation of causality, as the random allocation of genes at conception mimics the randomization in a clinical trial, and thus prevents the occurrence of confounding or reverse causation. This approach has originally been proposed almost three decades ago by Katan in order to study the causal relation between serum cholesterol and cancer[86].

FIGURE 1.4 Mendelian randomization approach.



A genetic variant is used as a proxy for a risk factor to test its effect on an outcome of interest. This association provides evidence for causality, as confounding or reverse causation are ruled out.

Iron status genetics can be used to identify genetic determinants of serum hepcidin

The known correlations between hepcidin and iron metabolism can be exploited in the search for genetic determinants of serum hepcidin. The genes that have previously been identified via GWAS for iron parameters are likely candidates for hepcidin. In addition, application of a *multivariate* GWAS, in which the association of genetic variants with multiple, potentially correlated traits is assessed simultaneously, may lead to improved power to actually identify associated genetic variants compared to a univariate GWAS[87-90].

Objectives of this thesis

The two main objectives are:

1 To identify biochemical correlates and genetic determinants of serum hepcidin in the general population (Part I).
2 To elucidate the effect of hepcidin and iron on atherosclerosis in the general population (Part II).

Population

The epidemiological studies described in this thesis are based on data from the Nijmegen Biomedical Study (NBS). The NBS is a population-based study that was initiated in 2001 by the Department for Health Evidence and the Department of Laboratory Medicine. It consists of several phases (NBS phase 1 to phase 5) in which detailed information on lifestyle and disease status of the participants was collected. Participants of each phase were invited to participate in the next phase, provided that they did not object against participation in follow-up studies.

For this thesis we used data from NBS phase 1 (NBS-1, 2003) and the NBS-2-NIMA (non-invasive measurements of atherosclerosis) substudy (2005-2008) (Figure 1.5). NBS-1 comprised a limited questionnaire about lifestyle, health status, and medical history and donation of a blood sample. A total of 9350 (43%) persons filled out the NBS-1 questionnaire, of which 6468 (69%) donated blood samples. In the NBS-2-NIMA, all NBS-2 participants aged 50-70 years (N=2114) were re-invited to fill out an additional QN, donate a fasting blood sample, and undergo anthropometric measurements and non-invasive measurements of atherosclerosis. A total of 1491 subjects participated in NBS-2-NIMA (response 71%). Approval to conduct the NBS and NBS-2-NIMA study was obtained from the Radboud university medical center Institutional Review Board. All participants gave written informed consent for participation in the NBS.

Outline of this thesis

PART I: Determinants of serum hepcidin

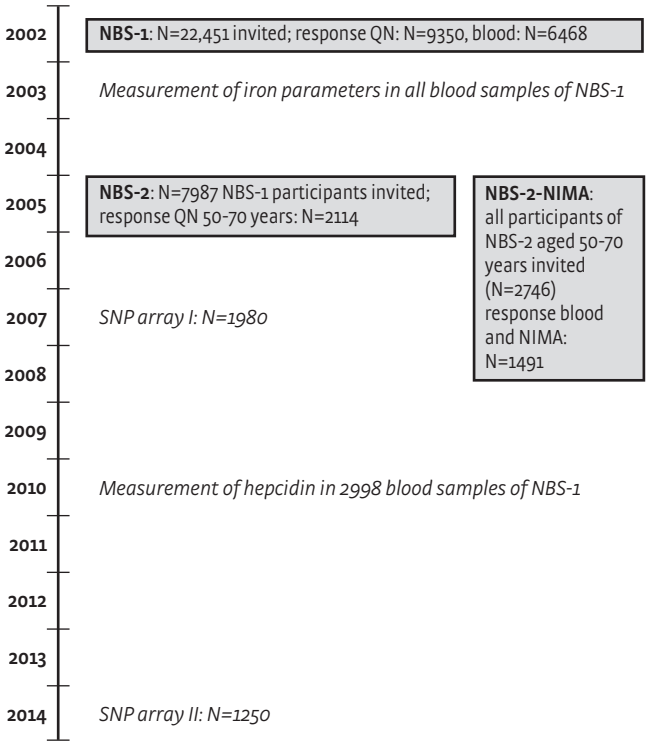
In **chapter 2**, we assess age- and sex-stratified reference ranges of serum hepcidin and study associations between hepcidin and (biochemical) variables in almost 3000 individuals of the general population. **Chapter 3** describes a candidate-gene association study in which we focus on the genes *HFE* and *TMPRSS6* and study the association of SNPs in these genes with hepcidin and the iron parameters, *i.e.* serum iron, ferritin, TIBC and TS. In **chapter 4**, we present a meta-analysis of genome-wide association studies (GWAS) on serum hepcidin. A simulation study comparing methods available for multivariate analysis of GWAS is presented in **chapter 5**.

PART II: Effects on atherosclerosis

In **chapter 6**, we study the associations of hepcidin and the iron parameters with non-invasive measurements of atherosclerosis (*i.e.* presence of plaque, intima-media thickness (IMT), and ankle-brachial index (ABI)). We evaluate these relationships from a genetic perspective using an MR approach in **chapter 7**.

A general discussion of the studies presented in this thesis and future perspectives are given in **chapter 8**.

FIGURE 1.5 Schematic overview of phase 1 and 2 of the Nijmegen Biomedical Study.



Schematic overview of the data collection within phase 1 and 2 of the Nijmegen Biomedical Study (NBS) and measurements of main variables used in this thesis (hepcidin, iron parameters and genome-wide single nucleotide polymorphism data).
SNP array I was applied to 1980 blood samples of NBS-1 (overlap with NBS-2-NIMA: N=598).
SNP array II was applied to 1250 blood samples of NBS-1 (overlap with SNP array I: N=200, overlap with NBS-2-NIMA: N=384).
NIMA indicates non-invasive measurements of atherosclerosis; SNP array I and II, measurement of genome-wide single nucleotide polymorphism data.

References

1 Krause A, Neitz S, Magert HJ, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147-50.

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

3 Isoda M, Hanawa H, Watanabe R, et al. Expression of the peptide hormone hepcidin increases in cardiomyocytes under myocarditis and myocardial infarction. J Nutr Biochem 2010;21:749-56.

4 Kulaksiz H, Theilig F, Bachmann S, et al. The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. J Endocrinol 2005;184:361-70.

5 Peslova G, Petrak J, Kuzelova K, et al. Hepcidin, the hormone of iron metabolism, is bound specifically to alpha-2-macroglobulin in blood. Blood 2009;113:6225-36.

6 Itkonen O, Stenman UH, Parkkinen J, et al. Binding of hepcidin to plasma proteins. Clin Chem 2012;58:1158-60.

7 Laarakkers CM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. PLoS One 2013;8:e75518.

8 Kroot JJ, Tjalsma H, Fleming RE, et al. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-69.

9 Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. Blood 2008;112:4292-7.

10 Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.

11 Delaby C, Pilard N, Goncalves AS, et al. Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. Blood 2005;106:3979-84.

12 Ramey G, Deschemin JC, Durel B, et al. Hepcidin targets ferroportin for degradation in hepatocytes. Haematologica 2010;95:501-4.

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

14 Roy CN, Mak HH, Akpan I, et al. Hepcidin antimicrobial peptide transgenic mice exhibit features of the anemia of inflammation. Blood 2007;109:4038-44.

15 Cohen LA, Gutierrez L, Weiss A, et al. Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. Blood 2010;116:1574-84.

16 Wang W, Knovich MA, Coffman LG, et al. Serum ferritin: Past, present and future. Biochim Biophys Acta 2010;1800:760-9.

17 Olthof AW, Sijens PE, Kreeftenberg HG, et al. Correlation between serum ferritin levels and liver iron concentration determined by MR imaging: impact of hematologic disease and inflammation. Magn Reson Imaging 2007;25:228-31.

18 Swinkels DW, Janssen MC, Bergmans J, et al. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. Clin Chem 2006;52:950-68.
19 Gao J, Chen J, Kramer M, et al. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab 2009;9:217-27.
20 Ganz T. Hepcidin and iron regulation, 10 years later. Blood 2011;117(17):4425-33.
21 Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24-38.
22 Fleming RE, Ponka P. Iron overload in human disease. N Engl J Med 2012;366:348-59.
23 Babitt JL, Huang FW, Xia Y, et al. Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest 2007;117:1933-9.
24 Kautz L, Meynard D, Monnier A, et al. Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood 2008;112:1503-9.
25 Andriopoulos B, Jr., Corradini E, Xia Y, et al. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 2009;41:482-7.
26 Babitt JL, Huang FW, Wrighting DM, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 2006;38:531-9.
27 Silvestri L, Pagani A, Nai A, et al. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab 2008;8:502-11.
28 Ahmad KA, Ahmann JR, Migas MC, et al. Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 2002;29:361-6.
29 Wallace DF, Summerville L, Lusby PE, et al. First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin. Gut 2005;54:980-6.
30 Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. Science 2008;320:1088-92.
31 Ganz T. Systemic iron homeostasis. Physiol Rev 2013;93:1721-41.
32 Kautz L, Jung G, Valore EV, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 2014;46:678-84.
33 Kautz L, Nemeth E. Molecular liaisons between erythropoiesis and iron metabolism. Blood 2014.
34 Pak M, Lopez MA, Gabayan V, et al. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 2006;108:3730-5.
35 Wrighting DM, Andrews NC. Interleukin-6 induces hepcidin expression through STAT3. Blood 2006;108:3204-9.
36 Guo W, Bachman E, Li M, et al. Testosterone administration inhibits hepcidin transcription and is associated with increased iron incorporation into red blood cells. Aging Cell 2013;12:280-91.
37 Latour C, Kautz L, Besson-Fournier C, et al. Testosterone perturbs systemic iron balance through activation of epidermal growth factor receptor signaling in the liver and repression of hepcidin. Hepatology 2014;59:683-94.

38 Hou Y, Zhang S, Wang L, et al. Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element. Gene 2012;511(2):398-403.
39 Ikeda Y, Tajima S, Izawa-Ishizawa Y, et al. Estrogen regulates hepcidin expression via GPR30-BMP6-dependent signaling in hepatocytes. PLoS One 2012;7:e40465.
40 Goodnough JB, Ramos E, Nemeth E, et al. Inhibition of hepcidin transcription by growth factors. Hepatology 2012;56:291-9.
41 van Dijk BA, Laarakkers CM, Klaver SM, et al. 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;142:979-85.
42 Nemeth E, Roetto A, Garozzo G, et al. Hepcidin is decreased in TFR2 hemochromatosis. Blood 2005;105:1803-6.
43 Casanovas G, Swinkels DW, Altamura S, et al. Growth differentiation factor 15 in patients with congenital dyserythropoietic anaemia (CDA) type II. J Mol Med (Berl) 2011;89:811-6.
44 Gardenghi S, Marongiu MF, Ramos P, et al. Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood 2007;109:5027-35.
45 Finberg KE. Iron-refractory iron deficiency anemia. Semin Hematol 2009;46:378-86.
46 Ruchala P, Nemeth E. The pathophysiology and pharmacology of hepcidin. Trends Pharmacol Sci 2014;35:155-61.
47 Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, et al. High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol 2009;146:317-25.
48 Kroot JJ, Hendriks JC, Laarakkers CM, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem 2009;389:124-9.
49 Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009;75:976-81.
50 Kemna EH, Tjalsma H, Podust VN, et al. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin Chem 2007;53:620-8.
51 Schaap CC, Hendriks JC, Kortman GA, et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin Chem 2013;59:527-35.
52 Peters HP, Laarakkers CM, Swinkels DW, et al. Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010;25:848-53.
53 Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271-6.
54 Roe MA, Collings R, Dainty JR, et al. Plasma hepcidin concentrations significantly predict interindividual variation in iron absorption in healthy men. Am J Clin Nutr 2009;89:1088-91.

55 Zimmermann MB, Troesch B, Biebinger R, et al. Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin. Am J Clin Nutr 2009;90:1280-7.
56 Young MF, Glahn RP, Ariza-Nieto M, et al. Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. Am J Clin Nutr 2009;89:533-8.
57 van Santen S, Kroot JJ, Zijderveld G, et al. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. Clin Chem Lab Med 2013;51:1395-401.
58 Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. Eur J Haematol 2010;85:345-52.
59 Liu B, Moloney A, Meehan S, et al. Iron promotes the toxicity of amyloid beta peptide by impeding its ordered aggregation. J Biol Chem 2011;286:4248-56.
60 Kunutsor SK, Apekey TA, Walley J, et al. Ferritin levels and risk of type 2 diabetes mellitus: an updated systematic review and meta-analysis of prospective evidence. Diabetes Metab Res Rev 2013;29:308-18.
61 Falk E. Pathogenesis of atherosclerosis. J Am Coll Cardiol 2006;47:C7-12.
62 Insull W, Jr. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. Am J Med 2009;122:S3-S14.
63 Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1:1293-4.
64 Kraml PJ, Klein RL, Huang Y, et al. Iron loading increases cholesterol accumulation and macrophage scavenger receptor I expression in THP-1 mononuclear phagocytes. Metabolism 2005;54:453-9.
65 Lapenna D, Pierdomenico SD, Ciofani G, et al. Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques. Free Radic Biol Med 2007;42:492-8.
66 Kiechl S, Willeit J, Egger G, et al. Body iron stores and the risk of carotid atherosclerosis: prospective results from the Bruneck study. Circulation 1997;96:3300-7.
67 Salonen JT, Tuomainen TP, Salonen R, et al. Donation of blood is associated with reduced risk of myocardial infarction. The Kuopio Ischaemic Heart Disease Risk Factor Study. Am J Epidemiol 1998;148:445-51.
68 Meyers DG, Jensen KC, Menitove JE. A historical cohort study of the effect of lowering body iron through blood donation on incident cardiac events. Transfusion 2002;42:1135-9.
69 Meyers DG, Strickland D, Maloley PA, et al. Possible association of a reduction in cardiovascular events with blood donation. Heart 1997;78:188-93.
70 Ascherio A, Rimm EB, Giovannucci E, et al. Blood donations and risk of coronary heart disease in men. Circulation 2001;103:52-7.
71 Zheng H, Cable R, Spencer B, et al. Iron stores and vascular function in voluntary blood donors. Arterioscler Thromb Vasc Biol 2005;25:1577-83.
72 Engberink MF, Geleijnse JM, Durga J, et al. Blood donation, body iron status and carotid intima-media thickness. Atherosclerosis 2008;196:856-62.

73 Peffer K, den Heijer M, Holewijn S, et al. The effect of frequent whole blood donation on ferritin, hepcidin, and subclinical atherosclerosis. Transfusion 2012;53:1468-74.
74 Sullivan JL. Do hemochromatosis mutations protect against iron-mediated atherogenesis? Circ Cardiovasc Genet 2009;2:652-7.
75 Sullivan JL. Macrophage iron, hepcidin, and atherosclerotic plaque stability. Exp Biol Med (Maywood) 2007;232:1014-20.
76 Saeed O, Otsuka F, Polavarapu R, et al. Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler Thromb Vasc Biol 2012;32:299-307.
77 Li JJ, Meng X, Si HP, et al. Hepcidin destabilizes atherosclerotic plaque via overactivating macrophages after erythrophagocytosis. Arterioscler Thromb Vasc Biol 2012;32:1158-66.
78 van der Weerd NC, Grooteman MP, Bots ML, et al. Hepcidin-25 is related to cardiovascular events in chronic haemodialysis patients. Nephrol Dial Transplant 2013;28:3062-71.
79 Kuragano T, Itoh K, Shimonaka Y, et al. Hepcidin as well as TNF-alpha are significant predictors of arterial stiffness in patients on maintenance hemodialysis. Nephrol Dial Transplant 2011;26:2663-7.
80 Benyamin B, McRae AF, Zhu G, et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60-5.
81 McLaren CE, Garner CP, Constantine CC, et al. Genome-wide association study identifies genetic loci associated with iron deficiency. PLoS One 2011;6:e17390.
82 Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. Blood 2010;115:94-6.
83 Oexle K, Ried JS, Hicks AA, et al. Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferrin receptor (sTfR) levels. Hum Mol Genet 2011;20:1042-7.
84 Pichler I, Minelli C, Sanna S, et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum Mol Genet 2011;20:1232-40.
85 Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;32(1):1-22.
86 Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. Lancet 1986;1:507-8.
87 Zhu W, Zhang H. Why Do We Test Multiple Traits in Genetic Association Studies? J Korean Stat Soc 2009;38:1-10.
88 Allison DB, Thiel B, St Jean P, et al. Multiple phenotype modeling in gene-mapping studies of quantitative traits: power advantages. Am J Hum Genet 1998;63:1190-201.
89 Klei L, Luca D, Devlin B, et al. Pleiotropy and principal components of heritability combine to increase power for association analysis. Genet Epidemiol 2008;32:9-19.
90 Chavali S, Barrenas F, Kanduri K, et al. Network properties of human disease genes with pleiotropic effects. BMC Syst Biol 2010;4:78.

PART I

Determinants of serum hepcidin

CHAPTER 2

Serum hepcidin: reference ranges and biochemical correlates in the general population

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ABSTRACT

To date, concentrations of the promising biomarker hepcidin have only been assessed in serum of relatively small series of healthy volunteers and patients. We assessed age and sex stratified reference ranges of serum hepcidin concentration in a selected reference set and performed regression analyses to study associations between hepcidin and (biochemical) variables in a large, well-phenotyped sample of the general population (n=2,998). All participants filled out a questionnaire on lifestyle, health status and medical history. Serum measurements of iron parameters, liver enzyme alanine aminotransferase (ALAT), creatinine and C-reactive protein (CRP) were available. Serum hepcidin concentrations were lower for premenopausal than for postmenopausal women (median 4.1 nM vs 8.5 nM, respectively). Hepcidin concentrations in men were constant over age (median 7.8 nM). Serum hepcidin was strongly associated with serum ferritin in men and women [β-coefficient of log-transformed variables (95% confidence interval): 0.78 (0.74 – 0.82) and 0.83 (0.78 – 0.88), respectively]. Additional significant, though less strong, associations were observed for CRP and total iron binding capacity (TIBC) in men and for TIBC, ALAT and glomerular filtration rate in women. Our study provides age and gender specific reference ranges of serum hepcidin concentration and indicates ferritin as the primary correlate of serum hepcidin concentration.

Introduction

Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis [1,2]. It is a 25-amino acid peptide hormone which is produced and secreted predominantly by hepatocytes, circulates in the blood stream and is excreted by the kidneys. By binding to the cellular iron exporter ferroportin and inducing its internalization and degradation, hepcidin regulates cellular iron efflux [3]. In this way, the absorption of dietary iron from the intestine and the release of recycled iron derived from senescent erythrocytes is controlled [1,2].

The synthesis of hepcidin is regulated by certain physiologic and pathologic processes. Hepcidin concentrations are decreased in situations that require increased concentrations of circulating iron. In case of increased erythropoiesis, for example in response to hypoxia, anemia, iron deficiency or conditions characterized by ineffective erythropoiesis (*e.g.* thalassemia major and intermedia), a decreased hepcidin concentration will result in the release of stored iron and in an increase in the dietary iron absorption [4-7]. On the other hand, infection and inflammation cause an increase in hepcidin synthesis [4,8-10], resulting in decreased availability of circulating iron, which is considered to represent a defense mechanism of the human body against extracellularly proliferating (iron-dependent) pathogens [1]. In chronic (low grade) inflammatory states, this ultimately leads to a deficiency of iron available for erythropoiesis called anemia of chronic disease [11]. Finally, hepcidin concentration is increased in situations of iron overload [12], except for situations in which mutations in genes encoding hepcidin or its upstream positive regulators are responsible for the surplus of iron by preventing hepcidin upregulation [6]. Although notable progression has been made in discovering the identities of hepcidin regulators involved in the aforementioned processes, we do not yet fully understand the mechanisms by which they influence hepcidin expression.

Since the discovery of hepcidin and the elucidation of its important role in iron homeostasis [1,2], hepcidin has been suggested as a promising diagnostic marker for iron-related disorders. Determination of serum hepcidin concentration may be a helpful tool in screening for hereditary hemochromatosis, thus preventing cumbersome procedures in the search for causative (rare) genetic variants. Furthermore, hepcidin concentrations have been suggested to negatively correlate with the severity of hemochromatosis and to determine the prognosis and need for stringency of the treatment protocol [13]. Hepcidin concentrations may also be used in the management of patients with iron loading anemias [5]. In addition, hepcidin is key in the diagnosis of iron refractory iron deficiency anemia [14], and might contribute to the diagnosis of iron deficiency in patients with anemia of chronic diseases [15]. Hepcidin might be a potential marker in the prediction of erythropoietin (EPO) response and to guide treatment with EPO and intravenous iron [16]. Finally, measurement of serum hepcidin concentration is of importance in the monitoring of novel therapies for iron disorders which target hepcidin, its upstream regulators or its downstream receptor ferroportin [17].

Until now, knowledge on how hepcidin exerts its regulatory function and on the molecular processes that regulate hepcidin production is largely based on animal studies and *in vitro* studies which often use hepcidin mRNA expression as a read out [2]. In addition, many studies of hepcidin in humans have relied on urinary hepcidin assays and on measurements in relatively small groups of healthy subjects or patient series with a variety of iron disorders [2,18], predominantly without making a distinction between gender and age, factors that most probably influence hepcidin concentration. Reference ranges of serum hepcidin concentration, based on a large subset from the general population, are instructive for the use of hepcidin as a diagnostic tool and therapeutic target in the future, but are currently not available. Furthermore, knowledge on the association between potential correlates, for example markers of infection, inflammation and iron status, and serum hepcidin concentration in humans is relatively scarce, although this information could greatly contribute to understanding of the mechanisms by which hepcidin expression is influenced *in vivo*. There are a number of studies which reported a high correlation between serum hepcidin and serum ferritin concentration in small samples of healthy persons [19-22], but other potentially important correlates have only been investigated in animal experiments or small healthy and diseased human populations. For example, transferrin has been shown to be a major determinant of hepcidin expression in hypotransferrinemic mice [23], and in multivariate analyses the estimated glomerular filtration rate (eGFR) was reported not to be a major independent correlate of serum hepcidin concentration in patients with chronic kidney disease [21]. However, the applicability of these and other findings to the general human population is unclear. To increase our insight in the distribution of hepcidin in human populations and to pave the way for its use in diagnostic medicine, we studied serum hepcidin concentrations and reference ranges and determined its association with selected (biochemical) parameters in a large, well-phenotyped sample of the general population.

Materials and methods

Study population

We included 2,998 participants from the Nijmegen Biomedical Study (NBS). Details of the NBS have been described before [24]. Briefly, the NBS is a population-based survey conducted by the Department of Epidemiology, Biostatistics, and HTA and the Department of Laboratory Medicine of the Radboud University Nijmegen Medical Centre, The Netherlands. Approval to conduct the study was obtained from the Institutional Review Board. Age and gender stratified randomly selected adult inhabitants of Nijmegen (n=22,451), a city located in the eastern part of The Netherlands, received an invitation to fill out a postal questionnaire on, for example, lifestyle, health status and medical history, and to donate an 8.5 ml blood sample in a serum separator tube and a 10 ml EDTA blood sample. A total of 9,350 (43%) individuals filled out the questionnaire, of which 6,468 (69%) responders donated blood samples between 8 am and 9 pm; time of blood sampling was recorded. Prior

to the procedure described above, a pilot study was performed to optimize the logistical procedures of the NBS. The pilot study entailed a random sample of 650 male and female inhabitants of Nijmegen aged ≥18 years; 342 subjects (53%) filled out the questionnaire and 262 (77%) of the responders donated a blood sample. All participants gave written informed consent for participation in the NBS.

For this study, serum hepcidin concentration was measured in 2,998 out of the total of 6,730 available serum samples. Serum measurements of iron, ferritin, total iron binding capacity (TIBC), liver enzyme alanine aminotransferase (ALAT), creatinine and C-reactive protein (CRP) were previously performed in all available serum samples.

The following variables were extracted from the self-administered questionnaire: length, weight [used to derive body-mass index (BMI)], age, use of iron supplements at time of blood donation for at least six months, presence of anemia determined by a physician, being a blood and/or plasma donor, pregnancy and presence of a regular menstruation.

Laboratory methods

Serum hepcidin concentration was measured with a competitive-ELISA as described before [25]. In short, 96-well plates were coated overnight at 4°C with goat-anti-rabbit IgG (Fc) antibody, washed and then blocked with bovine serum albumin (BSA) (2h, ambient temperature), washed and thereafter incubated with an in-house raised rabbit-anti-human hepcidin antibody [26] (2h, ambient temperature) and again washed. Next, the standards, study samples (20-fold diluted) and a reference sample were pipetted into the wells. Subsequently, biotinylated hepcidin-25 calibrator was added to all wells and the plates were incubated overnight at 4°C. After washing, the plates were incubated with Streptavidin-β-peroxidase (Streptavidin-POD) conjugate for 1h at ambient temperature, again washed and then o-phenylenediamine (OPD) substrate was added and incubated for 15 minutes in the dark. The color reaction was stopped with H_2SO_4 and optical density was measured at 492 nm in an automated c-ELISA reader. In each plate a standard curve and the reference preparation was present. This reference preparation was used for estimation of the accuracy of the method. In total, 86 microtiter plates were used in 15 different assay runs. The mean hepcidin concentration in this preparation was 6.87 nmol/L (nM), while the intra-assay variation, the between-plates variation and inter-assay variation were 6.3%, 5.5% and 11.9%, respectively. The intra-assay, inter-assay and between-plates variations were calculated from the results obtained in the reference preparation. In 86 plates the reference preparation was measured in duplicate and the calculated concentrations in each plate were used to establish the intra-assay variation. The mean hepcidin concentration per plate was used for calculation of the between-plates (inter-day) variation and the inter-assay variation. The analytical sensitivity, defined as the minimum hepcidin concentration evoking a response significantly different from that of the zero calibrator, was 8.96 pM. As the samples were 20-fold diluted, samples found to have a hepcidin concentration below 179 pM (20 x 8.96 pM)

were imputed with a random value out of a uniform distribution with a minimum of 0 nM and a maximum of 0.18 nM (n=12). Hepcidin concentrations are expressed in nmol/L; 1 nM serum hepcidin equals 2.79 µg/L.

Total serum iron was measured by colorimetric measurement using ascorbate/FerroZine reagents (Roche Diagnostics B.V.) on an Abbott Aeroset analyzer. Unsaturated iron binding capacity (UIBC) was measured by adding a known quantity of Fe^{3+} to the serum samples, reducing it with ascorbate to Fe^{2+} and measuring it with FerroZine as described above (Roche reagents on an Aeroset). TIBC was calculated by adding serum iron and UIBC. Serum transferrin saturation (TS) was computed by dividing serum iron by TIBC. Serum ferritin concentration was determined by a chemiluminescent microparticle immuno-assay on the Abbott Architect calibrated against the ferritin assay on the Immulite 2000 of Diagnostic Products Corporation (DPC, Los Angeles, CA).

Serum creatinine was measured by a kinetic alkaline picrate method on an Abbott Aeroset auto-analyser by exploiting Bromcresol purple (Jaffe method [27]). In view of the importance of interlaboratory and methodological differences in the creatinine assays on results of estimated glomerular filtration rate (eGFR), our creatinine data obtained by the Jaffe method were calibrated against creatinine values traceable to isotope dilution mass spectrometry [27,28]. For this purpose, the eGFR was calculated using a re-expressed MDRD (Modification of Diet in Renal Disease) formula: 175 x (standardized serum creatinine (in µmol/L) / 88.4)$^{-1.154}$ x (age (in years))$^{-0.203}$ x 0.742 (if female). As this formula is only valid for white individuals and we did not have information about race except for country of birth, we used this variable as a proxy and calculated the MDRD formula only for subjects born in The Netherlands and other western countries. Genetic analyses revealed that country of birth was highly associated with race (data not shown), supporting our assumption.

ALAT was measured using standard reagents in a reaction rate assay based on the conversion of NADH to NAD (Abbott Reagent on Aeroset). Samples with a measurement result of <3 U/L were set at 2 U/L.

CRP was quantified by immunologic agglutination detection with latex-coupled polyclonal anti-CRP antibodies (Abbott Reagent on Aeroset).

Statistical analysis

Statistical analyses were performed with SPSS for Windows, release 16.0.2 (SPSS Inc., Chicago, Illinois). Distributions of serum hepcidin, serum ferritin, ALAT and BMI were skewed towards higher values; logarithmic transformations were applied to normalize the distributions. For all continuous variables, median and 2.5th and 97.5th percentiles (P2.5 and P97.5, respectively) were calculated from original untransformed values. Blood sampling time and CRP were categorized into three groups based on clinically relevant cutoffs. For blood sam-

pling time, these were <12 pm, 12-5 pm and >5 pm, according to Dutch routine and in line with previously reported hepcidin concentration patterns throughout the day [22,29]. For CRP, cutoffs of <5 mg/L, 5-20 mg/L and >20 mg/L were used. Categorical variables were expressed in numbers and corresponding percentages.

Reference ranges for serum hepcidin concentration, stratified by 5-year age groups and gender, were constructed using the median, P2.5 and P97.5 per category. A reference subset was selected by excluding subjects that passed the following criteria at time of blood sampling: pregnant, ALAT>50 U/L, CRP>10 mg/L, eGFR<60 mL/min/1.73 m^2, use of iron supplements, presence of anemia or BMI>30 kg/m^2.

Univariable and multivariable least squares linear regression analyses were used to evaluate the associations between log-transformed serum hepcidin concentrations and selected (biochemical) variables, unadjusted and adjusted for age and time of blood sampling. The assumption of linearity between serum hepcidin concentrations and independent variables was confirmed using graphic methods. Resulting regression coefficients (β) express the change in log-transformed serum hepcidin that are associated with a 1-unit change in the independent variable. Some of the independent variables were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a β% change in serum hepcidin. The R^2 (adjusted for the number of explanatory variables in the model) was obtained to indicate the amount of variance in hepcidin concentration that was explained by the included variables. All analyses were stratified by gender.

Results

Characteristics of the study population

Forty-eight percent of the total study population (n=2,998) was male. Median age of men was 63 years; median age of women 54 years. CRP was increased (>20 mg/L) in 2% of the population. Serum ferritin concentration was considerably lower in women than in men, which is in concordance with the lower median iron concentration, higher TIBC, lower TS and larger number of anemic subjects observed in women compared to men. For most subjects, blood sampling was performed between 12 and 5 pm. Blood sampling time distribution over the age groups was dissimilar: older individuals underwent blood sampling earlier during the day (Supplemental Figure 2.1). Additional characteristics of the subjects included in the study, stratified by gender, are described in Table 2.1. A total of 1,948 subjects (1,066 men; 882 women) passed set criteria and were included in the reference set. The characteristics of the reference set are given in Supplemental Table 2.1.

TABLE 2.1 Characteristics of the study population (N=2,998).

Variable		Men (N=1,445)				Women (N=1,553)			
		N*	(%)	Median	P2.5-P97.5	N*	(%)	Median	P2.5-P97.5
Age, years		1,445	(100)	63	29-81	1,553	(100)	55	25-80
Ferritin, µg/L		1,443	(100)	164.1	17.8-620.2	1,553	(100)	81.6	8.7-368.6
Iron, µmol/L		1,434	(99)	18	8-32	1,544	(99)	16	6-30
TIBC, µmol/L		1,434	(99)	58	43-78	1,544	(99)	60	44-84
TS, %		1,434	(99)	30.2	13.1-56.9	1,544	(99)	26.9	9.3-50.0
ALAT, U/L		1,398	(97)	13	5-35	1,507	(98)	10	4-34
eGFR, mL/min/1.73 m^2		1,430	(99)	82.7	46.0-122.4	1,530	(99)	82.0	51.1-123.7
CRP	Less than 5 mg/L	1,160	(80)			1,201	(77)		
	5-20 mg/L	241	(17)			314	(20)		
	More than 20 mg/L	29	(2)			29	(2)		
	Unknown	15	(1)			9	(1)		
BMI	Less than 18 kg/m^2	4	(0)			15	(1)		
	18-25 kg/m^2	628	(44)			825	(53)		
	25-40 kg/m^2	786	(54)			662	(43)		
	More than 40 kg/m^2	3	(0)			13	(1)		
	Unknown	24	(2)			38	(2)		
Current use of iron supplements	Yes	12	(1)			25	(2)		
	No	1,264	(87)			1,349	(87)		
	Unknown	169	(12)			179	(12)		
Pregnant	Yes					12	(1)		
	No					1,494	(96)		
	Unknown					47	(3)		
Regular menstruation	Yes					505	(33)		
	No					1,001	(64)		
	Unknown					47	(3)		
Self-reported anemia	Yes	52	(4)			361	(23)		
	No	1,283	(89)			1,087	(70)		
	Unknown	110	(8)			105	(7)		
Current blood and/or plasma donor	Yes	496	(34)			399	(26)		
	No	934	(65)			1,125	(72)		
	Unknown	15	(1)			29	(2)		
Time of blood sampling	Before 12 pm	352	(24)			326	(21)		
	Between 12 pm and 5 pm	812	(56)			956	(62)		
	After 5 pm	273	(19)			263	(17)		
	Unknown	8	(1)			8	(1)		

N indicates number; P2.5, 2.5th percentile; P97.5, 97.5th percentile; TIBC, total-iron binding capacity; TS, transferrin saturation; ALAT, alanine aminotransferase; eGFR, glomerular filtration rate calculated using the modification of diet in renal disease formula 4; CRP, C-reactive protein; BMI, body mass index.

* Numbers are different from the total number of included persons due to missing values.

Age and gender specific reference ranges for serum hepcidin concentration in the reference set

Reference ranges of serum hepcidin concentration in the reference set per 5-year age group are given for men and women in Table 2.2 and Supplemental Figure 2.2. Serum hepcidin concentration in men was constant over age, with a median of 7.8 nM (P2.5-P97.5, 0.6-23.3 nM). Hepcidin concentrations in women trend upwards as they progress through menopause, with a median serum hepcidin concentration of 4.1 nM (P2.5-P97.5, 0.4-19.7) for women younger than 55 years and 8.5 nM (P2.5-P97.5, 1.2-24.8 nM) for women of 55 years and older. These results were confirmed by univariable regression analyses (Supplemental Table 2.2): age was not associated with serum hepcidin concentration in men [β 0.003 (95% CI, -0.001; 0.007)], but it was in women, whether age was defined as a continuous [β 0.029 (95% CI, 0.025; 0.032)] or dichotomous variable (<55 years vs ≥55 years, based on the distinction between pre- and postmenopausal state by using age as a proxy in the absence of information on menopausal state) [β 0.840 (95% CI, 0.745; 0.935)].

The lowest median hepcidin concentration [2.6 nM (P2.5-P97.5, 0.7-10.5 nM)] was found in the category of women aged 18-24 years, while the highest median concentration was observed in the category of women aged 80-84 years [11.9 nM (P2.5-P97.5, 1.6-19.2 nM)]. Serum hepcidin concentrations lower than the detection limit of 0.18 nM were observed for both men (n=4) and women (n=5). Serum hepcidin concentration varied substantially between subjects, which is reflected in wide reference ranges.

As hepcidin concentrations in serum follow a clear circadian rhythm [22,29,30], we considered the influence of time of blood sampling on serum hepcidin reference ranges by calculating reference ranges stratified for time of blood sampling divided in 3 categories: <12 pm, 12-5 pm and >5 pm (Supplemental Tables 2.3-2.5). Serum hepcidin concentration in men who underwent blood sampling in the morning (<12 pm) was lower compared to the concentration in men who donated blood after 12 pm [12-5 pm vs <12 pm: β 0.427 (95% CI, 0.315; 0.539); >5 pm vs <12 pm: β 0.366 (95% CI, 0.225; 0.508)] (Supplemental Table 2.2). This effect did not change by adjusting for age. In women, a similar effect was observed for hepcidin concentration in blood sampled between 12-5 pm as compared to morning samples [β 0.416 (95% CI, 0.287; 0.546)], but the hepcidin concentration was only slightly elevated in samples obtained after 5 pm compared to morning samples [β 0.080 (95% CI, -0.087; 0.247)]. However, adjusting for age increased the regression coefficient in women for samples obtained after 5 pm compared to morning samples to 0.305 (95% CI, 0.150; 0.460) (data not shown). The regression coefficient for samples obtained between 12-5 pm compared to morning samples did not change by adjusting for age. Hence, time of blood sampling has an effect on serum hepcidin concentration that is independent of age and gender.

Finally, we assessed serum hepcidin reference ranges after the additional exclusion of individuals with a serum ferritin concentration below 30 µg/L (Supplemental Table 2.6). Hepcidin reference ranges in these non-iron-deficient subjects were slightly elevated compared to the ranges presented in Table 2.2, especially in premenopausal women.

TABLE 2.2 Reference ranges for serum hepcidin (nM) per 5-year age group for men and women in the reference population.*

	Men (N=1,066)					Women (N=882)				
				95% reference range					95% reference range	
Age, years	N	(%)	Median	P2.5	P97.5	N	(%)	Median	P2.5	P97.5
18-24	10	(1)	9.1	2.3	17.8	21	(2)	2.6	0.7	10.5
25-29	16	(2)	8.4	0.5	24.2	28	(3)	3.1	0.6	11.0
30-34	18	(2)	7.4	0.8	25.0	24	(3)	3.9	0.2	21.0
35-39	22	(2)	6.4	0.7	19.4	36	(4)	3.3	0.5	16.0
40-44	19	(2)	10.2	1.6	17.8	65	(7)	4.8	0.3	24.2
45-49	76	(7)	8.2	1.3	21.0	110	(12)	3.5	0.3	14.6
50-54	106	(10)	7.0	0.3	22.0	140	(16)	5.4	0.4	22.8
55-59	173	(16)	7.7	0.4	24.8	129	(15)	8.5	0.8	21.7
60-64	179	(17)	7.9	0.3	22.7	137	(16)	8.2	1.2	27.3
65-69	186	(17)	9.0	0.5	22.2	95	(11)	8.4	1.4	22.6
70-74	133	(12)	8.4	1.0	26.9	62	(7)	8.7	1.0	37.8
75-79	99	(9)	6.8	0.8	25.5	16	(2)	9.2	2.1	29.0
80-84	22	(2)	6.8	3.5	20.1	10	(1)	11.9	1.6	19.2
≥85	7	(1)	11.3	3.4	20.5	9	(1)	6.7	1.2	24.5
All	1,066	(100)	7.8	0.6	23.3	882	(100)	6.5	0.5	23.2

N indicates number; P2.5, 2.5th percentile; and P97.5, 97.5th percentile.

* See Supplemental Table 2.6 for serum hepcidin reference ranges after the additional exclusion of individuals with a serum ferritin concentration below 30 µg/L.

Biochemical correlates of serum hepcidin concentration

The results of the univariable linear regression analyses are presented in Supplemental Table 2.2. Below, results of regression analyses after adjustment for age and time of blood sampling (Table 2.3) are discussed. These analyses revealed ferritin to be most strongly associated with serum hepcidin concentration. The relation between serum ferritin and serum hepcidin was positive for both men and women with adjusted regression coefficients of 0.806 (95% CI, 0.770; 0.843) and 0.853 (95% CI, 0.813; 0.892), meaning that a 1% change in serum ferritin in µg/L is associated with a 0.81% and 0.85% change in serum hepcidin concentration (nM), respectively. TIBC demonstrated a negative association with serum hepcidin [men: β -0.033 (95% CI, -0.038; -0.028); women: β -0.027 (95% CI, -0.032; -0.022)]. A positive association between BMI and serum hepcidin concentration was observed, but this association was not statistically significant in men. Additional statistically significant associations in men after adjustment for age and time of blood sampling were found for increasing serum hepcidin concentration and iron, TS and CRP (Table 2.3). ALAT and eGFR were not statistically significantly associated. In women, additional statistically significant associations adjusted for age and time of blood sampling were observed for iron, TS, ALAT, eGFR and CRP (Table 2.3).

A sex-specific multivariable model was constructed that included age and time of blood sampling and those variables that showed a p-value<0.05 for association with hepcidin concentration after adjustment for age and time of blood sampling. For men these were ferritin, iron, TIBC and CRP; for women these were BMI, ferritin, iron, TIBC, ALAT, eGFR and CRP (Table 2.3). TS was omitted as it is derived using TIBC and iron. In men, independent correlates in the multivariable model for serum hepcidin concentration were time of blood sampling, ferritin, TIBC and CRP (Table 2.4). In women, independent correlates for serum hepcidin concentration were age, time of blood sampling, ferritin, TIBC, ALAT and eGFR (Table 2.4). ALAT demonstrated a negative association with serum hepcidin concentration, which is opposite to the positive association observed by adjusting for age and time of blood sampling only, indicating confounding influences by other variables. The models explained 59.8% and 63.3% of total serum hepcidin variance in men and women, respectively. Note that unadjusted, univariable analyses revealed that serum ferritin concentration by itself explained 56.0% and 60.2% (Supplemental Table 2.2) for men and women, respectively, indicating that almost all explained variance in the multivariable model was attributable to variation in serum ferritin.

TABLE 2.3 Results of linear regression analyses for serum hepcidin concentrations (nM) adjusted for age and time of blood sampling.

		Men				Women			
			95% CI				95% CI		
Variable		Beta*	Lower limit	Upper limit	R^2, %	Beta*	Lower limit	Upper limit	R^2, %
BMI, kg/m² †		*0.307	-0.063	0.677	3.9	*0.528	0.223	0.832	20.1
Ferritin, µg/L		*0.806	0.770	0.843	58.7	*0.853	0.813	0.892	62.6
Iron, µmol/L		0.009	0.001	0.017	4.0	0.034	0.026	0.042	22.6
TIBC, µmol/L		-0.033	-0.038	-0.028	13.5	-0.027	-0.032	-0.022	25.5
TS, %		0.013	0.009	0.017	6.1	0.025	0.020	0.030	24.8
ALAT, U/L		*0.070	-0.026	0.165	3.4	*0.145	0.051	0.239	19.6
eGFR, mL/min/1.73 m²		-0.001	-0.004	0.001	3.9	-0.005	-0.008	-0.002	20.2
CRP	Less than 5 mg/L	ref	ref	ref		ref	ref	ref	
	5-20 mg/L	0.283	0.158	0.408	6.3	0.230	0.113	0.347	20.8
	More than 20 mg/L	0.775	0.450	1.099		0.712	0.369	1.055	

CI indicates confidence interval; BMI, body mass index; TIBC, total-iron binding capacity; TS, transferrin saturation; ALAT, alanine aminotransferase; eGFR, glomerular filtration rate calculated using the modification of diet in renal disease formula 4; CRP, C-reactive protein; and ref, reference category.

* The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the betas express the changes in log-transformed hepcidin (nM) that are associated with a 1-unit change in the dependent variable, except for betas indicated with an asterix (*), for which both the dependent and independent variable were log-transformed before inclusion in the linear model. Interpretation for these betas is as follows: a 1% change in the independent variable corresponds to a beta% change in the dependent variable.

† BMI was included as continuous variable in the regression models.

TABLE 2.4 Results of multivariable regression models for serum hepcidin concentrations (nM) stratified by gender.

		Men			Women		
			95% CI			95% CI	
Variable		Beta*	Lower limit	Upper limit	Beta*	Lower limit	Upper limit
BMI, kg/m^2 †		NA	NA	NA	*-0.042	-0.274	0.190
Ferritin, µg/L		*0.779	0.740	0.818	*0.830	0.783	0.877
Iron, µmol/L		-0.005	-0.010	0.001	0.003	-0.003	0.009
TIBC, µmol/L		-0.008	-0.012	-0.005	-0.005	-0.009	-0.001
ALAT, U/L		NA	NA	NA	*-0.111	-0.180	-0.043
eGFR, mL/min/1.73 m^2		NA	NA	NA	-0.003	-0.005	-0.001
CRP	Less than 5 mg/L	ref	ref	ref	ref	ref	ref
	5-20 mg/L	0.126	0.042	0.210	0.058	-0.030	0.147
	More than 20 mg/L	0.264	0.047	0.482	0.219	-0.023	0.462
Age, years		0.000	-0.004	0.004	-0.002	-0.007	0.002
Age, <55 vs ≥55 years		-0.101	-0.211	0.009	0.129	0.020	0.238
Time of blood sampling	Before 12 pm	ref	ref	ref	ref	ref	ref
	Between 12 pm and 5 pm	0.336	0.264	0.409	0.389	0.303	0.474
	After 5 pm	0.374	0.277	0.470	0.419	0.306	0.532

CI indicates confidence interval; BMI, body mass index; TIBC, total-iron binding capacity; ALAT, alanine aminotransferase; eGFR, glomerular filtration rate calculated using the modification of diet in renal disease formula 4; CRP, C-reactive protein; vs, versus; NA, not applicable; and ref, reference category.

* The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the betas express the changes in log-transformed hepcidin (nM) that are associated with a 1-unit change in the dependent variable, except for betas indicated with an asterix (*), for which both the dependent and independent variable were log-transformed before inclusion in the linear model. Interpretation for these betas is as follows: a 1% change in the independent variable corresponds to a beta% change in the dependent variable.

† BMI was included as continuous variable in the regression models.

Discussion

Our data provide age and gender specific reference ranges of serum hepcidin concentration in a large reference population. We demonstrated that serum hepcidin concentrations are lower for premenopausal than for postmenopausal women, but are constant over age in men. In addition, our results confirm previous reports about the circadian rhythm of hepcidin. Based on regression analyses results, we report a strong association between serum hepcidin concentration and serum ferritin in both men and women, which explained approximately 60% of total hepcidin variance. This finding withstood adjustment for other (biochemical) variables: BMI, age, iron, TIBC, ALAT, eGFR, CRP and time of blood sampling.

For the calculation of serum hepcidin concentration reference ranges, we selected a reference subset excluding individuals with characteristics evidently influencing hepcidin concentration and thus explicitly warranting specific serum hepcidin reference ranges, namely characteristics that indicate conditions with (a) low hepcidin concentration associated with increased iron demands or decreased synthesis (ALAT>50 U/L, pregnancy and anemia), and (b) increased hepcidin concentration associated with (low grade) inflammation (CRP>10 mg/L and BMI>30 kg/m^2), increased iron concentration (use of iron supplements) or decreased elimination (eGFR<60 mL/min/1.73 m^2) [1,2,8,10,19,21,31-34].

Variation in hepcidin concentration over age differed between men and women. Men showed a stable hepcidin concentration, although a non-significant trend for an age-related increase in serum hepcidin was previously reported based on 65 men [22]. In women, serum hepcidin concentration was substantially higher for postmenopausal than for premenopausal women, although we used age as a proxy in the absence of information on menopausal state of the women. Nevertheless, higher serum hepcidin concentration in postmenopausal women is in agreement with the observation that ferritin concentrations tend to increase sharply as women progress through menopause [35,36]. Serum hepcidin concentrations were elevated for men older than 85 years, supporting a role of hepcidin in anemia of the elderly [37]. However, an opposite effect was observed for women and the number of men and women in this age category may be too low (7 and 9, for men and women, respectively) to draw conclusions.

Our serum hepcidin reference ranges are in concordance with hepcidin concentrations in healthy controls previously reported by our group and others [20,26,29,38]. However, Ganz *et al.* [22] reported much higher median hepcidin concentrations for their series of healthy volunteers. In addition, they found a significant gender difference in serum hepcidin that was far more pronounced than the overall differences between men and women that we observed, which might be due to the lower median age for women (32.6 years) in their study compared to that in our population (55 years). The difference in *absolute* hepcidin concentrations between the two studies could have been caused by the application of different hepcidin assays [39]. In the

past few years, hepcidin assays have been developed on mass spectrometry (MS) platforms and more recently also immunochemical (IC) methods have become available. In patients with diseases associated with substantially elevated hepcidin concentrations or a decrease in renal clearance, we found that our IC assay measured relatively higher concentrations than MS methods, most likely because an IC method lacks the selectivity to distinguish hepcidin-25 from its isoforms hepcidin-20 and -22 that are prevalent in these diseases [25]. However, in our experience with the exploitation of TOF-MS techniques hepcidin isoforms are rare in reference populations [25]. Moreover, the study of Ganz *et al*. [22] and our current study used similar IC methodologies, although antibodies might differ. This apparent difference in absolute hepcidin concentrations between the various assays around the world [39] clearly illustrates the need for harmonization. In this context, it is worth mentioning that in the most recent world-wide send-out of samples (the so called *round robin 2*) which took place in the spring of 2010 among 18 centers, including 22 methods for measuring plasma hepcidin, we obtained data that will allow us to generate regression equations for hepcidin results measured by the various methods in various laboratories and thus to define hepcidin reference ranges for universal use (J.J.K., D.W.S., *et al*., manuscript in preparation).

We observed a trend of increasing hepcidin concentration during the day, consistent with previous reports [22,26,29,30,38]. In addition, this is in agreement with the observation that hepcidin expression is regulated by transcription factors such as Upstream Stimulatory Factor (USF) and c-Myc/Max through E-boxes, as genes that are regulated through E-boxes, including the Clock genes *period*, *timeless* and *clock*, tend to be under circadian rhythmic transcriptional control [40]. However, circadian variation in hepcidin could be secondary as well, *e.g.* driven by variation in iron intake during the day. There is currently no evidence to support either a primary or secondary circadian variation in hepcidin. Nevertheless, because of the daily variations in serum hepcidin concentration, we adjusted for time of blood sampling in our association analyses in order to correct for its potential confounding influence.

Serum ferritin was shown to be the most important correlate of serum hepcidin concentration. A positive regression coefficient was found, indicating that increased serum ferritin concentration is associated with increased serum hepcidin concentration, which has consistently been reported before [19-22]. Iron, TIBC and TS only showed moderate associations with serum hepcidin, but directions of the effects were in accordance with expectations. Increasing iron concentration and TS were associated with increased hepcidin concentrations. This may suggest that TS determines hepcidin concentration, confirming hepcidin's proposed role in counter-regulating increased body iron concentration by decreasing iron absorption and macrophage iron release. However, the cross-sectional design of our study does not allow assessment of causality. TIBC showed a negative relation with serum hepcidin, which corresponds to the observation that TIBC increases in situations of low iron status. The association between hepcidin on one side and serum iron concentration on the other disappeared after incorporation of variables which were significant in a model adjust-

ed for age and time of blood sampling: ferritin, TIBC and CRP for men and BMI, ferritin, TIBC, ALAT, eGFR and CRP for women. Similarly, the association between hepcidin and BMI in women became non-significant after inclusion of these variables in the model. CRP showed a positive and significant association with serum hepcidin concentration, both in men and women, which is in accordance with the theory that inflammation and infection cause an increase in hepcidin production [1,2,4,8-11]. However, this effect was substantially reduced in women after inclusion of BMI, ferritin, TIBC, ALAT and eGFR, indicating that the association between CRP and serum hepcidin was confounded by these variables. ALAT and serum hepcidin concentration showed a positive association when adjusted for age and time of blood sampling only, but this effect became negative after adjusting for BMI, ferritin, iron, TIBC, eGFR and CRP as well. ALAT is a marker of hepatocyte damage, thus it is not surprising that increasing ALAT concentrations are associated with decreasing hepcidin concentrations, since hepcidin is predominantly produced by these cells [2]. Notably, causal relationships cannot be deduced from the regression analyses of the current study.

Our study has some limitations. First, in the absence of a definition of "health" of a reference population, the exclusion criteria applied in this study to generate reference ranges could be debated, even though carefully selected. Moreover, hemoglobin (Hb) values were not measured in our study population, and therefore we used self-reported anemia as a proxy for low Hb concentrations as exclusion criterion for the reference set. In addition to data on presence or absence of anemia, information about BMI, pregnancy and use of iron supplements was also obtained by using self-administered questionnaires. Thus, individuals who were unaware of having one of the characteristics used for exclusion or who reported false answers, might mistakenly be included in the reference subset. However, as we used a large study population, we expect that this potential misclassification has not influenced the hepcidin reference ranges. Furthermore, we applied age as a proxy for menopausal state using a cutoff of 55 years. Finally, we included subjects in the reference set with missing values for exclusion variables, but this did not influence reference ranges (data not shown).

We observed a high degree of interindividual variance in serum hepcidin concentration. This implicates that population-based reference ranges may have limitations when used for the interpretation of individual hepcidin concentrations. It appears that hepcidin values, like other hormones, should be interpreted as (in)appropriate in the context of indices of iron metabolism.

In conclusion, we provide age and gender specific reference ranges of serum hepcidin concentration. Until reliable (commutable) calibrators become available for harmonization of the current differences in hepcidin results obtained by the various assays, our reference ranges can be made appropriate for universal use by exploiting algorithms derived from regular world-wide assay comparison studies. Universal hepcidin reference ranges enable translational scientists as well as physicians in clinical practice around the world to

compare hepcidin concentrations and to collectively define criteria for the use of hepcidin assays in diagnosis, staging, monitoring and assessing treatment indication of iron disorders. Furthermore, our data provide insight in (biochemical) correlates of serum hepcidin concentration, designating serum ferritin as by far the most important associate of serum hepcidin concentration. This association was robust to adjustment for other iron parameters and biochemical variables measured in this study. However, no inference can be made about causality based on our current study, thus it is impossible to state whether it is the serum ferritin concentration that determines the serum hepcidin concentration or the other way around. We emphasize the importance of additional studies that do allow elucidation of causality between hepcidin, ferritin and other (biochemical) variables that may be associated with serum hepcidin concentration but were not evaluated in this study.

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References

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

2 Kemna EHJM, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. Haematologica 2008;93:90-7.

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

4 Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037-44.

5 Nemeth E, Ganz T. Hepcidin and iron-loading anemias [editorial]. Haematologica 2006;91:727-32.

6 Ganz T, Nemeth E. Regulation of iron acquisition and iron distribution in mammals. Biochim Biophys Acta 2006;1763:690-9.

7 Pak M, Lopez MA, Gabayan V, et al. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 2006;108:3730-5.

8 Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271-6.

9 Kemna E, Pickkers P, Nemeth E, et al. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood 2005;106:1864-6.

10 Wessling-Resnick M. Iron homeostasis and the inflammatory response. Annu Rev Nutr 2010;30:105-22.

11 Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med 2005;352:1011-23.

12 Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276:7811-9.

13 Swinkels DW, Janssen MC, Bergmans J, Marx JJ. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. Clin Chem 2006;52:950-68.

14 Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008;40:569-71.

15 Theurl I, Aigner E, Theurl M, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. Blood 2009;113:5277-86.

16 Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? Nephrol Dial Transplant 2008;23:2450-3.

17 Nemeth E. Targeting the hepcidin-ferroportin axis in the diagnosis and treatment of anemias. Adv Hematol 2010;2010:750643.

18 Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: from research to clinic. World J Gastroenterol 2009;15:538-51.

19 Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009;75:976-81.
20 Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.
21 Peters HPE, Laarakkers CMM, Swinkels DW, Wetzels JFM. Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. Nephrol Dial Transplant 2010;25:848-53.
22 Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. Blood 2008;112:4292-7.
23 Bartnikas TB, Andrews NC, Fleming MD. Transferrin is a major determinant of hepcidin expression in hypotransferrinemic mice. Blood 2010;117:630-7.
24 Hoogendoorn EH, Hermus AR, de Vegt F, et al. 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.
25 Kroot JJC, Laarakkers CMM, Geurts-Moespot AJ, et al. Immunochemical and Mass-Spectrometry-Based Serum Hepcidin Assays for Iron Metabolism Disorders. Clin Chem 2010;56:1570-9.
26 Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJC, et al. High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol 2009;146:317-25.
27 Wetzels JFM, Kiemeney LALM, Swinkels DW, et al. Age- and gender-specific reference values of estimated GFR in Caucasians: the Nijmegen Biomedical Study. Kidney Int 2007;72:632-7.
28 Wetzels JFM, Willems HL, den Heijer M. Age- and gender-specific reference values of estimated glomerular filtration rate in a Caucasian population: Results of the Nijmegen Biomedical Study [letter]. Kidney Int 2008;73:657-8.
29 Kroot JJC, Hendriks JCM, Laarakkers CMM, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem 2009;389:124-9.
30 Kemna EHJM, Tjalsma H, Podust VN, Swinkels DW. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin Chem 2007;53:620-8.
31 Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. Eur J Haematol 2010;85:345-52.
32 Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. Gastroenterology 2006;131:788-96.
33 Ruivard M, Lainé F, Ganz T, et al. Iron absorption in dysmetabolic iron overload syndrome is decreased and correlates with increased plasma hepcidin. J Hepatol 2009;50(6):1219-25.
34 del Giudice EM, Santoro N, Amato A, et al. Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. J Clin Endocrinol Metab 2009;94:5102-7.

35 Koziol JA, Ho NJ, Felitti VJ, Beutler E. Reference centiles for serum ferritin and percentage of transferrin saturation, with application to mutations of the HFE gene. Clin Chem 2001;47:1804-10.
36 Cook JD, Finch CA, Smith NJ. Evaluation of the iron status of a population. Blood 1976;48:449-55.
37 Ferrucci L, Semba RD, Guralnik JM, et al. Proinflammatory state, hepcidin, and anemia in older persons. Blood 2010;115:3810-6.
38 Busbridge M, Griffiths C, Ashby D, et al. Development of a novel immunoassay for the iron regulatory peptide hepcidin. Br J Biomed Sci 2009;66:150-7.
39 Kroot JJC, Kemna EHJM, Bansal SS, et al. Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. Haematologica 2009;94:1748-52.
40 Bayele HK, McArdle H, Srai SKS. Cis and trans regulation of hepcidin expression by upstream stimulatory factor. Blood 2006;108:4237-45.

SUPPLEMENTAL TABLE 2.1 Characteristics of the selected reference population (N=2,869).

Variable		Men (N=1,066)				Women (N=882)			
		N*	(%)	Median	P2.5-P97.5	N*	(%)	Median	P2.5-P97.5
Age, years		1,066	(100)	62	30-80	882	(100)	55	25-77
Ferritin, µg/L		1,064	(100)	153.7	17.0-568.8	882	(100)	77.9	9.6-337.9
Iron, µmol/L		1,059	(99)	18	9-32	879	(100)	17	7-31
TIBC, µmol/L		1,059	(99)	58	44-78	879	(100)	60	45-83
TS, %		1,059	(99)	31.1	14.2-58.5	879	(100)	28.2	10.1-51.2
ALAT, U/L		1,032	(97)	13	5-32	862	(98)	10	4-27
eGFR, mL/min/1.73 m^2		1,055	(99)	84.5	62.6-124.7	872	(99)	82.9	62.3-119.8
CRP, mg/L	Less than 5 mg/L	937	(88)			767	(87)		
	5-20 mg/L	119	(13)			112	(13)		
	More than 20 mg/L								
	Unknown	10	(1)			3	(0)		
BMI, kg/m2	Less than 18 kg/m^2	1	(0)			8	(1)		
	18-25 kg/m^2	521	(49)			538	(61)		
	25-40 kg/m^2	524	(49)			310	(35)		
	More than 40 kg/m^2								
	Unknown	20	(2)			26	(3)		
Current use of iron supplements	Yes								
	No	956	(90)			764	(87)		
	Unknown	110	(10)			118	(13)		
Pregnant	Yes								
	No					851	(97)		
	Unknown					31	(4)		
Regular menstruation	Yes					305	(35)		
	No					546	(62)		
	Unknown					31	(4)		
Self-reported anemia	Yes								
	No	992	(93)			809	(92)		
	Unknown	74	(7)			73	(8)		
Current blood and/or plasma donor	Yes	375	(35)			240	(27)		
	No	681	(64)			628	(71)		
	Unknown	10	(1)			14	(2)		
Time of blood sampling	Before 12 pm	249	(23)			195	(22)		
	Between 12 pm and 5 pm	592	(56)			535	(61)		
	After 5 pm	220	(21)			151	(17)		
	Unknown	5	(0)			1	(0)		

N indicates number; P2.5, 2.5th percentile; P97.5, 97.5th percentile; TIBC, total-iron binding capacity; TS, transferrin saturation; ALAT, alanine aminotransferase; eGFR, glomerular filtration rate calculated using the modification of diet in renal disease formula 4; CRP, C-reactive protein; BMI, body mass index.

* Numbers are different from the total number of included persons due to missing values.

SUPPLEMENTAL TABLE 2.2 Results of unadjusted linear regression analyses for serum hepcidin concentrations (nM).

		Men				Women			
			95% CI				95% CI		
Variable		Beta*	Lower limit	Upper limit	R^2, %	Beta*	Lower limit	Upper limit	R^2, %
BMI, kg/m2 †		*0.400	0.027	0.773	0.2	*1.174	0.851	1.496	3.2
Ferritin, µg/L		*0.808	0.771	0.845	56.0	*0.876	0.841	0.912	60.2
Iron, µmol/L		0.007	0.000	0.015	0.2	0.025	0.016	0.034	1.8
TIBC, µmol/L		-0.033	-0.038	-0.028	9.7	-0.036	-0.041	-0.031	11.8
TS, %		0.012	0.007	0.016	2.0	0.024	0.019	0.029	5.3
ALAT, U/L		*0.056	-0.037	0.149	0.0	*0.215	0.112	0.318	1.0
eGFR, mL/min/1.73 m^2		-0.002	-0.005	0.000	0.1	-0.013	-0.015	-0.010	5.5
CRP	Less than 5 mg/L	ref	ref	ref		ref	ref	ref	
	5-20 mg/L	0.292	0.168	0.416	2.6	0.308	0.180	0.437	1.8
	More than 20 mg/L	0.784	0.454	1.113		0.633	0.253	1.014	
Age, years		0.003	-0.001	0.007	0.1	0.029	0.025	0.032	13.9
Age, <55 vs ≥55 years		0.091	-0.021	0.203	0.1	0.840	0.745	0.935	16.1
Time of blood sampling	Before 12 pm	ref	ref	ref		ref	ref	ref	
	Between 12 pm and 5 pm	0.427	0.315	0.539	3.7	0.416	0.287	0.546	3.1
	After 5 pm	0.366	0.225	0.508		0.080	-0.087	0.247	

CI indicates confidence interval; BMI, body mass index; vs, versus; TIBC, total-iron binding capacity; TS, transferrin saturation; ALAT, alanine aminotransferase; eGFR, glomerular filtration rate calculated using the modification of diet in renal disease formula 4; CRP, C-reactive protein; and ref, reference category.

* The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the betas express the changes in log-transformed hepcidin (nM) that are associated with a 1-unit change in the dependent variable, except for betas indicated with an asterix (*), for which both the dependent and independent variable were log-transformed before inclusion in the linear model. Interpretation for these betas is as follows: a 1% change in the independent variable corresponds to a beta% change in the dependent variable.

† BMI was included as continuous variable in the regression models

SUPPLEMENTAL TABLE 2.3

Reference values for serum hepcidin (nM) per 5-year age group for both men and women who donated blood before 12 pm.

	Men (N=249)					Women (N=195)				
				95% reference range					95% reference range	
Age, years	N	(%)	Median	P2.5	P97.5	N	(%)	Median	P2.5	P97.5
18-24	3	(1)	5.5	3.1	7.9	6	(3)	1.9	0.8	3.5
25-29	3	(1)	5.7	0.5	6.3	7	(4)	1.9	0.6	6.3
30-34	2	(1)	6.2	5.1	7.3	3	(2)	3.3	1.3	4.5
35-39	5	(2)	3.3	0.7	4.9	6	(3)	3.0	0.7	5.4
40-44	6	(2)	5.3	3.1	14.6	13	(7)	1.9	0.4	6.7
45-49	19	(8)	5.3	0.8	15.6	30	(15)	1.8	0.3	12.9
50-54	21	(8)	6.3	1.1	19.1	32	(16)	4.6	0.0	9.8
55-59	29	(12)	5.2	0.4	16.1	22	(11)	5.8	0.8	17.6
60-64	41	(16)	6.4	0.3	20.8	31	(16)	7.6	2.0	30.2
65-69	39	(16)	6.9	0.2	20.4	20	(10)	6.6	1.3	18.7
70-74	44	(18)	6.1	0.2	15.5	14	(7)	6.4	2.2	18.3
75-79	32	(13)	5.1	1.3	31.3	2	(1)	12.3	7.6	16.9
80-84	4	(2)	6.6	3.8	9.9	5	(3)	11.5	1.6	14.3
≥85	1	(0)	NA	NA	NA	4	(2)	5.7	3.3	10.4
All	249	(100)	5.9	0.5	18.8	195	(100)	4.5	0.4	18.5

N indicates number; P2.5, 2.5th percentile; P97.5, 97.5th percentile; and NA, not applicable.

SUPPLEMENTAL TABLE 2.4

Reference values for serum hepcidin (nM) per 5-year age group for both men and women who donated blood between 12 and 5 pm.

	Men (N=592)					Women (N=535)				
				95% reference range					95% reference range	
Age, years	N	(%)	Median	P2.5	P97.5	N	(%)	Median	P2.5	P97.5
18-24	3	(1)	11.6	2.3	17.8	8	(1)	2.8	0.7	10.5
25-29	6	(1)	7.5	4.4	18.5	10	(2)	3.7	1.8	10.9
30-34	6	(1)	9.8	0.8	25.0	14	(3)	3.3	0.2	21.0
35-39	7	(1)	7.2	1.1	13.5	22	(4)	3.7	0.5	16.0
40-44	7	(1)	12.8	1.6	17.8	39	(7)	5.1	0.3	27.0
45-49	28	(5)	10.0	2.5	29.3	49	(9)	3.9	0.0	14.2
50-54	48	(8)	7.5	1.4	22.4	79	(15)	5.9	0.5	31.9
55-59	79	(13)	9.7	0.2	26.8	83	(16)	9.1	0.8	27.1
60-64	116	(20)	8.0	0.6	24.1	93	(17)	7.8	1.1	27.3
65-69	130	(22)	9.7	0.8	22.5	68	(13)	9.8	2.2	23.5
70-74	83	(14)	9.2	1.0	28.5	47	(9)	9.6	0.7	40.5
75-79	57	(10)	8.5	0.7	25.1	14	(3)	9.2	2.1	29.0
80-84	17	(3)	6.9	3.5	20.1	5	(1)	13.5	7.5	19.2
≥85	5	(1)	14.1	3.4	20.5	4	(1)	6.4	1.2	24.5
All	592	(100)	9.0	0.8	24.0	535	(100)	7.4	0.5	24.8

N indicates number; P2.5, 2.5th percentile; and P97.5, 97.5th percentile.

SUPPLEMENTAL TABLE 2.5

Reference values for serum hepcidin (nM) per 5-year age group for both men and women who donated blood after 5 pm.

	Men (N=220)					Women (N=151)				
				95% reference range					95% reference range	
Age, years	N	(%)	Median	P2.5	P97.5	N	(%)	Median	P2.5	P97.5
18-24	4	(2)	10.8	2.9	13.8	7	(5)	2.8	1.1	8.6
25-29	7	(3)	9.8	5.6	24.2	11	(7)	4.8	0.6	11.0
30-34	10	(5)	7.4	2.1	15.8	7	(5)	5.4	1.6	9.0
35-39	10	(5)	9.2	3.4	19.4	8	(5)	2.3	0.9	8.9
40-44	6	(3)	9.3	1.9	15.0	12	(8)	5.0	0.1	11.0
45-49	29	(13)	6.9	1.4	20.4	31	(21)	4.3	0.4	23.4
50-54	37	(17)	6.3	0.0	27.3	29	(19)	7.0	0.6	23.7
55-59	63	(29)	8.2	0.4	27.5	24	(16)	9.0	2.3	21.1
60-64	22	(10)	9.7	0.3	20.7	13	(9)	10.8	5.5	21.2
65-69	16	(7)	11.2	0.3	17.9	7	(5)	3.8	0.5	9.5
70-74	6	(3)	10.7	2.0	18.1	1	(1)	NA	NA	NA
75-79	8	(4)	6.0	1.2	25.5	0	(0)	NA	NA	NA
80-84	1	(0)	NA	NA	NA	0	(0)	NA	NA	NA
≥85	1	(0)	NA	NA	NA	1	(1)	NA	NA	NA
All	220	(100)	8.4	0.4	24.1	151	(100)	6.1	0.6	21.2

N indicates number; P2.5, 2.5th percentile; P97.5, 97.5th percentile; and NA, not applicable.

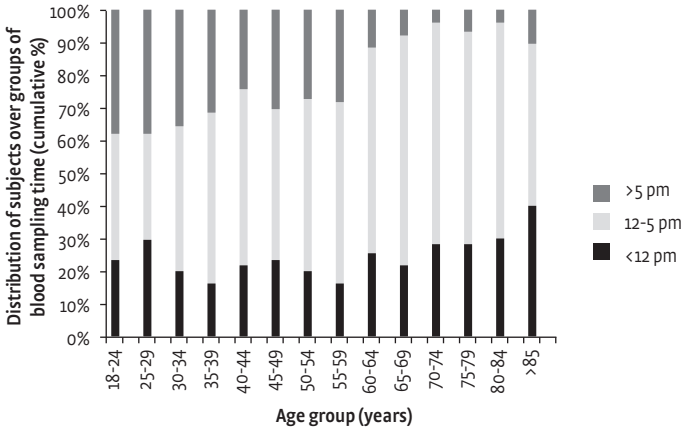
SUPPLEMENTAL TABLE 2.6

Reference ranges for serum hepcidin (nM) per 5-year age group for men and women in the reference population with additional exclusion of subjects with serum ferritin concentration below 30 µg/L.

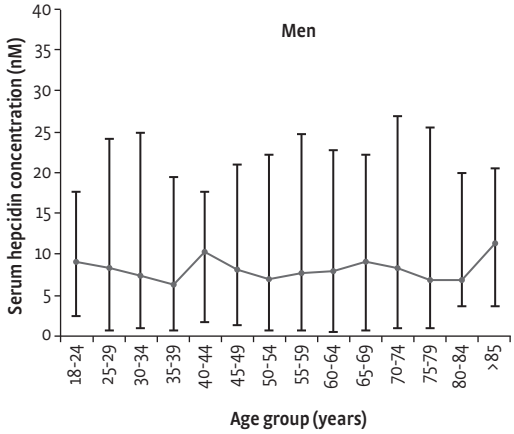
	Men (N=1,003)					Women (N=725)				
				95% reference range					95% reference range	
Age, years	N	(%)	Median	P2.5	P97.5	N	(%)	Median	P2.5	P97.5
18-24	10	(1)	9.1	2.3	17.8	13	(2)	3.0	1.7	10.5
25-29	15	(1)	9.2	4.4	24.2	16	(2)	5.5	1.1	11.0
30-34	17	(2)	7.5	0.8	25.0	17	(2)	5.4	1.3	21.0
35-39	21	(2)	7.1	0.7	19.4	22	(3)	5.2	1.1	16.0
40-44	18	(2)	10.6	1.9	17.8	43	(6)	5.6	1.7	25.8
45-49	71	(7)	7.1	1.9	22.1	73	(10)	5.2	1.0	16.0
50-54	96	(10)	7.7	1.5	22.2	115	(16)	7.0	1.4	24.6
55-59	161	(16)	8.3	1.9	25.0	120	(17)	9.1	1.8	21.7
60-64	165	(16)	8.3	1.5	23.1	125	(17)	9.1	3.4	27.4
65-69	175	(17)	9.5	1.5	22.3	89	(12)	8.5	2.2	22.8
70-74	129	(13)	8.5	1.5	27.4	58	(8)	9.2	1.7	38.5
75-79	96	(10)	7.4	1.0	25.5	16	(2)	9.2	2.1	29.0
80-84	22	(2)	6.8	3.5	20.1	10	(1)	11.9	1.6	19.2
≥85	7	(1)	11.3	3.4	20.5	8	(1)	7.1	3.3	24.5
All	1,003	(100)	8.4	1.6	23.7	725	(100)	7.6	1.7	23.9

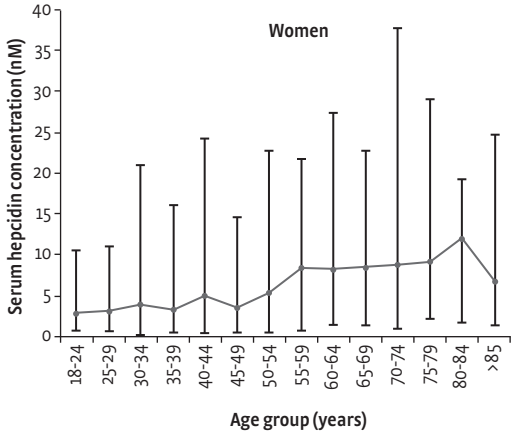
N indicates number; P2.5, 2.5th percentile; P97.5, 97.5th percentile; and NA, not applicable.

SUPPLEMENTAL FIGURE 2.1 Distribution of blood sampling time per 5-year age group.



SUPPLEMENTAL FIGURE 2.2 Serum hepcidin concentration (nM) per 5-year age group. Median values with 2.5th and 97.5th percentile are shown.





CHAPTER 3

Associations of common variants in *HFE* and *TMPRSS6* with iron parameters are independent of serum hepcidin in a general population: a replication study

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ABSTRACT

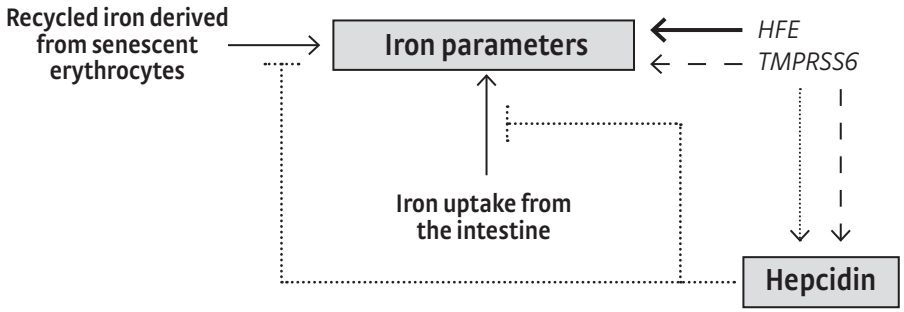
Genome-wide association studies have convincingly shown that single nucleotide polymorphisms (SNPs) in *HFE* and *TMPRSS6* are associated with iron parameters. It was commonly thought that these associations could be explained by the intermediate effect on hepcidin concentration. A recent study in an isolated Italian population, however, concluded that these associations were not exclusively dependent on hepcidin values. Here, we are the second to investigate the role of hepcidin in the associations between common variants in *HFE* and *TMPRSS6* with iron parameters. We extracted 101 SNPs in *HFE* and *TMPRSS6* from genome-wide imputed SNP data of 1832 individuals from the general population (Nijmegen Biomedical Study). Single locus and haplotype associations with serum iron parameters and hepcidin were studied using linear regression analyses. We found that *HFE* rs1800562 and *TMPRSS6* rs855791 are main determinants of *HFE* and *TMPRSS6*-related variation in serum iron, ferritin, transferrin saturation and total iron binding capacity. These SNPs are associated with the ratios hepcidin/ferritin ($p<1x10^{-5}$) and hepcidin/transferrin saturation ($p<1x10^{-3}$), but not with serum hepcidin ($p>0.2$). Adjustment for hepcidin or the ratio hepcidin/ferritin did not decrease the strength of the SNP-iron parameter associations. Our results do not support an intermediate role for hepcidin in the SNP–iron parameter associations, which confirms previous findings, and indicate a pleiotropic SNP effect on the hepcidin ratios and the iron parameters. Taken together, this suggests that there might be other, yet unknown, serum hepcidin-independent mechanisms which play a role in the association of *HFE* and *TMPRSS6* variants with serum iron parameters.

Introduction

Genome-wide association studies (GWAS) have shown that at a population level single nucleotide polymorphisms (SNPs) in the hemochromatosis gene (*HFE*) and in the transmembrane serine protease 6 gene (*TMPRSS6*) are associated with ferritin, iron, transferrin and transferrin saturation (TS) (*i.e.* iron parameters). These associations have been found for the SNPs rs1800562 in *HFE* (p.Cys282Tyr), rs855791 in *TMPRSS6* (p.Ala736Val) and rs4820268 in *TMPRSS6* (p.Asp521Asp) [1-6]. The proteins encoded by *HFE* and *TMPRSS6*, Hfe and matriptase 2 (MT2) respectively, have been suggested to play a role in the transcriptional regulation of the hepatic peptide hormone hepcidin, key regulator of iron homeostasis [7-12]. It was commonly thought that the reported GWAS-associations between the *HFE* and *TMPRSS6* SNPs and iron parameters could be explained by this intermediate effect on hepcidin concentration. However, serum hepcidin concentrations were not measured in these GWAS, preventing a definite evaluation of this assumption. Recently, Traglia and colleagues [3] analyzed serum hepcidin concentrations, measured by a mass spectrometry based method [13], in 1657 related individuals from the Val Borbera genetic isolate in Northern Italy. They explored relationships between hepcidin and a set of anthropometric, haematologic and iron parameters and performed a GWAS for hepcidin. In the same paper, they also focused on the association of two common variants *HFE* rs1800562 and *TMPRSS6* rs855791 with iron, erythrocyte parameters and hepcidin values in 1545 genotyped individuals. They reported that their study allowed to conclude that associations between these SNPs and iron parameters were not exclusively dependent on hepcidin values. This unexpected finding has not been replicated in other populations yet.

In this study, we aim to evaluate the role of hepcidin in the association between *HFE* and *TMPRSS6*-related SNP variation and iron parameters in a second, independent population. We used data from the Nijmegen Biomedical Study (NBS) to analyze the associations between common variants in and surrounding the *HFE* and *TMPRSS6* genes and iron parameters and hepcidin on a population level. More specifically, the first goal of our study was to replicate the associations previously found in the iron GWAS and to determine which SNPs in both genes are main determinants of iron parameters in our population. These analyses revealed that out of the studied SNPs, *HFE* rs1800562 and *TMPRSS6* rs855791 were most strongly associated with the iron parameters. Secondly, we focused on these SNPs and evaluated the role of serum hepcidin in the associations of *HFE* and *TMPRSS6* variants with iron parameters. We also included ratios of hepcidin to ferritin and TS given the known dependence of hepcidin expression on stored iron and circulating iron, respectively [14-19]. We (i) considered hepcidin and its ratio to ferritin as intermediate variables in the association between the SNPs and iron parameters, (ii) explored the presence of pleiotropy, *i.e.* whether the SNPs both independently affect hepcidin and the iron parameters, and (iii) evaluated the presence of an effect on iron parameters only (Figure 3.1).

FIGURE 3.1 Hypothetical roles of serum hepcidin in the associations of *HFE* and *TMPRSS6* variants with serum iron parameters.



(i) Hepcidin and its ratio to ferritin might be intermediate in the association between *HFE* and *TMPRSS6* variants and iron parameters (dotted arrow).
(ii) The variants might both independently affect hepcidin and the iron parameters (dashed arrows), *i.e.* presence of pleiotropy.
(iii) The variants may affect the iron parameters only and not hepcidin (bold arrow).

Methods

Study population

This study was performed in participants from the Nijmegen Biomedical Study (NBS). Details of the NBS have been described before [20]. Briefly, the NBS 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. Approval to conduct the study was obtained from the Radboud University Medical Centre Institutional Review Board. Age- and sex-stratified randomly selected adult inhabitants of Nijmegen (n=22,451), a city located in the eastern part of the Netherlands, received an invitation to fill out a postal questionnaire 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 questionnaire, of which 6468 (69%) responders donated blood samples. All participants gave written informed consent for participation in the NBS.

Genotype data were available for 1832 out of the 6468 blood samples, because they were selected to serve as controls in GWAS [21]. For all these samples, measurements of hepcidin, iron parameters (iron, ferritin, TS and TIBC), liver enzyme alanine aminotransferase (ALT), creatinine, and C-reactive protein (CRP) were available. In addition, the following variables were extracted from the self-administered questionnaire: length, weight (used to derive body mass index [BMI]), age, use of iron supplements for at least 6 months at time of blood donation, presence of anemia determined by a physician, and pregnancy.

Laboratory methods

Serum hepcidin, total serum iron, TIBC, TS and ferritin were measured as described before [22]. Serum hepcidin concentration was measured with an in house developed and validated competitive enzyme-linked immunosorbent assay [22,23]. In short, 96-well plates were coated with goat anti-rabbit IgG, blocked with bovine serum albumin and incubated with an in-house raised rabbit anti-human hepcidin antibody [24]. Standards, study samples and a reference sample were added and thereafter incubated with streptavidin-β-peroxidase conjugate. A color reaction was started by adding O-phenylenediamine substrate. Optical density was measured at 492 nm in an automated c-enzyme-linked immunosorbent assay reader as a read out. The analytical sensitivity, defined as the minimum hepcidin concentration evoking a response significantly different from that of the zero calibrator, was 8.96 pM. As the samples were 20-fold diluted, samples found to have a hepcidin concentration less than 179 pM (20 x 8.96pM) were imputed with a random value out of a uniform distribution with a minimum of 0 nM and a maximum of 0.18 nM (n=9). Hepcidin concentrations are expressed in nanomoles per liter; 1 nM serum hepcidin equals 2.79 µg/L.

Genotyping and selection of SNPs

A total of 1980 participants of the NBS were genotyped with the Illumina HumanHapCNV370-Duo BeadChip [21]. Pre-imputation quality control criteria included sample yield ≥96%, Caucasian ancestry ≥90% (based on Structure analysis), SNP yield ≥96%, minor allele frequency (MAF) ≥1%, and Hardy-Weinberg equilibrium (HWE) p-value $>10^{-6}$. This resulted in 1832 samples and 312,199 SNPs available for imputation, which was performed with CEU HapMap Phase II (release 22, NCBI build 36, dbSNP build 126) as a reference sample using IMPUTE software [25]. After imputation, a total of 2,542,995 SNPs was available. Genotype data for the SNPs within the genes *HFE* and *TMPRSS6* were extracted for the purpose of this study, including SNPs within the 10 kB surrounding region on each side of both genes. Imputed SNPs were transformed to hard calls using a genotype probability threshold of 0.9: genotypes that did not exceed the probability threshold of 0.9 were set to missing. Selection of SNPs with MAF ≥1% and a HWE p-value $>10^{-3}$ resulted in the inclusion of 35 SNPs (7 genotyped) for *HFE* and 66 SNPs (16 genotyped) for *TMPRSS6* (Supplemental Table 3.1). All imputed SNPs that were included in our study, had a quality of imputation of >0.4 as measured using the SNPtest proper-info measure.

Haplotype analyses were applied to uncover allelic interactions. We did not focus on the identification of associations with unmeasured variants in LD with the haplotypes for which the prior chance is low given the availability of high density imputed SNP data. In the haplotype analysis only non-synonymous SNPs and SNPs known to influence expression of *HFE* or *TMPRSS6* were included. With regard to the latter, SNPs were selected based on the following expression databases: SCAN [26], the Chicago eQTL browser from the Pritchard lab (http://eqtl.uchicago.edu), seeQTL [27] and GTEx (Genotype-Tissue Expression) eQTL Browser (http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi). We only included SNPs that were

found to influence expression in liver tissue samples or in blood samples converted into cell lines (*e.g.* HapMap CEU cell lines). The variant selection procedure resulted in the inclusion of the SNPs rs855791 (p.Ala736Val), rs2235324 (p.Lys253Glu) and rs2160906 for *TMPRSS6* and rs1800562 (p.Cys282Tyr), rs1799945 (p.His63Asp) and rs198853 for *HFE*. These SNPs are non-synonymous variants except for rs2160906 and rs198853, which are eQTL located in an intron of *TMPRSS6* and in the 10 kb flanking region of *HFE*, respectively. There was no linkage disequilibrium between the variants within the genes (r-squared < 0.2).

Statistical analysis

The variables hepcidin, ferritin and the ratios of hepcidin to ferritin and hepcidin to TS were skewed towards higher values and therefore log-transformed to normalize their distributions. Outliers, defined as values that differed more than three times the standard deviation (SD) from the mean, were reduced to mean ± 3 SD (maximal number of outliers per trait: 26). Two different subsets were created. The first one was selected based on the same criteria as used by Traglia *et al*.[3]: exclusion of persons with CRP >10 mg/L or ferritin <30 µg/L. The second subset was selected based on the same criteria as previously[22] excluding persons with characteristics evidently influencing hepcidin concentration. The exclusion criteria were: pregnancy at time of blood sampling, ALT >50 U/L, CRP >10 mg/L, eGFR <60 mL/min/1.73 m^2, use of iron supplements, presence of anemia, or BMI >30 kg/m^2. Results for the subset were compared with results for the whole cohort to investigate whether the results for the whole cohort were influenced by extreme values on these variables.

Association analyses were performed using Plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/)[28]. The associations between the SNPs and the traits were evaluated using linear regression analyses adjusted for age, gender and time of blood sampling, since these variables are independent determinants of serum hepcidin[22]. We coded the most frequent homozygote genotype as 0 (reference), heterozygotes as 1, and the less frequent homozygote genotype as 2 and applied a genotypic (2 degrees of freedom) test to allow for deviation from additivity. The resulting regression coefficients express the mean change in the independent variable for the heterozygous and minor homozygous genotype relative to the reference genotype. In case of log-transformation of the independent variables, regression coefficients express the mean change in the log-transformed variable relative to the reference genotype.

Haplotypes were constructed based on inclusion of all SNPs fulfilling selection criteria in one gene. Haplotypes with estimated frequencies of 1% or higher were included in the haplotype analyses. For each trait, a haplotype association model was compared to the allelic effect of *TMPRSS6* rs855791 or *HFE* rs1800562 in order to conclude whether there was an additional effect of the haplotypes on top of the marginal allelic effect of *TMPRSS6* rs855791 or *HFE* rs1800562 alone.

Conditional analyses to estimate the effect of a gene variant or haplotype independent of the effect of another variant was performed by including the latter as covariate in a multivariable regression model.

Due to multiple testing issues, the nominal significance level of 0.05 is not sufficient to maintain an overall study false-positive rate of 5%. Application of a Bonferroni correction for the number of tested SNPs (*i.e.* 35 + 66 = 101) would lead to a p-value threshold of significance of $5x10^{-4}$.

Results

Characteristics of the study population

Mean age of the 1832 individuals was 62 years. Additional characteristics of the individuals included in the study are shown in Table 3.1. Application of the exclusion criteria resulted in the inclusion of 1505 individuals in subset 1 and 1177 individuals in subset 2 (Supplemental Table 3.2). Characteristics of the subsets and the whole cohort were similar, except for the proportion of females (51% in the whole cohort versus 47% in subset 1 and 44% in subset 2) and geometric mean hepcidin and ferritin concentration in subset 1 (6.4 nM and 107.2 µg/L in the whole cohort and 7.7 nM and 131.5 µg/L in subset 1, respectively).

TABLE 3.1 Characteristics of the total study population (N=1832).

		N*	%	Mean (SD)
Gender	Males	906	49	NA
	Females	926	51	NA
Age, years		1832	100	61.5 (10.3)
Time of blood sampling	Before 12 pm	378	21	NA
	Between 12 pm and 5 pm	1172	64	NA
	After 5 pm	274	15	NA
	Unknown	8	0	NA
Hepcidin, nM†		1832	100	6.4 (2.6)
Ferritin, µg/L†		1830	100	107.2 (2.6)
Ratio hepcidin/ferritin, nmoles/µg†		1830	100	59.4 (1.9)
Ratio hepcidin/TS, nM/%†		1812	99	0.23 (2.6)
Iron, µM		1812	99	17.2 (5.6)
TS, %		1812	99	29.6 (10.3)
TIBC, µM		1812	99	59.2 (9.0)

N indicates number; SD, standard deviation; NA, not applicable.

* Numbers are different from the total number of included persons because of missing values.

† The variables hepcidin, ferritin, ratio hepcidin/ferritin and ratio hepcidin/TS were log-transformed, and therefore geometric mean and SD are given.

Associations of common variants in *HFE* and *TMPRSS6* with ferritin, iron, TS and TIBC

Single SNP association analyses revealed that the SNP *HFE* rs1800562 was the strongest associate of ferritin, iron, TS and TIBC of all variants in and surrounding *HFE* included in our study (p between $1x10^{-18}$ and $1x10^{-3}$) (Supplemental Table 3.3). For *TMPRSS6*, rs855791 was most strongly associated with both iron and TS of all variants tested ($p=3.4x10^{-12}$ and $8.5x10^{-14}$, respectively). In addition, this SNP was one of the strongest associates of TIBC and ferritin, although not significantly associated (p=0.13 and 0.19, respectively) (Supplemental Table 3.3). Besides these two SNPs, other common variants in *HFE* and *TMPRSS6* showed associations (using the stringent Bonferroni corrected significance threshold of $p<5x10^{-4}$) with iron and TS: 10 SNPs for *HFE* and iron, 11 SNPs for *HFE* and TS, 11 SNPs for *TMPRSS6* and iron and 22 SNPs for *TMPRSS6* and TS. However, all of these associations for *TMPRSS6* and most of the associations for *HFE* were dependent on *TMPRSS6* rs855791 and *HFE* rs1800562, respectively, as shown by conditional analyses (Supplemental Table 3.4). Only rs1799945 (p.His63Asp), rs6918586, rs198855 and rs198851 (all three in flanking regions) in *HFE* were significantly associated with both iron and TS after conditioning on *HFE* rs1800562, but their strength of association did not approach that of *HFE* rs1800562.

Evaluation of allelic interaction between non-synonymous and eQTL variants via haplotype analysis (Supplemental Tables 3.5-3.6) suggested the presence of a haplotype effect for *HFE* that was independent of the marginal effect of *HFE* rs1800562. Still, this SNP was the greatest contributor to the haplotype effect. There was no evidence for allelic interaction within *TMPRSS6*. These results confirm *HFE* rs1800562 and *TMPRSS6* rs855791 as most important variants within *HFE* and *TMPRSS6*, respectively, in their ability to affect the iron phenotypes.

Table 3.2 shows the associations between *TMPRSS6* rs855791 and *HFE* rs1800562 with the iron parameters. Both SNPs are c.G>A SNPs, with a MAF for the A allele of 0.455 for *TMPRSS6* rs855791 and 0.063 for *HFE* rs1800562 in the total study population. The SNPs showed the strongest association with iron and TS. The minor allele A of *TMPRSS6* rs855791 showed decreased iron concentration [beta (95% CI) AG vs. GG -1.5 (-2.1 to -0.9); AA vs. GG -2.5 (-3.2 to -1.8)] and TS [beta (95% CI) AG vs. GG -2.9 (-4.0 to -1.9); AA vs. GG -5.0 (-6.3 to -3.7)], while the minor allele A of *HFE* rs1800562 was associated with both increased iron concentrations [beta (95% CI) AG vs. GG 2.3 (1.5 to 3.0); AA vs. GG 10.7 (5.5 to 15.9)] and TS [beta (95% CI) AG vs. GG 5.4 (4.0 to 6.8); AA vs. GG 22.6 (13.2 to 32.0)]. Ferritin and TIBC were associated with *HFE* rs1800562, but not with *TMPRSS6* rs855791. The A allele of *HFE* rs1800562 is associated with an increase of ferritin and a decrease of TIBC, respectively.

Results for the two subsets were comparable to the results observed for the total study population, indicating that our findings are not driven by extremes on the exclusion variables (Supplemental Tables 3.7-3.8).

Role of hepcidin in the associations of *HFE* rs1800562 and *TMPRSS6* rs855791 with the iron parameters

Results of the association analyses of *HFE* rs1800562 and *TMPRSS6* rs855791 with serum hepcidin, the ratio of hepcidin to ferritin and the ratio of hepcidin to TS are presented in Table 3.2 (see Supplemental Tables 3.7-3.8 for results in the subsets). The SNPs were not associated with hepcidin ($p > 0.2$) but were by far the strongest associates of the ratios out of all variants tested in our study (Supplemental Table 3.9). *TMPRSS6* rs855791 and *HFE* rs1800562 were associated with an increase and decrease, respectively, in both log hepcidin/ferritin [*TMPRSS6* rs855791: beta (95%CI) AG vs. GG 0.04 (0.01 to 0.07), AA vs. GG 0.08 (0.05 to 0.12); *HFE* rs1800562: beta (95% CI) AG vs. GG -0.04 (-0.08 to -0.01), AA vs. GG -0.65 (-0.90 to -0.41)] and log hepcidin/TS [*TMPRSS6* rs855791: beta (95%CI) AG vs. GG 0.05 (0.01 to 0.09), AA vs. GG 0.12 (0.07 to 0.17); *HFE* rs1800562: beta (95% CI) AG vs. GG -0.07 (-0.13 to -0.02), AA vs. GG -0.58 (-0.96 to -0.20)]. Stratification of the study population by both *HFE* rs1800562 and *TMPRSS6* rs855791 and subsequent calculation of mean hepcidin concentrations per stratum indicated the presence of statistical interaction, but our sample size was not sufficient to reach statistical significance (Supplemental Table 3.10).

The associations of *TMPRSS6* rs855791 and *HFE* rs1800562 with ferritin, iron, TS and TIBC were not dependent on serum hepcidin: regression coefficients for the associations did not change after inclusion of serum hepcidin concentrations in the regression models (Table 3.2). P-values for the associations of the SNPs with iron, TS and TIBC before and after adjustment for serum hepcidin were similar, but smaller for the associations of the SNPs with log ferritin after adjustment for serum hepcidin. Inclusion of the hepcidin/ferritin ratio as covariate in the regression models did not change the associations either and only decreased the p-values for the associations of the SNPs with log ferritin (Table 3.2). Identical observations were done for subset 1 and 2 (Supplemental Tables 3.7-3.8). We did not correct the associations for the hepcidin/TS ratio, because TS was calculated by dividing serum iron by TIBC.

TABLE 3.2 Associations between *TMPRSS6* rs855791 and *HFE* rs1800562 with iron parameters for the total study population (N=1832). Associations are adjusted for age, gender, time of blood sampling and additionally for serum hepcidin or the ratio hepcidin/ferritin.

					AG		AA	
		Additionally adjusted for:	Total N*	*P*	N	Beta AG vs GG (95% CI)	N	Beta AA vs GG (95% CI)
TMPRSS6 rs855791†	Log(hepcidin)‡, nM	NA	1824	2.02E-01	928	0.01 (-0.03; 0.05)	365	0.05 (-0.01; 0.10)
	Log(ferritin)‡, µg/L	NA	1822	1.86E-01	926	-0.03 (-0.07; 0.01)	365	-0.04 (-0.09; 0.01)
		Log(hepcidin)	1822	1.36E-05	926	-0.04 (-0.06; -0.01)	365	-0.07 (-0.10; -0.04)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1822	8.60E-06	926	0.04 (0.01; 0.07)	365	0.08 (0.05; 0.12)
	Log(hepcidin/TS)‡, nM/%	NA	1804	3.42E-05	919	0.05 (0.01; 0.09)	358	0.12 (0.07; 0.17)
	Iron, µM	NA	1804	3.36E-12	919	-1.50 (-2.06; -0.93)	358	-2.53 (-3.24; -1.82)
		Log(hepcidin)	1804	6.71E-13	919	-1.51 (-2.08; -0.95)	358	-2.60 (-3.31; -1.90)
		Log(hepcidin/ferritin)	1804	7.90E-11	919	-1.42 (-1.98; -0.86)	358	-2.37 (-3.08; -1.66)
	TS, %	NA	1804	8.47E-14	919	-2.92 (-3.96; -1.89)	358	-4.96 (-6.26; -3.66)
		Log(hepcidin)	1804	1.48E-15	919	-2.98 (-3.99; -1.96)	358	-5.21 (-6.48; -3.94)
		Log(hepcidin/ferritin)	1804	1.54E-12	919	-2.81 (-3.84; -1.77)	358	-4.71 (-6.01; -3.41)
	TIBC, µM	NA	1804	1.27E-01	919	0.74 (-0.20; 1.68)	358	1.16 (-0.02; 2.33)
		Log(hepcidin)	1804	2.57E-02	919	0.82 (-0.07; 1.71)	358	1.51 (0.40; 2.63)
		Log(hepcidin/ferritin)	1804	7.87E-02	919	0.81 (-0.13; 1.75)	358	1.30 (0.11; 2.48)
HFE rs1800562†	Log(hepcidin)‡, nM	NA	1824	3.89E-01	217	0.00 (-0.06; 0.05)	4	-0.27 (-0.66; 0.12)
	Log(ferritin), µg/L‡	NA	1822	2.01E-04	216	0.04 (-0.01; 0.10)	4	0.69 (0.33; 1.04)
		Log(hepcidin)	1822	2.08E-15	216	0.05 (0.01; 0.08)	4	0.88 (0.66; 1.11)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1822	1.35E-07	216	-0.04 (-0.08; -0.01)	4	-0.65 (-0.90; -0.41)
	Log(hepcidin/TS)‡, nM/%	NA	1804	5.73E-04	212	-0.07 (-0.13; -0.02)	4	-0.58 (-0.96; -0.20)
	Iron, µM	NA	1804	1.39E-11	212	2.26 (1.50; 3.02)	4	10.71 (5.53; 15.88)
		Log(hepcidin)	1804	5.70E-12	212	2.26 (1.51; 3.02)	4	11.09 (5.94; 16.24)
		Log(hepcidin/ferritin)	1804	3.33E-10	212	2.18 (1.42; 2.93)	4	9.33 (4.15; 14.52)
	TS, %	NA	1804	7.00E-18	212	5.36 (3.98; 6.75)	4	22.60 (13.17; 32.04)
		Log(hepcidin)	1804	3.20E-19	212	5.37 (4.02; 6.73)	4	23.93 (14.68; 33.18)
		Log(hepcidin/ferritin)	1804	1.85E-16	212	5.24 (3.87; 6.62)	4	20.58 (11.1; 30.06)
	TIBC, µM	NA	1804	1.17E-06	212	-3.05 (-4.30; -1.80)	4	-9.58 (-18.13; -1.02)
		Log(hepcidin)	1804	6.81E-08	212	-3.06 (-4.25; -1.88)	4	-11.51 (-19.61; -3.41)
		Log(hepcidin/ferritin)	1804	3.44E-07	212	-3.13 (-4.38; -1.88)	4	-10.94 (-19.54; -2.33)

N indicates number; Beta AG vs GG, regression coefficient for AG genotype versus GG genotype; Beta AA vs GG, regression coefficient for AA genotype versus GG genotype; CI, confidence interval.
For *TMPRSS6* rs855791 (p.Ala736Val), minor allele is A with frequency 0.455 in the whole cohort. Therefore, genotype GG is used as the reference genotype.
For *HFE* rs1800562 (p.Cys282Tyr), minor allele is A with frequency 0.063 in the whole cohort. Therefore, genotype GG is used as the reference genotype.

* Numbers are different from the total number of included persons because of missing values.

† Both SNPs are genotyped.

‡ The dependent variables hepcidin, ferritin, hepcidin/ferritin and hepcidin/TS were log-transformed. Therefore, the regression coefficients express the changes in each log-transformed variable that are associated with each genotype relative to the reference genotype.

Discussion

Our results showed that *HFE* rs1800562 and *TMPRSS6* rs855791 are the strongest associates of these genes for iron parameters in our study population. These SNPs and their correlated SNP variants also emerged from six previously published GWAS on serum iron, transferrin, TS and ferritin [1-6]. We found that serum hepcidin was not statistically significantly associated with *HFE* rs1800562 or *TMPRSS6* rs855791 nor with any other SNP in *HFE* and *TMPRSS6*. However, the ratios of hepcidin to ferritin and hepcidin to TS did show association with the two SNPs. Adjustment for hepcidin did not result in a decrease of the strength of the associations between the SNPs and the iron parameters, neither did adjustment for the ratio of hepcidin to ferritin in the SNP association analyses for iron, TS and TIBC. Hence, our data do not support an intermediate role of hepcidin in the SNP-iron parameter associations nor do they support a pleiotropic effect of the HFE and TMPRSS6 SNPs on iron parameters and hepcidin (Figure 3.1). However, we did find evidence for an independent, pleiotropic effect of the *HFE* and *TMPRSS6* SNPs on iron parameters and ratios of hepcidin to ferritin and TS.

Our findings confirm the results found by Traglia *et al*. in an isolated Italian population [3]. They replicated associations of *HFE* rs1800562 with serum iron, transferrin and TS and of *TMPRSS6* rs855791 with serum iron and TS in their cohort of 1545 related individuals and reported a borderline genome-wide significant association for serum ferritin and *HFE* rs1800562. *TMPRSS6* rs855791 association with serum ferritin was only nominally significant. Conform our results, Traglia *et al*. did not find an association of these SNPs with hepcidin, and use of hepcidin as covariate in their association analysis of the SNPs with the iron parameters did not change the associations. In contrast, we observed both for the total study population and for the subsets a remarkable stronger association between ferritin and the SNPs after adjustment for serum hepcidin, which was not observed in the data of Traglia *et al*. Nevertheless, estimates of regression coefficients were comparable between our study and the study of Traglia *et al*. Finally, Traglia *et al*. reported that *HFE* rs1800562 and *TMPRSS6* rs855791 were associated with the ratio of hepcidin to ferritin in subset 1. We observed this association both in our total study population and in our two subsets. Traglia *et al*. did not study the ratio of hepcidin to TS.

Our results and the results of Traglia *et al*. are in contrast to the generally accepted idea that *HFE* and *TMPRSS6* affect hepcidin transcription, thereby adapting the hepcidin expression in response to the systemic iron concentration. Indeed, there is only limited evidence from animal and *in vitro* studies for a *direct* relation between the Hfe protein and (intracellular) iron homeostasis in different cell types [16,29-32]. For example, it was shown that *HFE* mutations can directly affect iron accumulation in hereditary hemochromatosis macrophages, independently of the presence of hepcidin [29,30]. On the other hand, evidence for *HFE* and *TMPRSS6* affecting hepcidin transcription is abundant. Mice experiments and observational studies in humans have shown that defects in *HFE* result in insufficient expression of hep-

cidin in the liver and lower serum hepcidin levels relative to the body iron stores, leading to the iron storage disorder hereditary haemochromatosis [7-11]. Mutations in *TMPRSS6* have been associated to inappropriately high urine and serum hepcidin concentration for the setting of systemic iron deficiency, which has been suggested to cause iron-refractory iron deficiency anemia. Furthermore, a recent *in vitro* study by Nai *et al*. showed that the G allele of *TMPRSS6* rs855791 inhibits hepcidin more efficiently than the A allele [33]. In this same publication, it was also reported that hepcidin was significantly lower in *TMPRSS6* rs855791 GG homozygotes than in AA homozygotes in normal subjects after exclusion of iron-deficient individuals (serum ferritin <30 ng/mL) and individuals with clinically relevant inflammatory conditions (C-reactive protein >1 mg/dL) [33]. We and Traglia *et al*. did not observe an association between hepcidin and *TMPRSS6* rs855791 in our data, even though we also excluded iron-deficient individuals and individuals with clinically relevant inflammatory conditions in subset 1. In addition, the difference in results between Nai *et al*. and Traglia *et al*. is striking, because Nai *et al*. used a selection of unrelated individuals (N=545) of the same population used by Traglia *et al*. For *HFE* rs1800562, van Dijk *et al*. demonstrated in a small population of first-degree family members of clinically diagnosed *HFE* rs1800562 homozygous probands that hepcidin was lower in *HFE* rs1800562 AA homozygotes compared to AG heterozygotes and GG homozygotes combined ($p < 0.01$) [11], but we did not find a significant association between hepcidin and *HFE* rs1800562 in our sample of the general population. On the other hand, our findings for the ratios of hepcidin to ferritin and hepcidin to TS corroborate with the results of both Nai *et al*. and van Dijk *et al*. [11,33].

Reasons that may have masked an intermediate role of hepcidin in the association between the two SNP variants and iron parameters in our study and that of Traglia *et al*. include the reliance on measurement of hepcidin in serum and the potential omission of environmental and genetic factors that may cause variation in serum hepcidin levels independent of iron regulation. In other words, the hepcidin as measured in our study (by ELISA) and that of Traglia *et al*. (by mass-spectrometry) may not be a correct reflection of the hepcidin that is intermediate in the Hfe and MT2 regulation of iron parameters. This could also be true for TS as a proxy for circulating iron, as TS does not necessarily reflect the concentration of the (putative) hepcidin signaling form of the molecule, *i.e*. differic transferrin [34]. Besides, we used serum levels of hepcidin as well as serum levels of iron and ferritin, but the associations that we studied might be cell type or tissue-specific. Hepcidin is predominantly produced in hepatocytes [35-37], whereas the Hfe protein is expressed in a whole range of tissues and cell types [38,39], and MT2 is expressed primarily in the liver [40]. The identified associations between the *HFE* and *TMPRSS6* variants and ratios of hepcidin to ferritin and hepcidin to TS, a better reflection of the long-term balance between hepcidin and body iron status, may support this hypothesis. Nevertheless, adjustment for the ratio of hepcidin to ferritin in the association analyses for the SNPs with iron, TS and TIBC did not change the effects of the SNPs on the iron parameters either, indicating an independent, pleiotropic effect of the SNPs on both the hepcidin ratios and the iron parameters.

In summary, we can confirm that *HFE* rs1800562 and *TMPRSS6* rs855791 are main determinants of *HFE* and *TMPRSS6*-related variation in iron, ferritin, TS and TIBC in the general population. These SNPs are not associated with serum hepcidin itself, but do influence ratios reflecting hepcidin relative to circulating iron and iron stores, measured by the iron parameters TS and ferritin, respectively. Our study can confirm that serum hepcidin, whether corrected for iron stores or not, is not the intermediate variable in the associations of the SNPs with the iron parameters, thereby confirming the results of Traglia *et al*. In addition, our data indicate that the SNPs exert a pleiotropic effect on both the hepcidin ratios and the iron parameters. Taken together, our study points at a direct SNP effect on certain serum iron parameters, rather than an indirect effect entirely dependent upon a change in serum hepcidin levels. We call for additional functional studies in a controlled setting that do allow further elucidation of the role of hepcidin in the associations between the SNPs and iron parameters, results which will contribute to elucidation of underlying mechanisms and which will eventually facilitate the development of interventions for iron disorders.

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References

1 Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009;41:1173-5.

2 Benyamin B, McRae AF, Zhu G, et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60-5.

3 Traglia M, Girelli D, Biino G, et al. 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.

4 Middelberg RP, Ferreira MA, Henders AK, et al. Genetic variants in LPL, OASL and TOMM40/APOE-C1-C2-C4 genes are associated with multiple cardiovascular-related traits. BMC Med Genet 2011;12:123.

5 Pichler I, Minelli C, Sanna S, et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum Mol Genet 2011;20:1232-40.

6 Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. Blood 2010;115:94-6.

7 Ahmad KA, Ahmann JR, Migas MC, et al. Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 2002;29:361-6.

8 Bridle KR, Frazer DM, Wilkins SJ, et al. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 2003;361:669-73.

9 Muckenthaler M, Roy CN, Custodio AO, et al. Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. Nat Genet 2003;34:102-7.

10 Nicolas G, Viatte L, Lou DQ, et al. Constitutive hepcidin expression prevents iron overload in a mouse model of hemochromatosis. Nat Genet 2003;34:97-101.

11 van Dijk BA, Laarakkers CM, Klaver SM, et al. 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;142:979-85.

12 Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. Science 2008;320:1088-92.

13 Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.

14 Corradini E, Meynard D, Wu Q, et al. Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. Hepatology 2011;54:273-84.

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

16 Ramos E, Kautz L, Rodriguez R, et al. Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. Hepatology 2011;53:1333-41.

17 Feng Q, Migas MC, Waheed A, et al. Ferritin upregulates hepatic expression of bone morphogenetic protein 6 and hepcidin in mice. Am J Physiol Gastrointest Liver Physiol 2012;302:G1397-404.
18 Fleming RE, Ponka P. Iron overload in human disease. N Engl J Med 2012;366:348-59.
19 Finberg KE. Iron-refractory iron deficiency anemia. Semin Hematol 2009;46:378-86.
20 Hoogendoorn EH, Hermus AR, de Vegt F, et al. 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.
21 Kiemeney LA, Thorlacius S, Sulem P, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. Nat Genet 2008;40:1307-12.
22 Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.
23 Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570-9.
24 Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, et al. High-sensitive radioimmunoassay for human serum hepcidin. British journal of haematology. 2009 Aug;146(3):317-25.
25 Marchini J, Howie B, Myers S, et al. A new multipoint method for genome-wide association studies by imputation of genotypes. Nature genetics. 2007 Jul;39(7):906-13.
26 Gamazon ER, Zhang W, Konkashbaev A, et al. SCAN: SNP and copy number annotation. Bioinformatics. 2010 Jan 15;26(2):259-62.
27 Xia K, Shabalin AA, Huang S, et al. seeQTL: a searchable database for human eQTLs. Bioinformatics. 2012 Feb 1;28(3):451-2.
28 Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.
29 Jacolot S, Yang Y, Paitry P, et al. Iron metabolism in macrophages from HFE hemochromatosis patients. Mol Genet Metab 2010;101:258-67.
30 Makui H, Soares RJ, Jiang W, et al. Contribution of Hfe expression in macrophages to the regulation of hepatic hepcidin levels and iron loading. Blood 2005;106:2189-95.
31 Gao J, Zhao N, Knutson MD, Enns CA. The hereditary hemochromatosis protein, HFE, inhibits iron uptake via down-regulation of Zip14 in HepG2 cells. J Biol Chem 2008;283:21462-8.
32 Ramos P, Guy E, Chen N, et al. Enhanced erythropoiesis in Hfe-KO mice indicates a role for Hfe in the modulation of erythroid iron homeostasis. Blood 2011;117:1379-89.
33 Nai A, Pagani A, Silvestri L, et al. TMPRSS6 rs855791 modulates hepcidin transcription *in vitro* and serum hepcidin levels in normal individuals. Blood 2011;118:4459-62.
34 DiRusso SC, Check IJ, Hunter RL. Quantitation of apo-, mono-, and diferric transferrin by polyacrylamide gradient gel electrophoresis in patients with disorders of iron metabolism. Blood 1985;66:1445-51.
35 Krause A, Neitz S, Magert HJ, Schulz, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147-50.

36 Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276:7811-9.
37 Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-69.
38 Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996;13:399-408.
39 Parkkila S, Niemela O, Britton RS, et al. Molecular aspects of iron absorption and HFE expression. Gastroenterology 2001;121:1489-96.
40 Velasco G, Cal S, Quesada V, et al. Matriptase-2, a membrane-bound mosaic serine proteinase predominantly expressed in human liver and showing degrading activity against extracellular matrix proteins. J Biol Chem 2002;277:37637-46.

Supplemental material

Supplemental Tables 3.1, 3.3, 3.4 and 3.9 can be found online:
http://jmg.bmj.com/content/50/9/593/suppl/DC1

SUPPLEMENTAL TABLE 3.2 Characteristics of subset 1 (N=1505) and subset 2 (N=1177).

		Subset 1 (N=1505)			Subset 2 (N=1177)		
		N*	%	Mean (SD)	N*	%	Mean (SD)
Gender	Males	793	53	NA	660	56	NA
	Females	712	47	NA	517	44	NA
Age, years		1505	100	62.5 (9.7)	1177	100	61.6 (10.0)
Time of blood sampling	Before 12 pm	314	21	NA	248	21	NA
	Between 12 pm and 5 pm	970	65	NA	740	63	NA
	After 5 pm	216	14	NA	186	16	NA
	Unknown	5	0	NA	3	0	NA
Hepcidin, nM†		1505	100	7.7 (2.0)	1177	100	6.2 (2.5)
Ferritin, µg/L†		1503	100	131.5 (2.0)	1175	100	107.1 (2.5)
Ratio hepcidin/ferritin, nmoles/µg†		1503	100	58.8 (1.9)	1175	100	58.0 (1.9)
Ratio hepcidin/TS, nM/%†		1486	99	0.26 (2.1)	1167	99	0.21 (2.5)
Iron, µM		1486	99	18.0 (5.3)	1167	99	17.9 (5.6)
TS, %		1486	99	31.3 (9.7)	1167	99	31.0 (10.5)
TIBC, µM		1486	99	58.2 (8.1)	1167	99	59.0 (8.9)

Subset 1 is selected based on the following exclusion criteria: CRP >10 mg/L or ferritin <30 µg/L.

Subset 2 is selected based on the following exclusion criteria: pregnancy at time of blood sampling, ALT >50 U/L, CRP >10 mg/L, eGFR <60 mL/min/1.73 m2, use of iron supplements, presence of anemia, or BMI >30 kg/m2.

N indicates number; SD, standard deviation; NA, not applicable.

* Numbers are different from the total number of included persons because of missing values.

† The variables hepcidin, ferritin, ratio hepcidin/ferritin and ratio hepcidin/TS were log-transformed, and therefore geometric mean and SD are given.

SUPPLEMENTAL TABLE 3.5 Haplotype association results for haplotypes in *TMPRSS6* with iron traits compared to the effect of *TMPRSS6* rs855791 alone for the total study population (N=1832). Associations are adjusted for age, gender and time of blood sampling.

Haplotype				Beta (95% CI)							
				Log(ferritin), µg/L*		Iron, µM		TS, %		TIBC, µM	
rs855791	rs2235324	rs2160906	Frequency	Haplotype model	Marginal model	Haplotype model	Marginal model	Haplotype model	Marginal model	Haplotype model	Marginal model
A	T	A	0.19	REF	REF	REF	REF	REF	REF	REF	REF
A	C	G	0.08	0.00 (-0.06; 0.06)	REF	0.63 (-0.19; 1.45)	REF	1.23 (-0.27; 2.73)	REF	0.18 (-1.18; 1.54)	REF
A	T	G	0.18	0.02 (-0.03; 0.06)	REF	0.34 (-0.29; 0.96)	REF	0.72 (-0.43; 1.87)	REF	0.01 (-1.04; 1.05)	REF
G	T	A	0.05	0.06 (-0.01; 0.13)	| |	1.25 (0.22; 2.28)	| |	2.42 (0.53; 4.31)	| |	-0.56 (-2.28; 1.16)	| |
G	C	G	0.29	0.03 (-0.01; 0.06)	0.02 (-0.00; 0.05)	1.47 (0.94; 2.0)	1.29 (0.94; 1.64)	3.06 (2.09; 4.03)	2.52 (1.88; 3.16)	-0.70 (-1.58; 0.18)	-0.59 (-1.18; -0.01)
G	T	G	0.20	0.02 (-0.02; 0.06)	| |	1.71 (1.14; 2.28)	| |	3.17 (2.12; 4.22)	| |	-0.34 (-1.30; 0.61)	| |
F-statistic†				0.4		1.1		1.1		0.2	
P				8.2E-01		3.7E-01		3.4E-01		9.6E-01	

All SNPs are genotyped.

CI indicates confidence interval; REF, reference.

* The dependent variable ferritin was log-transformed. Therefore, the regression coefficients express the changes in log-transformed ferritin (µg/L) for each haplotype relative to the reference haplotype.

† The F-statistic compares the haplotype model to the marginal model of only *TMPRSS6* rs855791 and indicates whether there is an additional effect of the haplotypes on top of the marginal allelic effect of *TMPRSS6* rs855791 alone.

SUPPLEMENTAL TABLE 3.6

Haplotype association results for haplotypes in *HFE* with iron traits compared to the effect of *HFE* rs1800562 alone for the total study population (N=1832). Associations are adjusted for age, gender and time of blood sampling.

Haplotype				Beta (95% CI)							
				Log(ferritin), µg/L†		Iron, µM		TS, %		TIBC, µM	
rs1799945*	rs1800562	rs198853*	Frequency	Haplotype model	Marginal model	Haplotype model	Marginal model	Haplotype model	Marginal model	Haplotype model	Marginal model
C	G	C	0.34	REF	REF	REF	REF	REF	REF	REF	REF
G	G	T	0.15	0.02 (-0.02; 0.06)	REF	1.19 (0.67; 1.71)	REF	2.51 (1.56; 3.46)	REF	-0.71 (-1.57; 0.16)	REF
C	G	T	0.45	-0.02 (-0.05; 0.01)	REF	0.15 (-0.24; 0.55)	REF	0.11 (-0.61; 0.83)	REF	0.32 (-0.34; 0.98)	REF
C	A	T	0.06	0.06 (0.01; 0.11)	0.07 (0.02; 0.12)	2.78 (2.01; 3.55)	2.49 (1.76; 3.22)	6.30 (4.89; 7.7)	5.81 (4.48; 7.14)	-3.13 (-4.41; -1.85)	-3.18 (-4.39; -1.98)
F-statistic‡				2.6		10.7		15.5		2.9	
P				7.1E-02		2.4E-05		2.2E-07		5.8E-02	

CI indicates confidence interval; REF, reference.

* SNPs indicated with an asterix (*) are imputed.

† The dependent variable ferritin was log-transformed. Therefore, the regression coefficients express the changes in log-transformed ferritin (µg/L) for each haplotype relative to the reference haplotype.

‡ The F-statistic compares the haplotype model to the marginal model of only *HFE* rs1800562 and indicates whether there is an additional effect of the haplotypes on top of the marginal allelic effect of *HFE* rs1800562 alone.

SUPPLEMENTAL TABLE 3.7 Associations between *TMPRSS6* rs855791 and *HFE* rs1800562 with iron parameters for subset 1 (N=1505). Associations are adjusted for age, gender, time of blood sampling and additionally for serum hepcidin or the ratio hepcidin/ferritin.

					AG		AA	
		Additionally adjusted for:	Total N*	*P*	N	Beta AG vs GG (95% CI)	N	Beta AA vs GG (95% CI)
TMPRSS6 rs855791†	Log(hepcidin)‡, nM	NA	1500	4.12E-02	752	0.00 (-0.04; 0.03)	309	0.05 (0.00; 0.09)
	Log(ferritin)‡, µg/L	NA	1498	1.38E-01	750	-0.03 (-0.06; 0.01)	309	-0.04 (-0.08; 0.00)
		Log(hepcidin)	1498	1.82E-04	750	-0.02 (-0.05; 0.00)	309	-0.07 (-0.10; -0.04)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1498	1.43E-05	750	0.02 (-0.00; 0.05)	309	0.08 (0.05; 0.12)
	Log(hepcidin/TS)‡, nM/%	NA	1481	1.79E-07	743	0.05 (0.01; 0.08)	303	0.13 (0.08; 0.17)
	Iron, µM	NA	1481	2.35E-14	743	-1.71 (-2.30; -1.12)	303	-2.83 (-3.55; -2.10)
		Log(hepcidin)	1481	2.94E-14	743	-1.71 (-2.30; -1.12)	303	-2.82 (-3.54; -2.09)
		Log(hepcidin/ferritin)	1481	7.42E-13	743	-1.66 (-2.24; -1.08)	303	-2.63 (-3.36; -1.91)
	TS, %	NA	1481	1.01E-16	743	-3.29 (-4.35; -2.22)	303	-5.60 (-6.92; -4.28)
		Log(hepcidin)	1481	4.37E-17	743	-3.29 (-4.35; -2.22)	303	-5.68 (-7.00; -4.36)
		Log(hepcidin/ferritin)	1481	4.31E-15	743	-3.19 (-4.25; -2.14)	303	-5.26 (-6.57; -3.94)
	TIBC, µM	NA	1481	1.01E-01	743	0.62 (-0.33; 1.57)	303	1.28 (0.10; 2.46)
		Log(hepcidin)	1481	4.93E-02	743	0.62 (-0.32; 1.57)	303	1.46 (0.29; 2.63)
		Log(hepcidin/ferritin)	1481	1.08E-01	743	0.61 (-0.34; 1.57)	303	1.27 (0.08; 2.45)
HFE rs1800562†	Log(hepcidin)‡, nM	NA	1500	5.80E-02	182	-0.03 (-0.07; 0.02)	4	-0.31 (-0.59; -0.02)
	Log(ferritin)‡, µg/L	NA	1498	2.37E-06	181	0.05 (0.00; 0.09)	4	0.65 (0.37; 0.92)
		Log(hepcidin)	1498	1.01E-16	181	0.06 (0.03; 0.09)	4	0.84 (0.63; 1.05)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1498	4.68E-10	181	-0.07 (-0.11; -0.03)	4	-0.65 (-0.88; -0.42)
	Log(hepcidin/TS)‡, nM/%	NA	1481	1.84E-07	177	-0.10 (-0.15; -0.05)	4	-0.60 (-0.90; -0.29)
	Iron, µM	NA	1481	9.11E-15	177	2.78 (2.00; 3.55)	4	10.15 (5.29; 15.00)
		Log(hepcidin)	1481	1.15E-14	177	2.77 (1.99; 3.55)	4	10.10 (5.23; 14.96)
		Log(hepcidin/ferritin)	1481	1.53E-12	177	2.62 (1.84; 3.40)	4	8.61 (3.74; 13.49)
	TS, %	NA	1481	2.08E-20	177	5.97 (4.56; 7.37)	4	21.45 (12.67; 30.23)
		Log(hepcidin)	1481	7.88E-21	177	6.00 (4.60; 7.41)	4	21.97 (13.18; 30.75)
		Log(hepcidin/ferritin)	1481	8.10E-18	177	5.70 (4.29; 7.10)	4	18.79 (9.97; 27.61)
	TIBC, µM	NA	1481	2.10E-04	177	-2.23 (-3.49; -0.97)	4	-9.05 (-16.90; -1.20)
		Log(hepcidin)	1481	5.44E-05	177	-2.32 (-3.57; -1.07)	4	-10.27 (-18.05; -2.48)
		Log(hepcidin/ferritin)	1481	2.21E-04	177	-2.25 (-3.51; -0.98)	4	-9.19 (-17.12; -1.25)

N indicates number; Beta AG vs GG, regression coefficient for AG genotype versus GG genotype; Beta AA vs GG, regression coefficient for AA genotype versus GG genotype; CI, confidence interval.
For *TMPRSS6* rs855791 (p.Ala736Val), minor allele is A with frequency 0.455 in the whole cohort. Therefore, genotype GG is used as the reference genotype.
For *HFE* rs1800562 (p.Cys282Tyr), minor allele is A with frequency 0.063 in the whole cohort. Therefore, genotype GG is used as the reference genotype.

* Numbers are different from the total number of included persons because of missing values.

† Both SNPs are genotyped.

‡ The dependent variables hepcidin, ferritin, hepcidin/ferritin and hepcidin/TS were log-transformed. Therefore, the regression coefficients express the changes in each log-transformed variable that are associated with each genotype relative to the reference genotype.

SUPPLEMENTAL TABLE 3.8 Associations between *TMPRSS6* rs855791 and *HFE* rs1800562 with iron parameters for subset 2 (N=1177). Associations are adjusted for age, gender, time of blood sampling and additionally for serum hepcidin or the ratio hepcidin/ferritin.

					AG		AA	
		Additionally adjusted for:	Total N*	*P*	N	Beta AG vs GG (95% CI)	N	Beta AA vs GG (95% CI)
TMPRSS6 rs855791†	Log(hepcidin)‡, nM	NA	1174	3.55E-01	583	-0.01 (-0.06; 0.05)	234	0.04 (-0.03; 0.10)
	Log(ferritin)‡, µg/L	NA	1172	1.42E-01	581	-0.04 (-0.09; 0.01)	234	-0.05 (-0.11; 0.01)
		Log(hepcidin)	1172	1.89E-04	581	-0.04 (-0.07; 0.01)	234	-0.08 (-0.12; -0.04)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1172	1.59E-04	580	0.04 (0.01; 0.07)	232	0.09 (0.05; 0.13)
	Log(hepcidin/TS)‡, nM/%	NA	1164	3.38E-04	575	0.05 (0.00; 0.10)	229	0.13 (0.06; 0.189)
	Iron, µM	NA	1164	5.49E-11	576	-1.71 (-2.39; -1.02)	231	-2.89 (-3.75; -2.04)
		Log(hepcidin)	1164	1.27E-11	576	-1.70 (-2.37; -1.02)	231	-2.97 (-3.82; -2.12)
		Log(hepcidin/ferritin)	1164	3.87E-10	576	-1.66 (-2.34; -0.97)	231	-2.78 (-3.64; -1.92)
	TS, %	NA	1164	1.05E-12	576	-3.48 (-4.75; -2.20)	231	-5.80 (-7.39; -4.20)
		Log(hepcidin)	1164	3.37E-14	576	-3.45 (-4.68; -2.21)	231	-6.03 (-7.59; -4.48)
		Log(hepcidin/ferritin)	1164	8.41E-12	576	-3.39 (-4.66; -2.11)	231	-5.59 (-7.20; -3.99)
	TIBC, µM	NA	1164	5.30E-02	576	1.12 (-0.03; 2.26)	231	1.63 (0.21; 3.06)
		Log(hepcidin)	1164	1.61E-02	576	1.08 (0.00; 2.16)	231	1.93 (0.57; 3.28)
		Log(hepcidin/ferritin)	1164	4.11E-02	576	1.15 (0.01; 2.30)	231	1.72 (0.28; 3.16)
HFE rs1800562†	Log(hepcidin)‡, nM	NA	1174	4.46E-01	129	-0.01 (-0.08; 0.06)	4	-0.24 (-0.62; 0.14)
	Log(ferritin)‡, µg/L	NA	1172	2.99E-04	128	0.03 (-0.04; 0.09)	4	0.71 (0.36; 1.06)
		Log(hepcidin)	1172	1.48E-14	128	0.03 (-0.01; 0.07)	4	0.89 (0.67; 1.11)
	Log(hepcidin/ferritin)‡, nmoles/µg	NA	1172	7.82E-07	128	-0.03 (-0.08; 0.02)	4	-0.65 (-0.89; -0.40)
	Log(hepcidin/TS)‡, nM/%	NA	1164	1.04E-03	127	-0.09 (-0.16; -0.02)	4	-0.54 (-0.91; -0.17)
	Iron, µM	NA	1164	6.84E-11	127	2.79 (1.83; 3.75)	4	10.09 (5.00; 15.18)
		Log(hepcidin)	1164	1.97E-11	127	2.79 (1.84; 3.74)	4	10.57 (5.53; 15.61)
		Log(hepcidin/ferritin)	1164	4.40E-10	127	2.75 (1.79; 3.70)	4	9.22 (4.08; 14.36)
	TS, %	NA	1164	4.25E-14	127	5.93 (4.15; 7.71)	4	21.51 (12.06; 30.96)
		Log(hepcidin)	1164	1.47E-15	127	5.94 (4.21; 7.66)	4	22.98 (13.79; 32.16)
		Log(hepcidin/ferritin)	1164	3.49E-13	127	5.86 (4.08; 7.63)	4	19.98 (10.44; 29.53)
	TIBC, µM	NA	1164	2.06E-03	127	-2.23 (-3.82; -0.64)	4	-9.70 (-18.16; -1.24)
		Log(hepcidin)	1164	3.02E-04	127	-2.24 (-3.75; -0.74)	4	-11.51 (-19.50; -3.51)
		Log(hepcidin/ferritin)	1164	1.35E-03	127	-2.27 (-3.86; -0.68)	4	-10.47 (-19.03; -1.91)

N indicates number; Beta AG vs GG, regression coefficient for AG genotype versus GG genotype; Beta AA vs GG, regression coefficient for AA genotype versus GG genotype; CI, confidence interval.

For *TMPRSS6* rs855791 (p.Ala736Val), minor allele is A with frequency 0.455 in the whole cohort. Therefore, genotype GG is used as the reference genotype.

For *HFE* rs1800562 (p.Cys282Tyr), minor allele is A with frequency 0.063 in the whole cohort. Therefore, genotype GG is used as the reference genotype.

* Numbers are different from the total number of included persons because of missing values.

† Both SNPs are genotyped.

‡ The dependent variables hepcidin, ferritin, hepcidin/ferritin and hepcidin/TS were log-transformed. Therefore, the regression coefficients express the changes in each log-transformed variable that are associated with each genotype relative to the reference genotype.

SUPPLEMENTAL TABLE 3.10 Mean serum hepcidin concentrations (nM) stratified by genotypes of *TMPRSS6* rs855791 and *HFE* rs1800562 in the total study population (N=1832) and in Subset 2 (N=1177).

			All				Subset 2			
			TMPRSS6 rs855791				*TMPRSS6* rs855791			
			GG	AG	AA	*P**	GG	AG	AA	*P**
HFE rs1800562	GG	N	467	811	330	1.38E-01	315	511	218	2.70E-01
		Geometric mean (SD)†	6.08 (2.60)	6.38 (2.64)	6.97 (2.54)		6.08 (2.55)	6.13 (2.56)	6.86 (2.54)	
	AG	N	64	117	38	9.48E-01	40	71	18	9.54E-01
		Geometric mean (SD)†	6.35 (2.67)	6.25 (2.40)	6.62 (2.65)		6.25 (2.69)	5.96 (2.19)	6.20 (1.93)	
	AA	N	2	2	1	6.38E-01	2	2	0	5.19E-01
		Geometric mean (SD)†	2.98 (1.73)	4.34 (1.50)	5.34 (NA)		2.98 (1.73)	4.34 (1.50)	NA	
	*P**		5.40E-01	8.36E-01	9.12E-01		5.54E-01	8.47E-01	6.55E-01	

N indicates number; NA, not applicable.

* P-value derived from one-way ANOVA using the log-transformed serum hepcidin concentration as dependent variable.

† Geometric means are presented, because we used log-transformed serum hepcidin as dependent variable conform other analyses.

CHAPTER 4

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 pathways 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 4.1). 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 4.2-4.4. 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 age^2, 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

Six 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

both in NBS and PREVEND samples 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 4.1-4.4) revealed two loci that were genome-wide significantly associated (p<5E-08) with serum hepcidin (Table 4.1 and Supplemental Figures 4.1-4.8). 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 4.8). Conditional analysis in NBS data showed no additional independent signals at the chromosome 2 locus after adjustment for rs354202 (Supplemental Figures 4.9-4.10).

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 4.5). No novel statistically significant loci for hepcidin/ferritin were identified; the novel 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 4.1). 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 4.6). 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-06). 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 4.2). The p-value for discovery and replication analyses combined became stronger for rs354202 only.

TABLE 4.1 Top hits (p-value < 1E-06) for hepcidin in all individuals and in the subset. Genome-wide significant associations (p-value < 5E-08) are indicated in bold.

						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	0.10	0.14	0.03	2.83E-05	+++	6096	0.10	0.19	0.04	1.69E-07	+++	5051
rs80098840	2	54918152	*EML6*	A	G	0.89	-0.14	0.03	5.73E-06	---	6096	0.89	-0.18	0.03	1.51E-07	---	5051
rs76949049	**2**	**54965697**	***EML6***	**T**	**C**	**0.10**	**0.16**	**0.03**	**1.14E-06**	**+++**	**6096**	**0.10**	**0.20**	**0.04**	**2.15E-08**	**+++**	**5051**
rs354202	**2**	**54970943**	***EML6***	**A**	**G**	**0.90**	**-0.17**	**0.03**	**7.02E-08**	**---**	**6096**	**0.90**	**-0.20**	**0.03**	**1.21E-08**	**---**	**5051**
rs354204	2	54971385	*EML6*	A	G	0.86	-0.12	0.03	4.92E-06	---	6096	0.86	-0.16	0.03	5.30E-08	---	5051
rs9973793	2	54998516	*EML6*	T	C	0.13	0.12	0.03	2.11E-05	+++	6096	0.13	0.16	0.03	2.84E-07	+++	5051
rs2033823	**2**	**55057740**	***EML6***	**T**	**C**	**0.90**	**-0.13**	**0.03**	**1.11E-05**	**---**	**6096**	**0.90**	**-0.19**	**0.03**	**2.64E-08**	**---**	**5051**
rs13420395	**2**	**55058720**	***EML6***	**A**	**G**	**0.10**	**0.13**	**0.03**	**1.32E-05**	**+++**	**6096**	**0.10**	**0.19**	**0.03**	**2.79E-08**	**+++**	**5051**
rs7592363	2	55060479	*EML6*	T	C	0.10	0.13	0.03	1.39E-05	+++	6096	0.10	0.18	0.03	6.11E-08	+++	5051
rs6747033	2	55061294	*EML6*	C	G	0.89	-0.14	0.03	6.65E-06	---	6096	0.89	-0.18	0.03	1.10E-07	---	5051
rs56281245	5	145007639	*PRELID2*‡	T	C	0.95	0.17	0.05	6.00E-04	+++	6096	0.95	0.26	0.05	6.66E-07	+++	5051
rs11388147	7	71647721	*CALN1*	G	GA	0.27	-0.15	0.03	9.63E-07	?--	3279	0.26	-0.13	0.03	7.98E-05	?--	2695
rs118031191	**10**	**129582469**	***FOXI2***‡	**A**	**G**	**0.03**	**-0.38**	**0.07**	**1.59E-08**	**---**	**6096**	**0.03**	**-0.33**	**0.08**	**1.41E-05**	**---**	**5051**
rs12289793	11	21348000	*NELL1*	A	G	0.79	0.10	0.03	1.60E-04	+++	6096	0.79	0.14	0.03	9.91E-07	+++	5051
rs117568227	12	66447376	*LLPH*‡,§	A	G	0.01	-1.03	0.21	7.82E-07	?-?	1479	0.01	-1.19	0.24	5.85E-07	?-?	1206
rs150188223	13	42844491	*AKAP11*	T	C	0.01	-0.57	0.18	1.41E-03	-??	1800	0.01	-1.00	0.20	5.10E-07	??-	1489
rs141939445	20	36896818	*KIAA1755*‡,§	T	C	0.99	0.71	0.16	6.87E-06	+?+	3279	0.99	0.91	0.18	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 4.2 Results of the replication analyses and discovery and replication combined.

						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	0.02	0.03	0.38	++	3770	0.06	0.02	7.01E-04	+++	9866
	Subset	A	G	0.78	0.74	0.03	0.03	0.31	++	3072	0.09	0.02	1.49E-05	+++	8123
rs1835473	All	A	G	0.68	0.70	0.03	0.02	0.32	++	3754	0.07	0.02	1.48E-05	+++	9850
	Subset	A	G	0.68	0.70	0.02	0.03	0.49	+-	3059	0.05	0.02	2.27E-03	++-	8110
rs56281245	All	T	C	0.95	0.95	0.06	0.05	0.24	++	3798	0.12	0.04	8.40E-04	+++	9894
	Subset	T	C	0.95	0.96	0.04	0.06	0.56	++	3092	0.16	0.04	3.83E-05	+++	8143
rs118031191	All	A	G	0.03	0.03	0.00	0.07	1.00	-+	3821	-0.18	0.05	9.12E-05	--+	9917
	Subset	A	G	0.03	0.03	0.00	0.07	0.96	-+	3115	-0.16	0.05	2.60E-03	--+	8166
rs12441903	All	A	G	0.89	0.87	-0.04	0.04	0.33	--	3816	-0.10	0.02	3.13E-05	---	9912
	Subset	A	G	0.89	0.87	-0.03	0.04	0.41	--	3108	-0.10	0.03	6.29E-05	---	8159
rs354202‡	All	A	G	NA	0.89	-0.15	0.10	0.12	-	524	-0.17	0.03	2.11E-08	--	6620
	Subset	A	G	NA	0.88	-0.11	0.11	0.30	-	444	-0.19	0.03	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.

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*., in press). 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. Of these 12 SNPs, only rs1800562 and rs1799945 in *HFE* and rs855791 in *TMPRSS6* showed association with the hepcidin ratios, whereas the other 9 SNPs did not associate with either hepcidin or the hepcidin ratios ($p > 0.05$, data not shown). 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 adjusted for age and gender 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.

References

1 Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24-38.

2 Ganz T. Systemic iron homeostasis. Physiol Rev 2013;93:1721-41.

3 Jiang R, Manson JE, Meigs JB, et al. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 2004;291:711-7.

4 Montonen J, Boeing H, Steffen A, et al. Body iron stores and risk of type 2 diabetes: results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study. Diabetologia 2012;55:2613-21.

5 Cherayil BJ. Iron and immunity: immunological consequences of iron deficiency and overload. Arch Immunol Ther Exp (Warsz) 2010;58:407-15.

6 Altamura S, Muckenthaler MU. Iron toxicity in diseases of aging: Alzheimer's disease, Parkinson's disease and atherosclerosis. J Alzheimers Dis 2009;16:879-95.

7 Kroot JJ, Tjalsma H, Fleming RE, et al. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-69.

8 Ganz T, Nemeth E. Hepcidin and iron homeostasis. Biochim Biophys Acta 2012;1823:1434-43.

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

10 Swinkels DW, Janssen MC, Bergmans J, et al. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. Clin Chem 2006;52:950-68.

11 Ahmad KA, Ahmann JR, Migas MC, et al. Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 2002;29:361-6.

12 Nemeth E, Roetto A, Garozzo G, et al. Hepcidin is decreased in TFR2 hemochromatosis. Blood 2005;105:1803-6.

13 van Dijk BA, Laarakkers CM, Klaver SM, et al. 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;142:979-85.

14 Bridle KR, Frazer DM, Wilkins SJ, et al. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 2003;361:669-73.

15 Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008;40:569-71.

16 De Falco L, Sanchez M, Silvestri L, et al. Iron refractory iron deficiency anemia. Haematologica 2013;98:845-53.

17 Traglia M, Girelli D, Biino G, et al. 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.

18 Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. 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 2013;50:593-8.
19 Fleming RE, Ponka P. Iron overload in human disease. N Engl J Med 2012;366:348-59.
20 Nai A, Pagani A, Silvestri L, et al. TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. Blood 2011;118:4459-62.
21 Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 2010;26:2190-1.
22 Kutyavin IV, Milesi D, Belousov Y, et al. A novel endonuclease IV post-PCR genotyping system. Nucleic Acids Res 2006;34:e128.
23 Tang Y, Katuri V, Dillner A, et al. Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. Science 2003;299:574-7.
24 Babitt JL, Huang FW, Wrighting DM, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 2006;38:531-9.
25 Mleczko-Sanecka K, Roche F, da Silva AR, et al. Unbiased RNAi screen for hepcidin regulators links hepcidin suppression to the proliferative Ras/RAF and the nutrient-dependent mTOR signaling pathways. Blood 2014;123:1574-85.
26 Machnicka B, Czogalla A, Hryniewicz-Jankowska A, et al. Spectrins: a structural platform for stabilization and activation of membrane channels, receptors and transporters. Biochim Biophys Acta 2014;1838:620-34.
27 Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.
28 Schaap CC, Hendriks JC, Kortman GA, et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin Chem 2013;59:527-35.
29 Bridle K, Cheung TK, Murphy T, et al. Hepcidin is down-regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. Alcohol Clin Exp Res 2006;30:106-12.
30 van Santen S, Kroot JJ, Zijderveld G, et al. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. Clin Chem Lab Med 2013;51:1395-401.

Supplemental material

Supplemental Tables 4.5 and 4.6 can be requested by email:
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SUPPLEMENTAL TABLE 4.1 Cohort information and acknowledgements.

Cohort name	Cohort description and references	Financial support	Acknowledgements
Nijmegen Biomedical Study (NBS)	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 center Institutional Review Board. All participants gave written informed consent for participation in the NBS. For this study we used the subset of 1819 NBS participants that was selected to serve as controls in GWAS[2].	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 center. Principal investigators of the Nijmegen Biomedical Study are L.A.L.M. Kiemeney, A.L.M. Verbeek, D.W. Swinkels and B. Franke. We thank Doorlène van Tienoven for performing the serum hepcidin measurements.
The Prevention of REnal and Vascular ENd stage Disease study (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. 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).	
Val Borbera (VB)	The INGI-Val Borbera population is a collection of 1,785 genotyped samples collected in the Val Borbera Valley, a geographically isolated valley located within the Appennine Mountains in Northwest Italy[3,4]. 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 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.

SUPPLEMENTAL TABLE 4.2 Laboratory measurements.

Cohort	Time of blood sampling	Serum hepcidin	Serum ferritin	Serum iron	Transferrin or TIBC	TS	CRP
NBS	Blood was sampled between between 8 AM and 9 PM; not fasting.	Serum hepcidin was measured in February 2010 in 2998 samples with an in house developed and validated competitive enzyme-linked immunosorbent assay as described before [1,2]. Detection limit: 0.18 nmoles/L (number of samples below detection limit=9).	Serum ferritin concentration was determined by a chemiluminescent microparticle immunoassay on the Abbott Architect calibrated against the ferritin assay on the Immulite 2000 of Diagnostic Products Corporation.	Colorimetric measurement using ascorbate/FerroZine reagents (Roche Diagnostics) on an Abbott Aeroset analyzer.	Unsaturated iron binding capacity was measured by adding a known quantity of Fe^{3+} to the serum samples, reducing it with ascorbate to Fe^{2+} and measuring it with FerroZine as described for total serum iron (Roche reagents on an Aeroset). TIBC was calculated by adding serum iron and unsaturated iron-binding capacity.	Serum transferrin saturation (TS) was computed by dividing serum iron by TIBC.	CRP was quantified by immunologic agglutination detection with latex-coupled polyclonal anti-CRP antibodies (Abbott Reagent on Aeroset).
PREVEND	Fasting blood samples in the morning.	Hepcidin was measured in 6607 samples between October 2012 and March 2013 using the same assay as described for the NBS. Detection limit: 0.5 nmoles/L (number of samples below detection limit=420).	Modular E170, Roche, Mannheim, Germany Immunoassay (Sandwich) Measuring range 0.50-2000 µg/L LOD 0.50 µg/L	Colorimetric assay, Roche Modular P	Immunoturbidimetric assay, Roche Modular P	Sandwich immunoassay, Roche Modular E	Nephelometry (BNII N; Dade Behring, Marburg, Germany).
VB	Fasting blood samples (about 20 ml) were obtained in separate sessions, in the early morning after an overnight fast.	Serum hepcidin was measured with a validated mass spectrometry based method as described before [4]: surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDITOF-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, with recent technical improvements. The lower limit of detection was 0.55 nM (number of samples below detection limit=175).	Standard methods	Standard methods	Standard methods	Standard methods	Standard methods

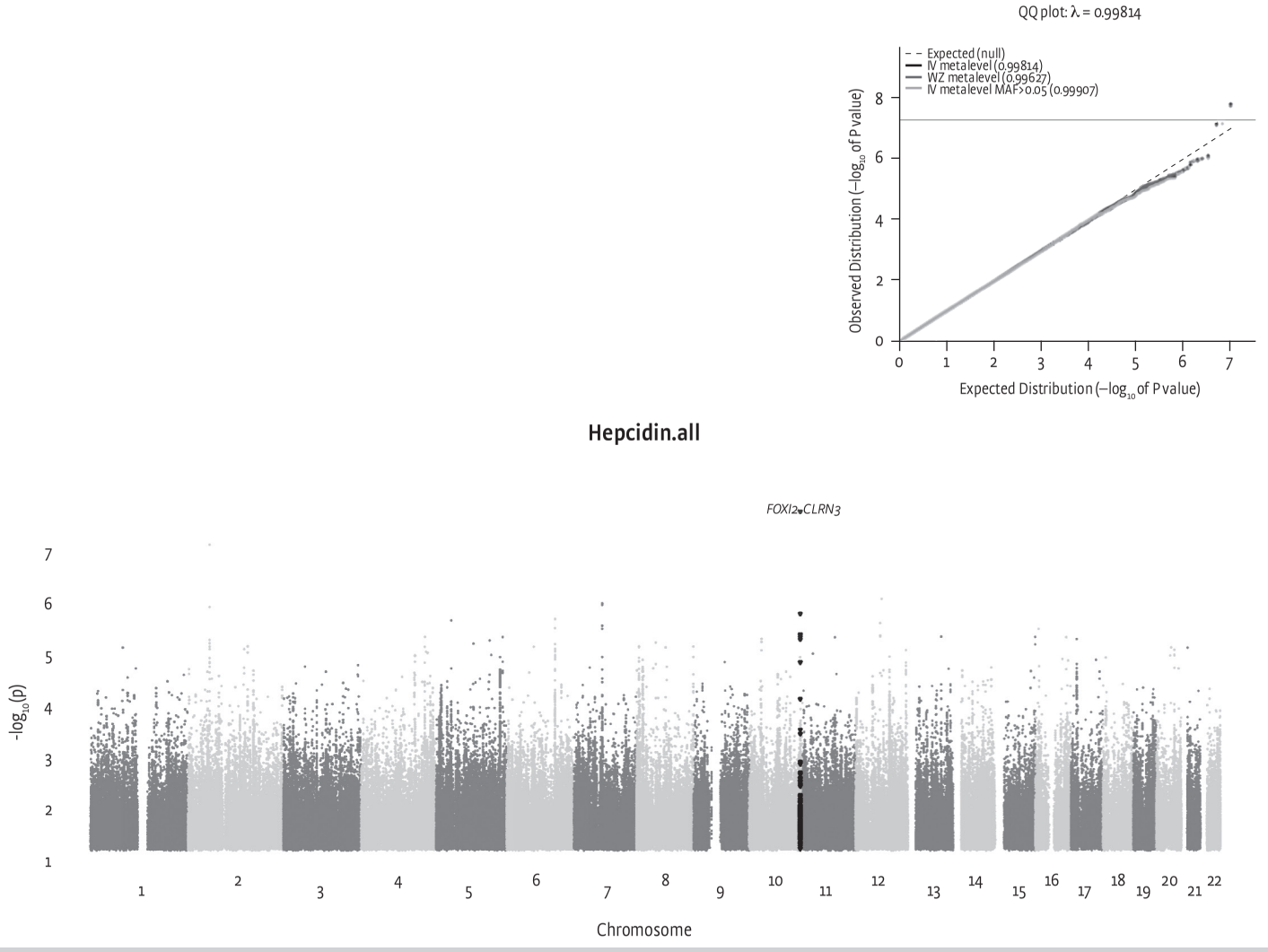
SUPPLEMENTAL TABLE 4.3 Phenotype information.

Discovery/ replication	Cohort	Sex	N	Age (years)	Hepcidin (nmoles/L)	Hepcidin/ferritin (µmoles/µg)	Hepcidin/TS (µmoles/L/%)	Ferritin (µg/L)	Serum iron (µmoles/L)	TIBC (µmoles/L)	TS (%)	CRP (mg/L)
Discovery	NBS	M	900	66 (55 - 77)	8.5 (1.2 - 23.2)	46.6 (17.1 - 120.6)	0.26 (0.04 - 0.92)	174.2 (29.5 - 532.5)	18.0 (10.0 - 28.0)	58.0 (45.0 - 73.0)	30.8 (16.1 - 51.0)	<4 (<4 - 15)
		F	918	57 (39 - 74)	6.7 (0.8 - 21.4)	75.7 (29.7 - 203.1)	0.24 (0.04 - 1.0)	84.0 (12.4 - 266.7)	16.0 (8.0 - 26.0)	60.0 (47.0 - 77.0)	26.6 (12.6 - 44.5)	<4 (<4 - 13)
	PREVEND	M	1495	50 (28 - 75)	4.6 (0.2 - 60.9)	30.7 (1.8 - 215.7)	1.9 (0.0 - 23.4)	179.6 (0.0 - 1925.0)	16.5 (0.0 - 45.0)	63.2 (37.7 - 103.0)	26.3 (2.9 - 65.2)	2.3 (0.2 - 51.8)
		F	1407	48 (28 - 75)	3.2 (0.2 - 32.7)	45.9 (0.7 - 631.7)	1.3 (0.1 - 19.2)	87.0 (3.0 - 1414.0)	15.0 (2.0 - 40.0)	66.7 (35.2 - 118.0)	23.1 (1.9 - 63.2)	3.0 (0.2 - 76.3)
	VB	M	688	56 (18 - 93)	11.9 (0.7 - 77.1)	92.8 (3.3 - 674.1)	0.4 (0.009 - 8.2)	161.3 (4 - 1283)	105 (18 - 321)	45.5 (9.1 - 136.4)	32.1 (6.4 - 96.1)	0.02 (0.01 - 0.47)
		F	792	56 (18 - 98)	9.8 (0.7 - 145)	201.5 (9.7 - 6530.6)	0.4 (0.018 - 7.1)	64.9 (2 - 696)	91.5 (7 - 199)	38 (2.1 - 127.4)	26.7 (1.5 - 89.7)	0.28 (0.01 - 1.58)
Replication	NBS	M	263	55 (34 - 83)	7.7 (1.4 - 19.6)	51.9 (18.3 - 128.5)	0.25 (0.05 - 0.82)	147.7 (28.8 - 468.6)	18.0 (9.0 - 31.8)	58.0 (48.0 - 74.0)	30.3 (15.8 - 54.4)	<4 (<4 - 11)
		F	261	57 (32 - 78)	7.1 (0.8 - 20.1)	77.8 (30.2 - 202.0)	0.26 (0.05 - 0.82)	93.5 (14.5 - 270.3)	16.0 (8.0 - 25.0)	60.0 (47.0 - 78.0)	28.1 (13.4 - 44.6)	<4 (<4 - 12)
	PREVEND	M	1580	50 (28 - 75)	4.4 (0.2 - 9.1)	29.2 (1.8 - 526.8)	1.8 (0.1 - 25.9)	182.8 (0.0 - 1636.0)	16.8 (0.0 - 46.0)	63.8 (40.2 - 143.1)	27.0 (2.0 - 73.0)	2.4 (0.2 - 76.2)
		F	1671	48 (28 - 75)	3.2 (0.2 - 50.4)	43.8 (1.3 - 416.7)	1.3 (0.1 - 26.5)	90.9 (0.0 - 975.0)	15.2 (2.0 - 44.0)	66.3 (32.6 - 120.5)	23.3 (2.0 - 60.5)	2.6 (0.2 - 94.0)

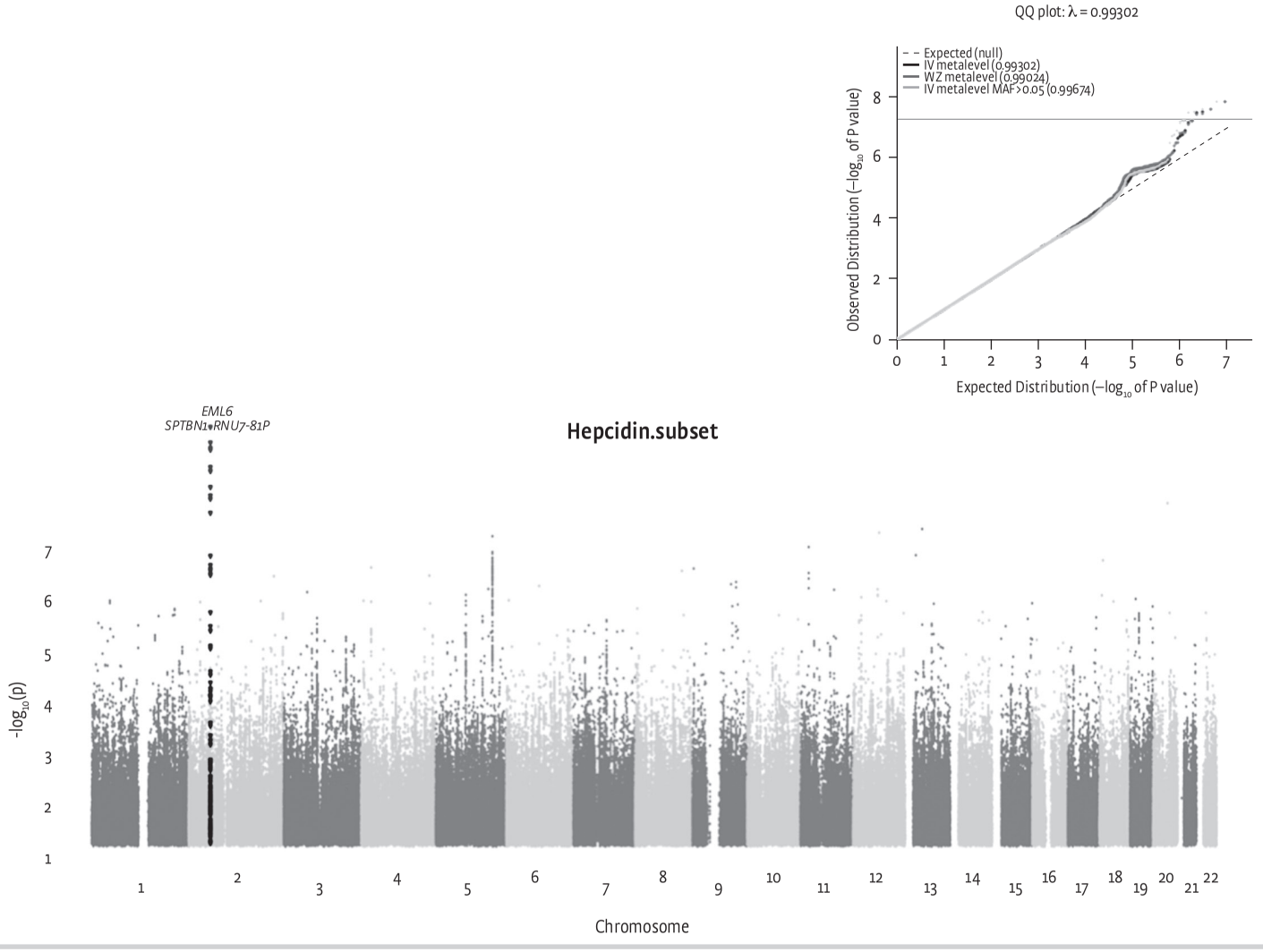
SUPPLEMENTAL TABLE 4.4 Information about genotyping, imputation and quality control.

Discovery/ replication	Cohort	Genotyping platform	Exclusion criteria	N of clean SNPs and individuals	Imputation			Statistical analysis
					N of imputed SNPs	Reference panel	Software	
Discovery	NBS	Illumina HumanHap-370CNV-Duo BeadChip	Sample yield ≥96% (after exclusion of intensity-only markers (n=23,573)), Caucasian ancestry ≥89% (based on Structure analysis), SNP yield ≥96%, MAF ≥1%, and HWE p-value >10^{-6}	1819 samples and 323,414 SNPs	38,037,370	1000genomes phase1 integrated version 3	IMPUTE2	SNPTEST v2.4.1
	PREVEND	Illumina Cyto SNP12 v2	Population stratification was assessed by principal component analysis, Z-score > 3 for the first 5 principal components were excluded. Callrate <95%, duplicate samples and sex discrepancies were also excluded. Markers with call rate <95%, pHWE<0.00001, MAF ≥1% were included.	3,649 samples and 232,571 SNPs	12,862,598	1000genomes phase1 integrated version 3	Minimac	SNPTEST v2.4.1
	VB	Illumina 370 Quad-CNV array, v3	call rate >=90%, MAF >=1%, HWE p-value >10^{-6}	1785 samples and 332887 (for 1664 individuals with Illumina 370k chip);648130 (for 121 individuals with Illumina OmniExpress 700k)	38,043,574	1000genomes phase1 integrated version 3	SHAPEITv2for 1664 individuals with Illumina 370K chip;none for 121 individuals with illumina OmniExpress 700k; IMPUTE version 2.2.2	R, GEMMA SNPTEST v2.4.1
In silico replication	NBS	HumanOmniExpress-12v1-1_B	Sample yield Country of birth The Netherlands or self-reported ethnicity Caucasian, ≥96%, SNP yield ≥96%, MAF ≥1%, and HWE p-value >10^{-6}	1212 samples (524 samples with hepcidin measurements) and 632,968 SNPs	38,037,370	1000genomes phase1 integrated version 3	IMPUTE2	SNPTEST v2.4.1

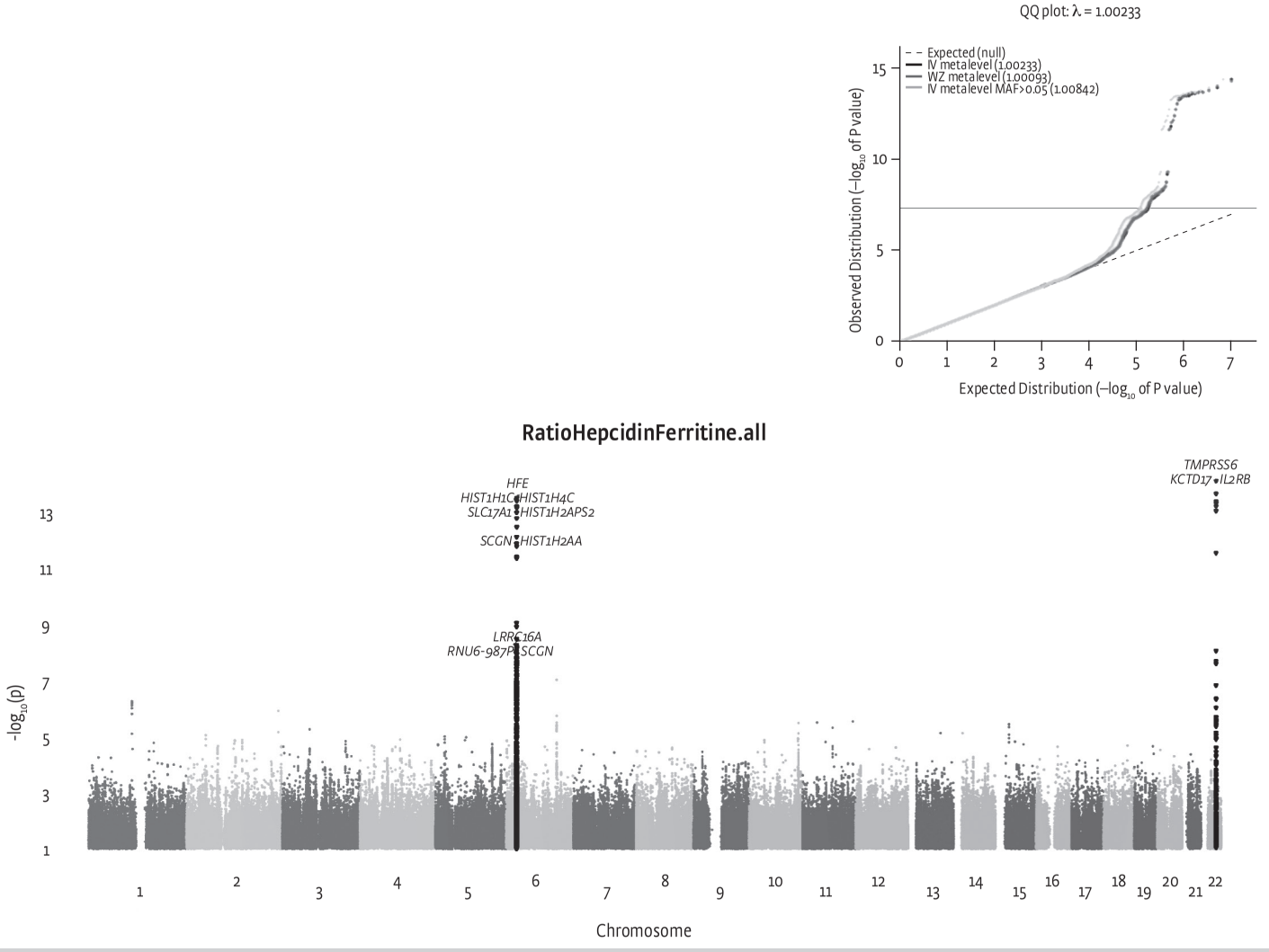
SUPPLEMENTAL FIGURE 4.1 Manhattan plot and QQ plot for the meta-analysis results for hepcidin in all individuals.



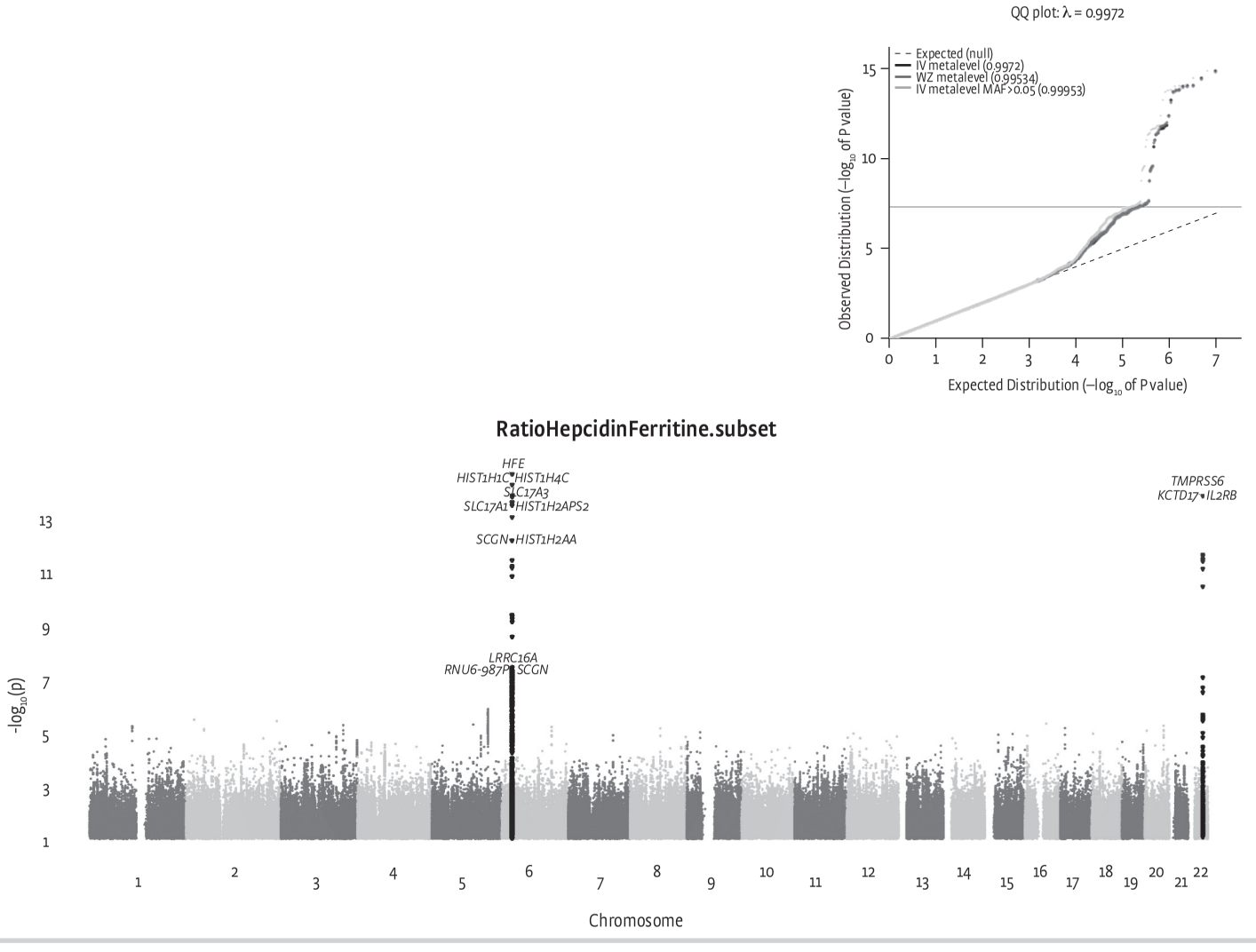
SUPPLEMENTAL FIGURE 4.2 Manhattan plot and QQ plot for the meta-analysis results for hepcidin in the subset.



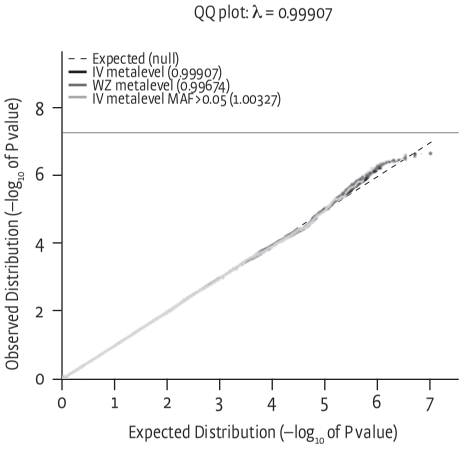
SUPPLEMENTAL FIGURE 4.3 Manhattan plot and QQ plot for the meta-analysis results for the ratio hepcidin/ferritin in all individuals.

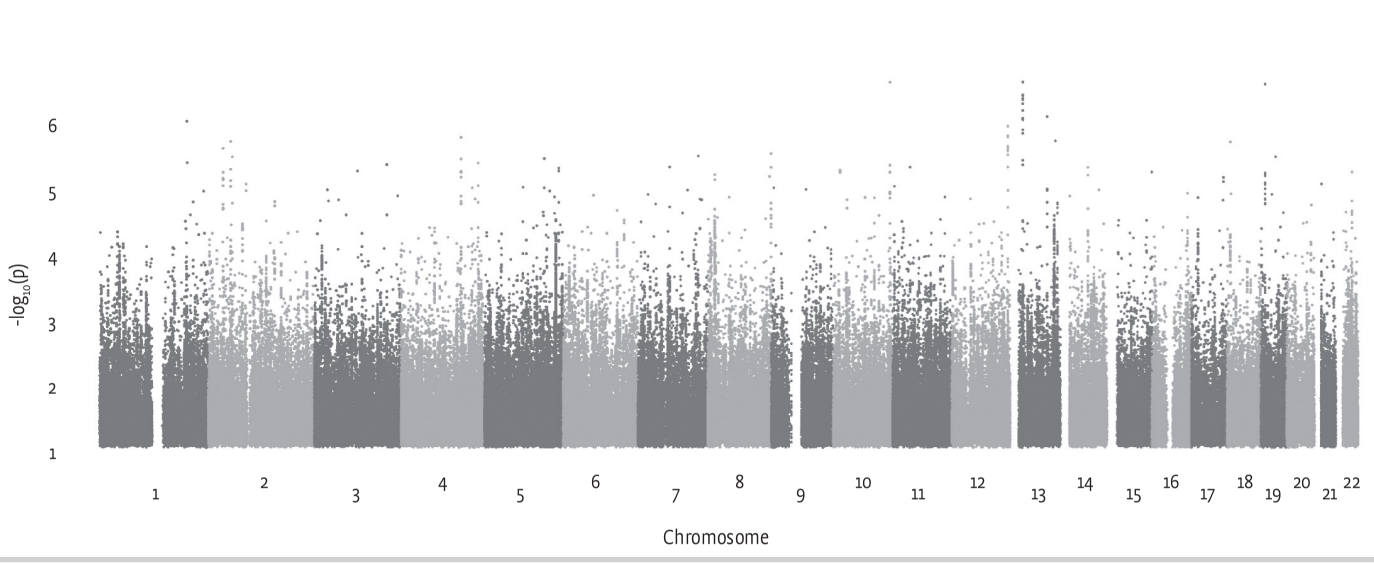


SUPPLEMENTAL FIGURE 4.4 Manhattan plot and QQ plot for the meta-analysis results for the ratio hepcidin/ferritin in the subset.

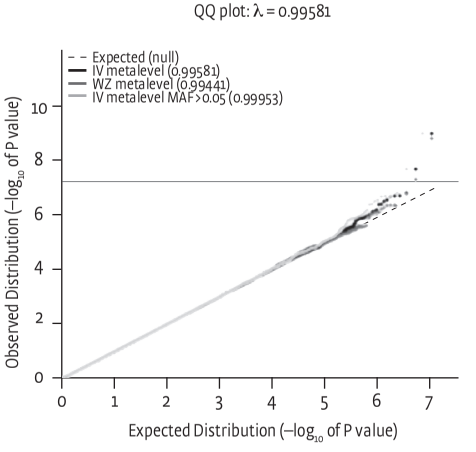


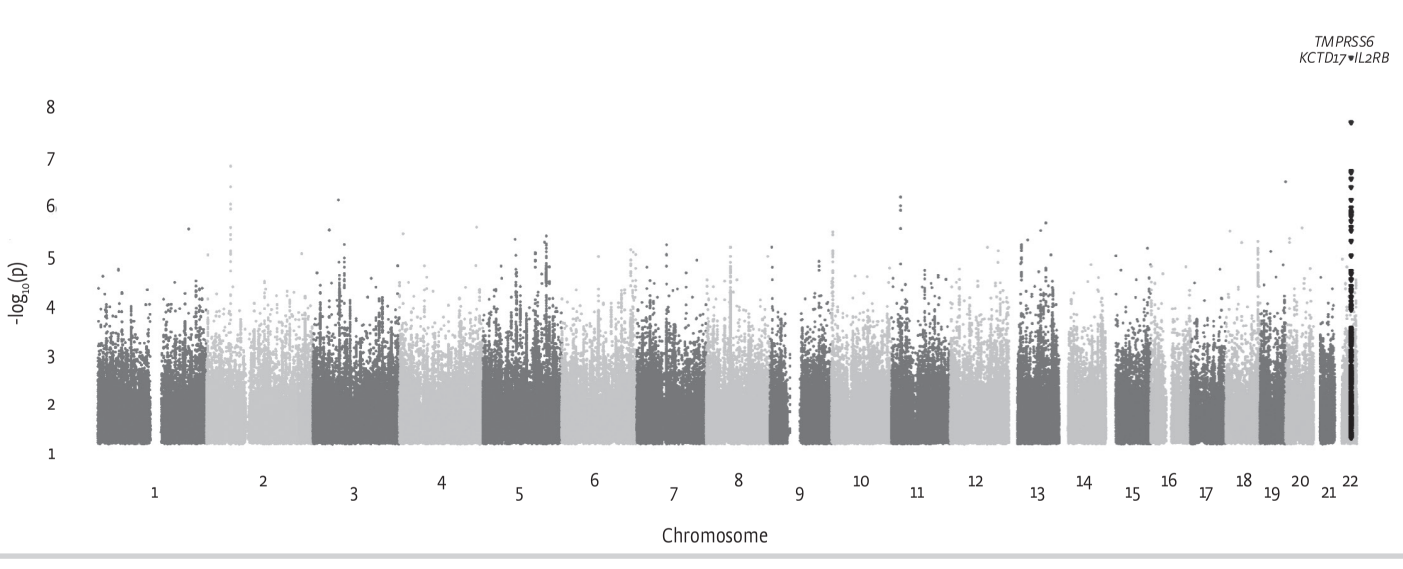
SUPPLEMENTAL FIGURE 4.5 Manhattan plot and QQ plot for the meta-analysis results for the ratio hepcidin/TS in all individuals.



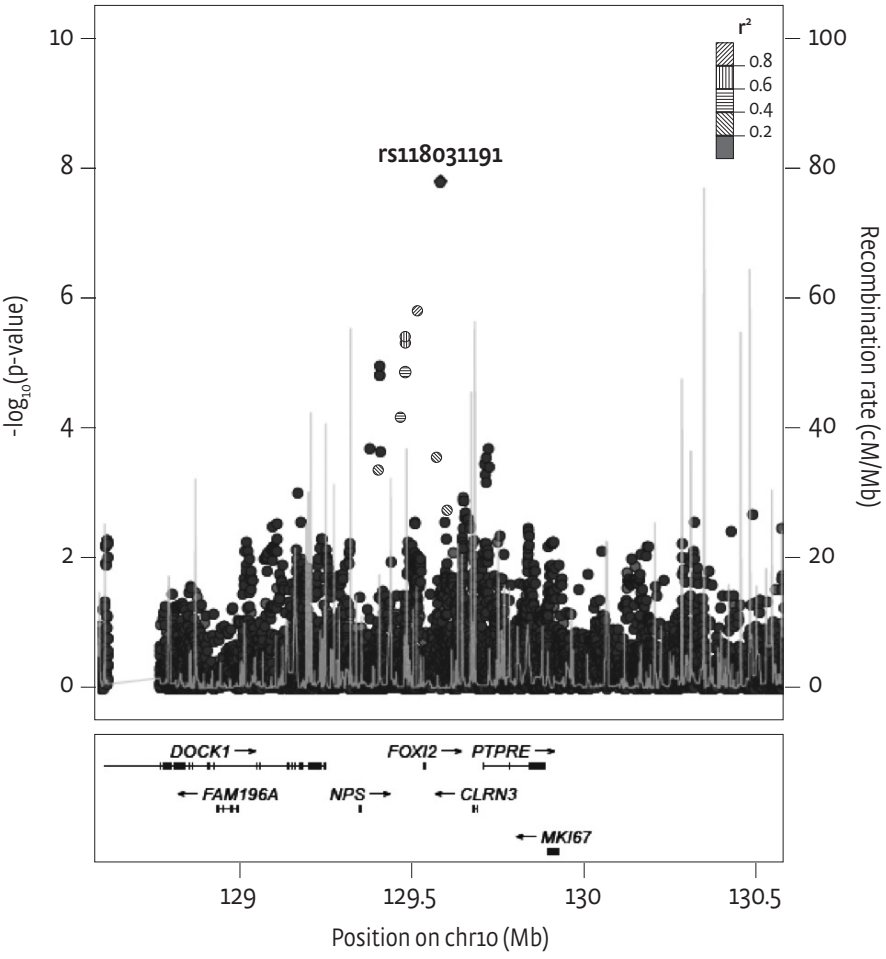


SUPPLEMENTAL FIGURE 4.6 Manhattan plot and QQ plot for the meta-analysis results for the ratio hepcidin/TS in the subset.

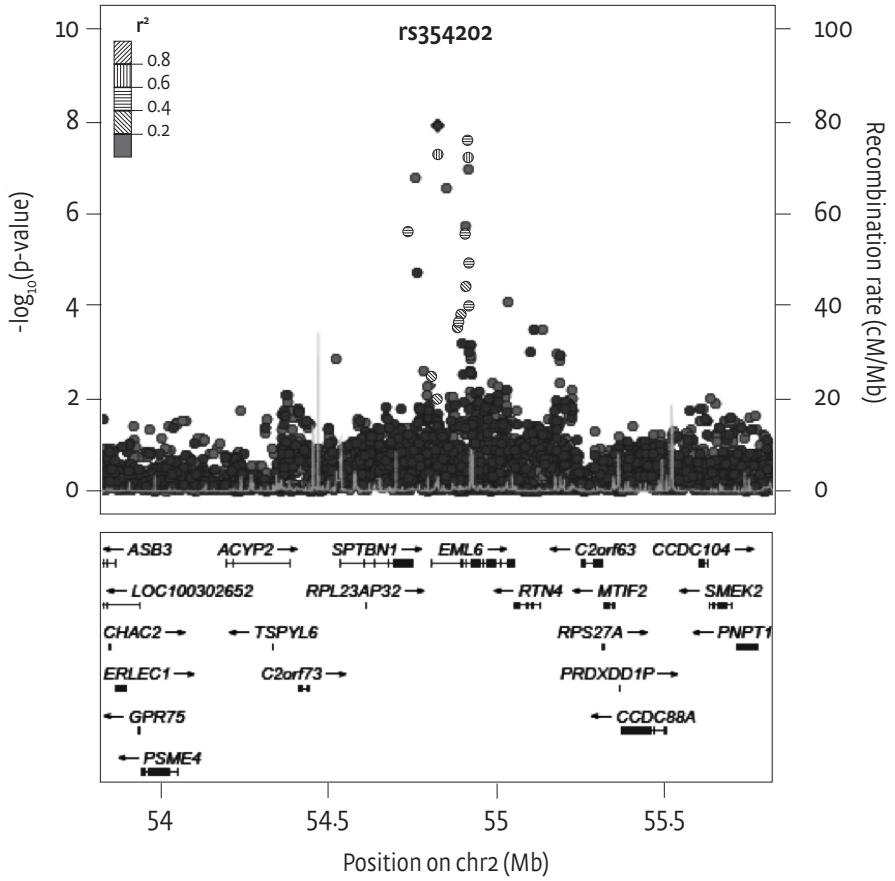




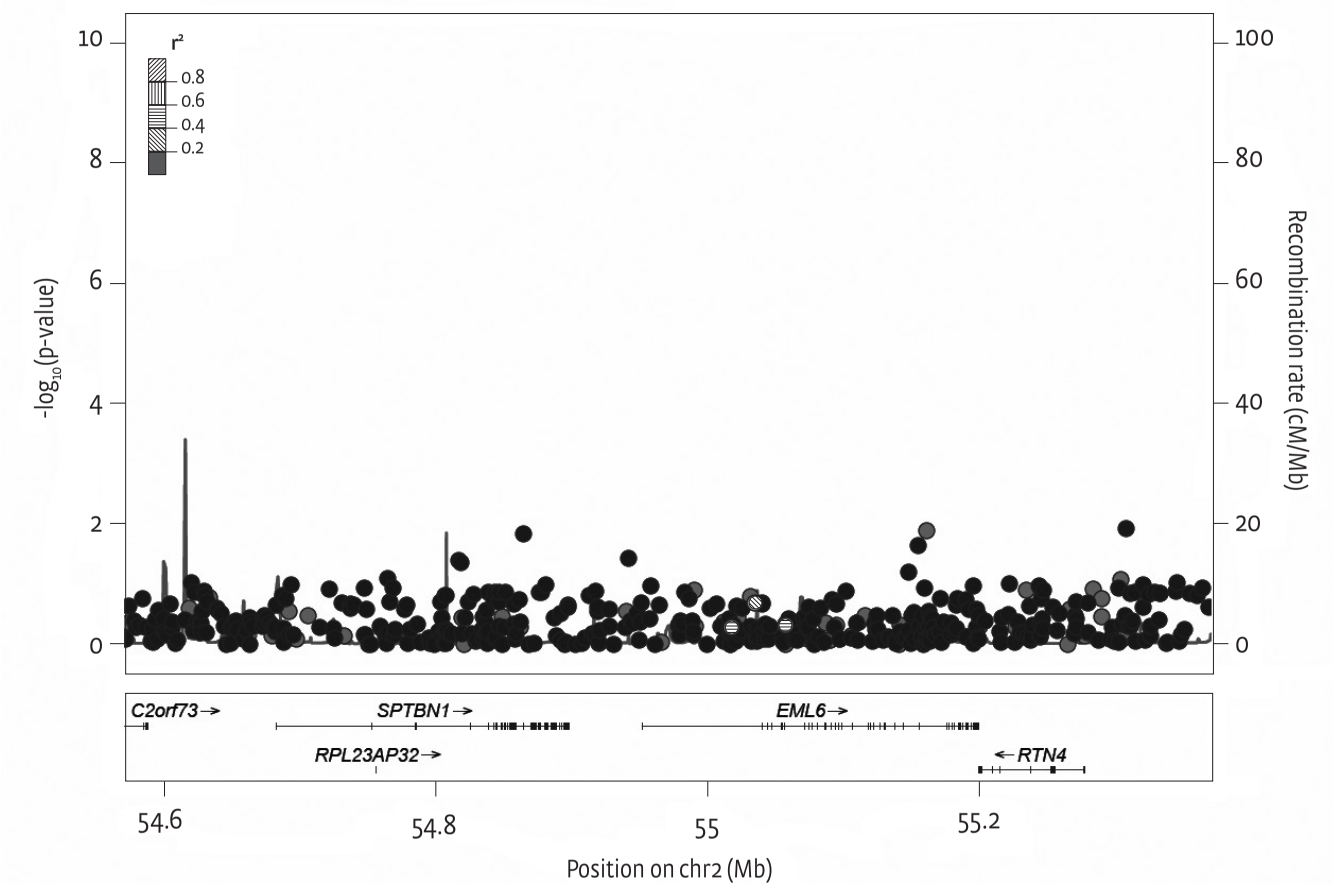
SUPPLEMENTAL FIGURE 4.7 Regional association plot for rs118031191 with serum hepcidin in all individuals.



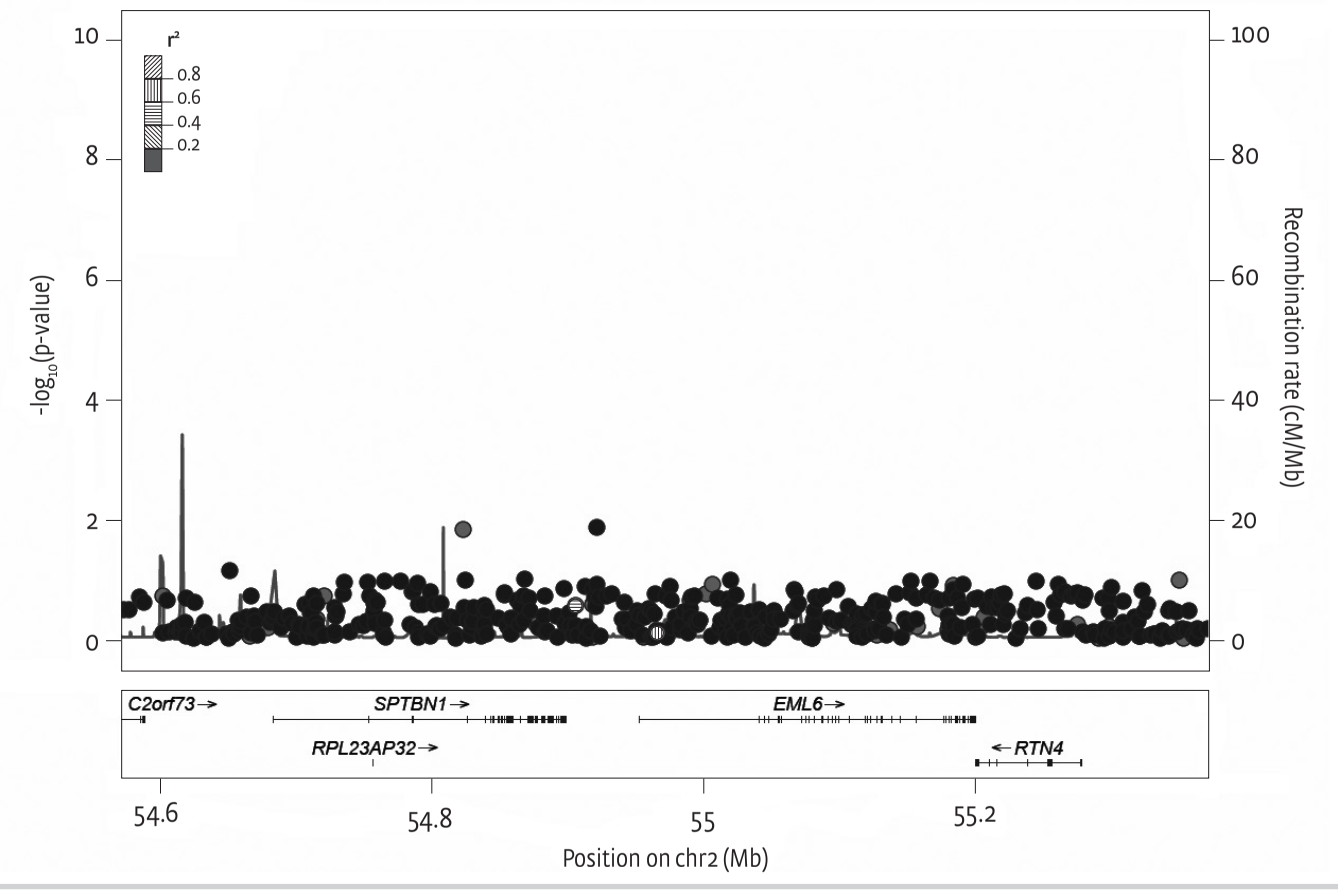
SUPPLEMENTAL FIGURE 4.8 Regional association plot for rs354202 with serum hepcidin in the subset.



SUPPLEMENTAL FIGURE 4.9 Regional association plot for the chromosome 2 locus with serum hepcidin conditioned on rs354202 in all individuals (NBS data only).



SUPPLEMENTAL FIGURE 4.10 Regional association plot for the chromosome 2 locus with serum hepcidin conditioned on rs354202 in the subset (NBS data only).



Supplemental references

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

2 Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570-9.

3 Colonna V, Pistis G, Bomba L, et al. Small effective population size and genetic homogeneity in the Val Borbera isolate. Eur J Hum Genet 2013;21:89-94.

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

CHAPTER 5

A comparison of multivariate genome-wide association methods

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ABSTRACT

Joint association analysis of multiple traits in a genome-wide association study (GWAS), *i.e.* a multivariate GWAS, offers several advantages over analyzing each trait in a separate GWAS. In this study we directly compared a number of multivariate GWAS methods using simulated data. We focused on six methods that are implemented in the software packages PLINK, SNPTEST, MultiPhen, BIMBAM, PCHAT and TATES, and also compared them to standard univariate GWAS, analysis of the first principal component of the traits, and meta-analysis of univariate results. We simulated data (N=1,000) for three quantitative traits and one bi-allelic quantitative trait locus (QTL), and varied the number of traits associated with the QTL (explained variance 0.1%), minor allele frequency of the QTL, residual correlation between the traits, and the sign of the correlation induced by the QTL relative to the residual correlation. We compared the power of the methods using empirically fixed significance thresholds (α=0.05). Our results showed that the multivariate methods implemented in PLINK, SNPTEST, MultiPhen and BIMBAM performed best for the majority of the tested scenarios, with a notable increase in power for scenarios with an opposite sign of genetic and residual correlation. All multivariate analyses resulted in a higher power than univariate analyses, even when only one of the traits was associated with the QTL. Hence, use of multivariate GWAS methods can be recommended, even when genetic correlations between traits are weak.

Introduction

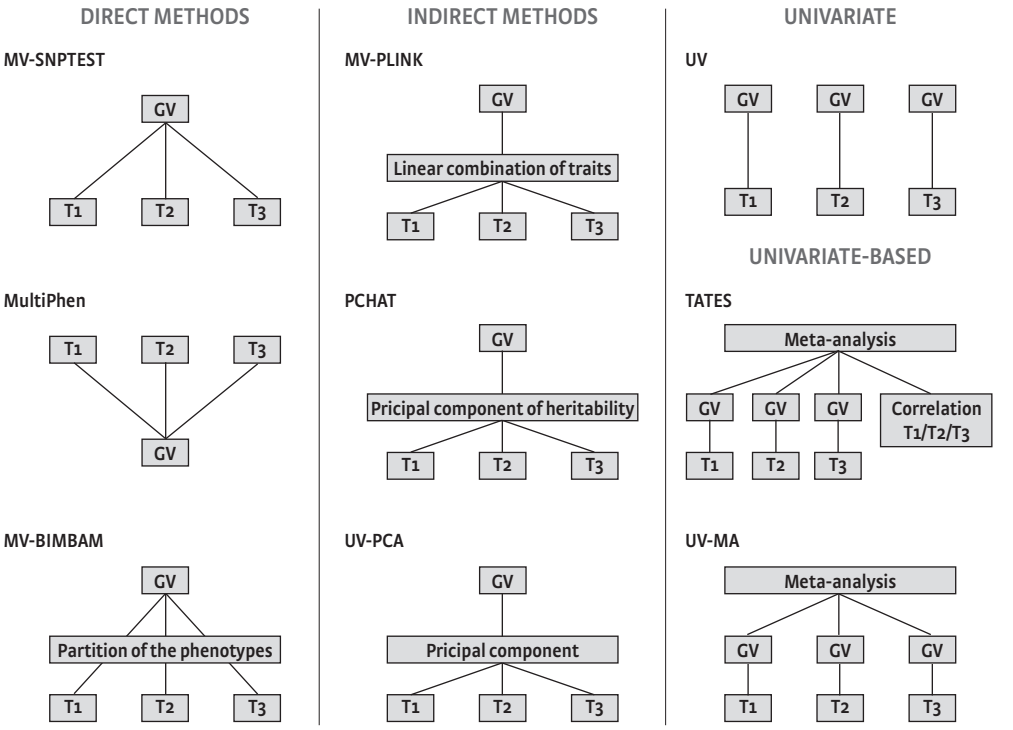
Genome-wide association studies (GWAS) have been very successful in the identification of common genetic variants associated with complex traits. Usually, information on a set of related traits is collected in populations sampled for GWAS. These traits are typically analyzed separately, *i.e.* in a univariate manner, for association to genome-wide DNA markers. This is often followed by an informal comparison of evidence for association at particular loci across the studied traits (*e.g.* [1]). However, a joint analysis of multiple, potentially correlated traits, *i.e.* a multivariate analysis, could be very advantageous for a number of reasons. First, a multivariate analysis has increased power in case of presence of genetic correlation between the different traits; the extra information that is provided by the cross-trait covariance is ignored in univariate analyses [2,3]. Secondly, most multivariate procedures can perform a single test for association with a set of traits. This reduces the number of performed tests and alleviates the multiple testing burden compared to analyzing all traits separately [2,4]. Finally, in case of presence of pleiotropy, where a single genetic variant is associated with multiple traits, a multivariate GWAS is more consistent with biology compared to cross-trait comparison of univariate analyses [5].

A number of methods for simultaneous analysis of multiple traits in population-based GWAS have been published (*e.g.* [4,6-19]). Although a few of the methods have been compared to newly proposed methods [12,15] and some of the methods have been compared to univariate analysis [4,7,12], little is known about their relative performances. Here, we performed the first direct comparison of several multivariate (MV) GWAS methods using simulated data. We included six methods, with a focus on methods already implemented in freely available software: the multivariate test of association MQFAM implemented in the genetic association analysis software PLINK (MV-PLINK) [7], a Bayesian multiple phenotype test implemented in SNPTEST (MV-SNPTEST) [20], the R package MultiPhen (MultiPhen) [12], a Bayesian model comparison and model averaging for multivariate regression in BIMBAM (MV-BIMBAM) [21,22], the Principal Component of Heritability Association Test (PCHAT) [4], and a Trait-based Association Test that uses Extended Simes procedure (TATES) [15]. These can be classified into direct, indirect and univariate-based methods (Figure 5.1). MV-SNPTEST, MultiPhen and MV-BIMBAM are direct MV methods, in which the effects of the genetic variant are modeled directly on the traits without changing the general format and nature of the trait data. MV-SNPTEST [20] and MV-BIMBAM [22] are both based on a Bayesian multivariate regression analysis, but MV-BIMBAM additionally partitions the traits into three groups: 1) traits that are unaffected by the genetic variant, 2) traits that are directly affected by the genetic variant, and 3) traits that are indirectly affected by the genetic variant through directly affected traits. MultiPhen identifies the linear combination of traits most associated with each genetic variant by applying a reversed ordinal regression, such that genotype (allele count) is regressed on a collection of traits [12]. MV-PLINK [7], PCHAT [4] and UV-PCA are indirect methods based on a reduction of the trait dimension. In MV-PLINK the association between a set of traits and a

genetic variant is assessed using canonical correlation analysis. Specifically, the linear combination of traits that maximizes the covariance between the genetic variant and all traits is extracted. PCHAT is based on extracting the principal component of heritability that is the optimal linear combination of the traits from a heritability point of view [4]. In TATES [15], the observed correlation structure between the traits is taken into account in the meta-analysis approach.

We compared the power of the methods under empirically fixed type I errors to one another and to standard univariate (UV) analysis, univariate analysis of the first principal component of the traits (UV-PCA), and meta-analysis of univariate results (UV-MA). In UV-PCA, the first principal component of a standard principal component analysis is extracted and used in a univariate analysis. In UV-MA, p-values obtained in standard UV GWAS analyses are combined in a meta-analysis approach. Our goal was to provide researchers with insights that will guide the application of the methods to real data.

FIGURE 5.1 Schematic representation of the included methods.



GV indicates genetic variant; MV, multivariate; PCHAT, Principal Component of Heritability Association Test; T1, trait 1; T2, trait 2; T3, trait 3; TATES, Trait-based Association Test that uses Extended Simes procedure; UV-MA, meta-analysis of univariate results; UV-PCA, univariate analysis of first principal component.

Methods

Data simulation

We simulated genotype and phenotype data for 1,000 individuals. Simulations were performed in R.

Genotype data were simulated for one bi-allelic quantitative trait locus (QTL) with minor allele frequency *q* and major allele frequency *p*. Genotypes were generated by sampling two alleles independently from a binomial distribution as 0 or 1, using two trials and a probability of success of each trial equal to *q*. The genotype is the sum of the two alleles, which can be 0, 1 or 2. Because alleles were sampled independently, genotypes were in Hardy Weinberg equilibrium.

Phenotype simulation was based on work by Saint-Pierre and colleagues [14]. For each individual, three quantitative traits Y_j (j=1, 2, 3) were simulated. The trait-specific QTL heritabilities (the relative variance of Y_j explained by the QTL, h^2_j), MAF of the QTL *q*, and residual correlation between the traits excluding the QTL effect (rE_{jj}) were controlled.

First, the effect of the QTL on the individual traits, a_j, was determined from h^2_j and *q* using the following formula [14]: $a_j = \sqrt{h_j^2 / 2pq}$

Secondly, the traits were constructed by adding up the trait-specific effect of the QTL and a residual component. Here, the trait-specific effect of the QTL was assumed to be additive and obtained by multiplying a_j with the number of effect (minor) alleles. The residual component (e_1,e_2,e_3) was simulated from a multivariate normal distribution with mean zero and with variance-covariance structure:

$$Var\begin{pmatrix} e_1 \\ e_2 \\ e_3 \end{pmatrix} = \begin{bmatrix} 1\text{-}h_1^2 & rE_{12}\sqrt{1\text{-}h_1^2}\sqrt{1\text{-}h_2^2} & rE_{13}\sqrt{1\text{-}h_1^2}\sqrt{1\text{-}h_3^2} \\ & 1\text{-}h_2^2 & rE_{23}\sqrt{1\text{-}h_2^2}\sqrt{1\text{-}h_3^2} \\ symm & & 1\text{-}h_3^2 \end{bmatrix}$$

where $(1 - h^2_j)$ is the trait-specific proportion of the variance not explained by h^2_j and rE_{jj} is the residual correlation between the traits excluding the QTL effect. The correlated residuals were generated using the function mvrnorm from the R package MASS. Third, all traits were centered and scaled to have zero mean and unit variance.

Application of methods

MV-PLINK: The command used for association testing with MV-PLINK [7,23] (https://genepi.qimr.edu.au/staff/manuelF/multivariate/main.html) was: *plink.multivariate --noweb --file geno --mqfam --mult-pheno pheno.phen --out output*. We applied an additive model. MV-

PLINK produces an F-statistic and a p-value per genetic variant analyzed. This p-value of multivariate association was extracted from the output. Note that the canonical correlation analysis (CCA) applied by MV-PLINK is similar to multivariate analysis of variance (MANOVA) as CCA is applied to a single genetic variant at a time.

MV-SNPTEST: The command used to perform additive association testing with MV-SNPTEST [20] (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html#multiple_phenotype_tests) was: *snptest -data geno.gen pheno.sample -o output -bayesian 1 -method expected -mpheno T1 T2 T3 -prior_qt_mean_b 0 -prior_qt_V_b 0.02 -prior_mqt_c 4 -prior_mqt_Q 6.*

An inverse Wishart prior [IW(6,4)] was set on the error covariance matrix ∑ and a matrix normal prior [N(0.02,∑)] on the vector of parameters, according to recommendations of the authors. Method 'expected' was applied, which results in the use of expected genotype counts (~dosages) in the analyses. The output file contains a $\log_{10}$ Bayes Factor (BF) per genetic variant, which was extracted for the purpose of this study.

MultiPhen: MultiPhen is an R package available from CRAN (http://cran.r-project.org/web/packages/MultiPhen/MultiPhen.pdf) for the R software (http://www.r-project.org/) [12]. The test for association is a likelihood ratio test (LRT) for model fit, testing whether all regression coefficients in the model are jointly significantly different from zero. We analyzed the simulation data using the mPhen function, specifying the genotype data, phenotype data and JointModel=TRUE. This results in a p-value per trait and a p-value for the LRT. The latter was extracted from the output.

MV-BIMBAM: The BIMBAM software [22] can be run in two different ways: 1) using option –mph 1, which tests for association between the multivariate traits, all partitioned in the group of directly affected traits, and genotype; and 2) using option –mph2, which considers all the different possible partitions of traits into the different categories of traits (directly affected, indirectly affected, unaffected). We applied option –mph2 under the additive model using the following command: *bimbam -g geno.txt -p pheno.txt -o output -f 3 -mph2 -A 0.1 -A 0.2.*

According to recommendations of the authors, the prior for the genetic effect A was set at 0.1 and 0.2. The association results are summarized by a $\log_{10}$ BF that evaluates presence of any association between the QTL and the traits averaging over all possible partitions of the traits into the different groups. This value was extracted from the output.

PCHAT: In PCHAT [4] (http://www.wpic.pitt.edu/wpiccompgen/PCHAT/PCHAT.htm) the sample is split in a training set, which is used to construct the optimal linear combination of traits from a heritability point of view, and a test set, which is used for association testing

between genotype and the optimal linear combination of traits. In this way, use of the same data for both estimation of the optimal linear combination of traits and association testing is avoided. In addition, so called 'bagging' is performed, in which bootstrap samples are drawn from the training sample and the optimal linear combination of traits is averaged across bootstrap samples. The null distribution of the test statistic is obtained in the same way, using permutation of the data. We applied the additive model and set input parameters to values recommended by the authors: 50 subsets and bagging subsets for the determination of the distribution of the PCHAT test statistic under the null hypothesis; 200 and 50 subsets and bagging subsets, respectively, for testing the association of a genetic variant with the trait; 150 individuals for the subsets; and 1000 simulations for determination of the distribution of the test statistic under the null hypothesis. The option "both" was used for the analysis, resulting in a permutation experiment to determine the null distribution of the test statistic, after which the association test is performed using the standard deviation and degrees of freedom obtained in the permutation test. PCHAT produces 11 output files. In one of them, the association result is expressed as a p-value, which was extracted for this study.

TATES: TATES [15] (http://ctglab.nl/software) requires a correlation matrix of the traits and univariate association results as input. Full, symmetrical correlation matrices were generated using the corr option in R. UV analyses for the traits were performed by fitting linear models using the lm function in R. TATES was run in R using the freely available script specifying three traits and one genetic variant. The output contains the TATES trait-based p-value corrected for the correlations between the traits, which was extracted for this study.

Univariate (UV) analysis, meta-analysis of univariate results (UV-MA) and UV-PCA: UV analyses were performed as described under 'TATES'. Resulting p-values were extracted for the purpose of this study. UV-MA was performed with METAL [24] (http://www.sph.umich.edu/csg/abecasis/Metal/), using univariate results per trait as input files and the analysis scheme 'scheme samplesize', which uses p-value and direction of effect as input for the MA and weighs according to sample size. PCA was performed in R using the princomp command. UV-PCA was executed using the first principal component (PC) in a univariate analysis as described above.

Empirical significance thresholds (Ho simulations) and power

For each simulation scenario (see below) 1,000 datasets were simulated. Empirical significance thresholds for all methods were derived based on permutation of the traits generated in these simulation datasets, resulting in the null distribution of the test statistic. We generated 10 permuted datasets per simulation dataset, resulting in a total of 10,000 permuted replicates per scenario. These replicates were analyzed with the multivariate methods, resulting in a p-value or $\log_{10}$ BF for each replicate per method per scenario. Significance thresholds were set in such a way that 5% of the 10,000 replicates per method yielded a significant result (5% false-positive rate). This was done by sorting the 10,000 association

measures in ascending (p-values) or descending order (Bayes Factors) and defining the empirical significance threshold as the mean of the 500th and 501st association measure.

UV analysis results in one p-value per trait. We adjusted the significance thresholds for three UV association tests, ensuring that alpha was fixed at 5% for all traits combined. For each null model, we first determined which trait was most strongly associated with the QTL per replicate, *i.e.* which trait resulted in the smallest p-value. These 10,000 p-values were sorted in ascending order and the mean of the 500th and 501st p-value was set as the threshold.

The power is defined as the percentage of 1,000 replicates for which the extracted p-value was smaller or $\log_{10}$ BF was larger than the empirical significance thresholds, ensuring an equal type I error rate of 5% for all methods.

Simulation scenarios

Simulations were focused on three main scenarios in which one, two or three out of the three traits were associated with the QTL. Within these main scenarios, data sets were generated for a given combination of parameter values (rE_{jj}, h^2_j, rG and q) as shown in Table 5.1. This resulted in a total of 30 simulation scenarios.

We simulated positive residual correlations between the traits and studied scenarios with a relatively high and low residual correlation (rE_{jj}=0.7 and rE_{jj}=0.3, respectively). The QTL was fixed to explain 0.1% of the trait variances. By varying the sign of a_1, we created a QTL induced correlation (rG) between trait 1 and traits 2 and 3 which was either positive or negative, enabling us to study the influence of a negative genetic correlation.

Note that due to the fixed trait-specific QTL heritabilities, the resulting QTL effects on the individual traits are larger for smaller q and vice versa. This fits with the scenario one would expect in real data [25].

TABLE 5.1 Simulation scenarios.

# traits associated with QTL	Heritability (h^2_j)	Effect size (a_j)	rG	rE	MAF (q)
1	h^2_1 = 0.1%, $h^2_2 = h^2_3$ = 0	a_1>0, $a_2=a_3$=0	0	3x0/3x0.3/3x0.7	0.01/0.4
2	$h^2_1 = h^2_2$ = 0.1%, h^2_3 = 0	$a_1=a_2$, a_3=0	+	3x0/3x0.3/3x0.7	0.01/0.4
	$h^2_1 = h^2_2$ = 0.1%, h^2_3 = 0	$-a_1=a_2$, a_3=0	-	3x0/3x0.3/3x0.7	0.01/0.4
3	$h^2_1 = h^2_2 = h^2_3$ = 0.1%	$a_1=a_2=a_3$	+	3x0/3x0.3/3x0.7	0.01/0.4
	$h^2_1 = h^2_2 = h^2_3$ = 0.1%	$-a_1=a_2=a_3$	-	3x0/3x0.3/3x0.7	0.01/0.4

MAF indicates minor allele frequency; j, trait; QTL, quantitative trait locus; rE, residual correlation; rG, genetic correlation.

Results

Empirical significance thresholds

Supplemental Table 5.1 shows the empirical significance thresholds for all methods for every simulation scenario. Thresholds were around 5% for MV-PLINK, MultiPhen, TATES and UV-PCA. Significance thresholds for PCHAT were slightly increased to approximately 6%, indicating slight deflation of type I error rate under the null. MV-SNPTEST and MV-BIMBAM showed $\log_{10}$ BF significance thresholds between -0.05 and 0.44. Significance thresholds for UV-MA were highly dependent on the residual correlation between the traits: around 5% for scenarios with uncorrelated traits and 0.2-0.3% for scenarios with high residual correlation, thus indicating high inflation of type I error rate under the null for the latter scenarios. Thresholds for UV analysis were around 5%/3=1.7% for scenarios with no residual correlation and slightly increased with increasing residual correlation.

Power comparison

One out of three traits associated with the QTL (Figure 5.2 A)

All MV methods resulted in higher power than UV analysis. Power of MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM was similar: between 10% and 15% for scenarios with a residual correlation of 0 or 0.3 and 20-25% for scenarios with rE=0.7. PCHAT outperformed MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM for scenarios with rE=0 and 0.3, but not for scenarios with rE=0.7, although power of PCHAT increased for rE=0.7 as well. TATES showed a power between 11-14% for all scenarios and performed slightly better than UV-PCA and UV-MA, which showed a similar performance as UV analysis of trait 1, the trait associated with the QTL.

Two out of three traits associated with the QTL (Figure 5.2 B)

MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM showed the best and similar performance, with higher power with increasing residual correlation. This was most noticeable when the correlation induced by the QTL was negative.

PCHAT and TATES showed a robust performance relatively independent of the residual correlation, but their power never exceeded that of MV-PLINK, MV-SNPTEST, MultiPhen or MV-BIMBAM.

UV-PCA and UV-MA showed the same performance for scenarios in which there was a residual correlation between the traits. For scenarios with no residual correlation, UV-PCA performed better under negative genetic correlation and UV-MA under positive genetic correlation. They were outperformed by UV analysis of trait 1 and 2 only in case of a negative genetic correlation.

Three out of three traits associated with the QTL (Figure 5.2 C)

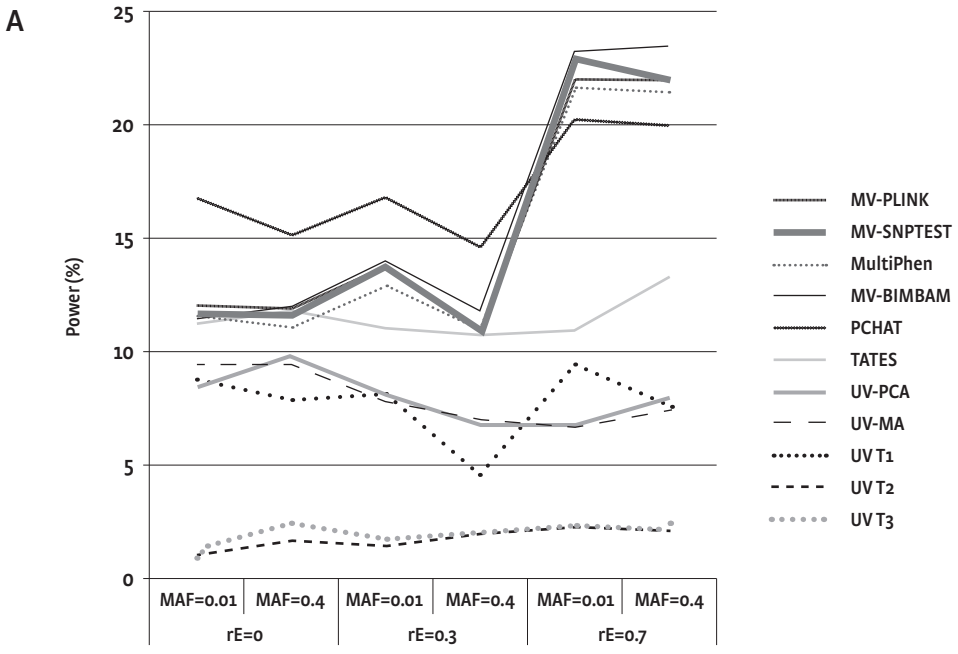
Again, MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM performed similar and best

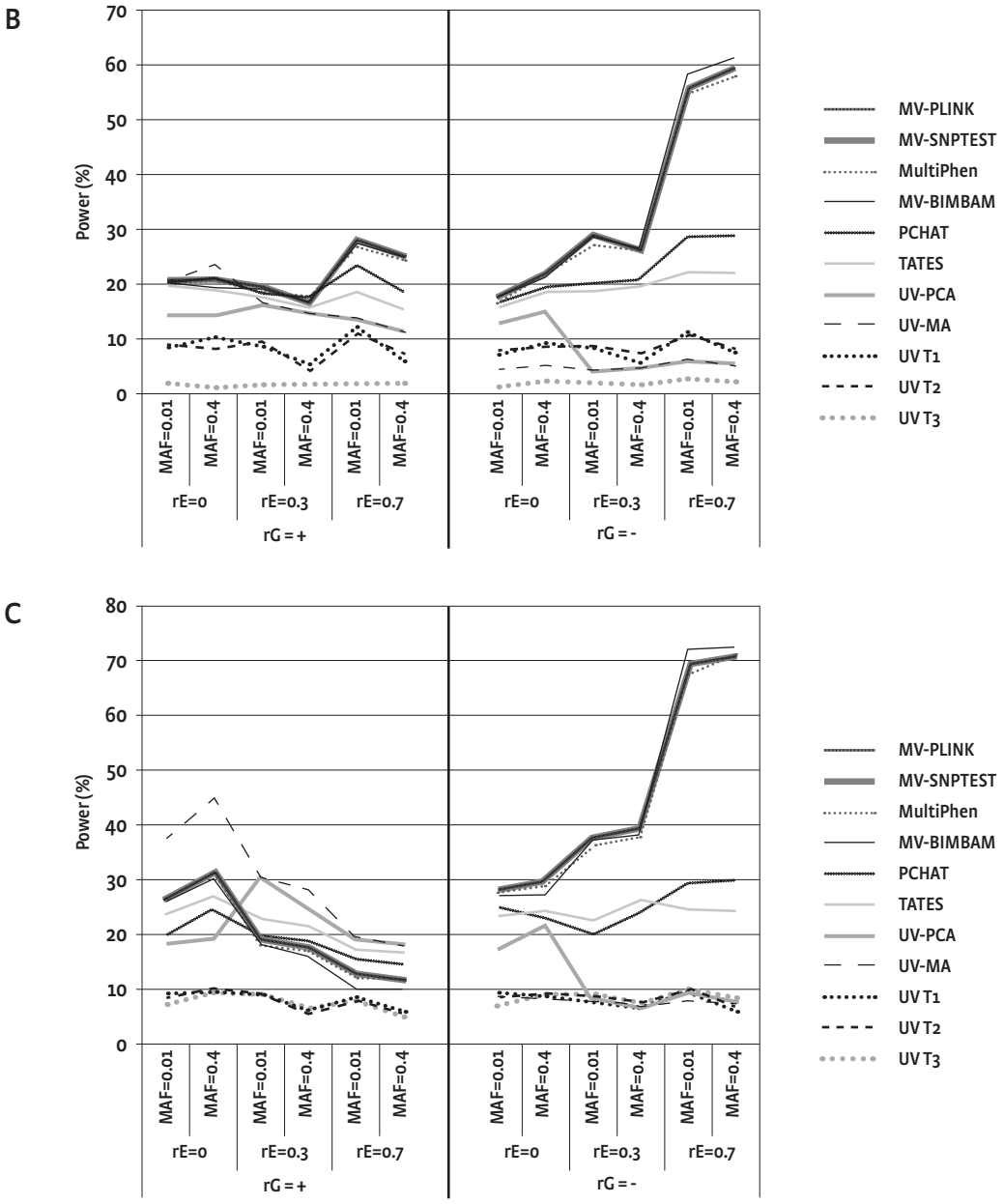
under simulation scenarios with a negative genetic correlation; in this case, their power increased with increasing residual correlation. However, for scenarios with a positive genetic correlation, power of MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM increased with decreasing residual correlation.

PCHAT and TATES showed a comparable performance over all simulation scenarios. Also here, power slightly increased with decreasing residual correlation in case of a positive genetic correlation, and slightly increased with increasing residual correlation for a negative genetic correlation.

Similar to simulation scenarios with two out of three traits associated to the QTL, UV-PCA and UV-MA showed comparable power for all scenarios with rE>0. Again, for scenarios with rE=0, UV-PCA performed better in case of a negative genetic correlation and UV-MA performed better in case of a positive genetic correlation. UV-MA outperformed all methods when rE was 0 and the genetic correlation was positive; for rE=0.3 and a positive genetic correlation, UV-PCA and UV-MA performed best of all methods. Power of UV-MA was the same as that of UV analysis for all scenarios with a negative genetic correlation.

FIGURE 5.2 Power of the methods for scenarios with one of three traits associated with the QTL (A), two of three traits associated with the QTL (B) and with all three traits associated with the QTL (C).





The explained variance of the QTL was fixed at 0.1%. For clarity reasons, we have not provided errors bars. Confidence ranges for the power estimates are all between 1 and 5%; exact values are provided in Supplemental Tables 5.3-5.5.

MAF, minor allele frequency; MV, multivariate; PCHAT, Principal Component of Heritability Association Test; QTL, quantitative trait locus; rE, residual correlation; rG, genetic correlation induced by the QTL; TATES, Trait-based Association Test that uses Extended Simes procedure; UV-MA, meta-analysis of univariate results; UV-PCA, univariate analysis of first principal component; UV T1, univariate analysis of trait 1; UV T2, univariate analysis of trait 2; UV T3, univariate analysis of trait 3.

Results for simulation scenarios with different heritabilities for the three traits (h^2_1=0.001, h^2_2=0.002, h^2_3 = 0.0005) are comparable to the results presented in Figure 5.2C (Supplemental Table 5.2).

Results for MAF=0.25, 0.10, and 0.05 are shown in Supplemental Tables 5.3-5.5. As expected, power was similar for all MAF scenarios. Low MAF was not a problem for the methods, except for MultiPhen which experienced convergence problems of the underlying R function 'polr' when MAF was equal to or lower than 5%.

Run time

Run time was measured on a Linux cluster using one core on a node equipped with 24 GB RAM and two Intel Xeon L5520 processors running on 2.26 GHz. Time for performing association analyses for 1000 subjects and 1000 replicates (similar to 1000 genetic variants in our study) was recorded. For TATES and UV-MA, run time also included the time used for UV association analyses of the three traits. MV-BIMBAM was the fastest method using 9 seconds, while PCHAT needed 437 minutes and 15 seconds. Run times for MV-PLINK, MV-SNPTEST, MultiPhen, TATES, UV-PCA and UV-MA were 23 seconds, 1 minute and 10 seconds, 3 minutes and 18 seconds, 23 seconds, 23 seconds and 19 seconds, respectively.

Discussion

In this study, we used simulated data to compare the performance of six multivariate genome-wide association methods (MV-PLINK, MV-SNPTEST, MultiPhen, MV-BIMBAM, PCHAT and TATES) and standard univariate analysis, univariate PCA, and meta-analysis of univariate analyses. Our results showed that there is not a single method that performed best under all simulation scenarios. However, all six multivariate methods resulted in a higher power than UV analysis, even when only one of the traits was associated with the QTL. UV-MA only outperformed all methods when all traits were associated with the QTL and the genetic correlation was positive.

Use of multivariate GWAS can be recommended even when genetic correlations between traits are expected to be weak. Indeed, even when only one of the traits was associated with the QTL and thus in the absence of genetic correlation and pleiotropy, MV analyses resulted in higher power than UV analyses. This was described before by Liu *et al*. for bivariate analyses and is due to the differences in the penalty for multiple testing [9]. Note that this penalty is commonly not applied in multiple UV analyses of real data.

The influence of the strength of residual correlation, *i.e.* the relative amount of shared genetics, and sign of genetic correlation, *i.e.* difference in sign of QTL effect, on power varied across the different methods. For MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM,

higher power was observed with increasing residual correlation in case of a single QTL trait and when two or all three traits were associated with the QTL with a negative genetic correlation. The latter is due to the resulting opposite sign of genetic and residual correlation. Indeed, we observed a similar increase in power when simulating a positive genetic correlation and negative residual correlation (data not shown). This effect has been described before for these and other methods [7,9,12,14] and was demonstrated analytically by Evans for bivariate linkage analysis [26]. In contrast, when residual and genetic correlation were in the same direction, power of these four methods decreased with increasing residual correlation between the traits, which also corroborates previous findings [7,12]. PCHAT and TATES were relatively independent of the underlying (genetic) correlations of the traits. For PCHAT, this can be explained by the fact that it constructs the optimal linear combination of traits from an heritability point of view, thereby essentially removing the influence of residual correlation on power [4]. For TATES, it was described that the power was not influenced by opposite effects of the QTL on the traits, because of its reliance on p-value information [15]. UV-MA did however severely suffer from a negative genetic correlation between the traits; indeed, in this scenario it performed equal or worse than standard UV analysis. These findings are not unexpected; a negative genetic correlation between the traits is disastrous for the power of a MA, because the direction of effect is taken into account. An alternative meta-analysis approach is Fisher's method [27]. As it combines univariate p-values into one test statistic, similar to TATES, it does not suffer from a differential sign of effect. For scenarios with a negative genetic correlation, Fisher's method performed better than UV-MA and also better than TATES, except for scenarios with a residual correlation of 0.7: here it was outperformed by TATES but not by UV-MA (data not shown). The reduced performance for an opposite QTL effect was observed for UV-PCA as well, but not for scenarios with no residual trait correlation. In these scenarios, the first PC reflects the negative genetic correlation between the traits. Power is thus increased compared to UV since there is no need to multiple testing penalty.

We would like to emphasize that all methods were compared based on empirically derived significance levels, adjusting each method to an exact 5% type I error rate. Null simulations illustrated that for MV-PLINK, MV-SNPTEST, MultiPhen, MV-BIMBAM, TATES and UV-PCA these empirical significance levels were all close to the nominal level of 0.05 for p-values or between 0.01-1 for $\log_{10}$ BF [28]. PCHAT was slightly conservative based on our observations. Thresholds for UV analysis were around 1.7% (*i.e.* 5 divided by 3) in case of uncorrelated traits, which is in line with a Bonferroni correction for three independent tests. As expected, for correlated traits the adjusted threshold was less stringent and somewhere between 1.7 and 5%. We found that significance thresholds for UV-MA were highly dependent on trait correlations with increased stringency with increase in correlation. Trait correlations result in longer tails for the test statistic distribution, and therefore a more stringent threshold must be applied to keep the type I error at 5%. Thus, use of the UV-MA can potentially lead to a high number of false-positive findings if traits are highly correlated and the significance threshold is not appropriately adjusted.

Some of the methods included in our study have been compared to one another before. MV-PLINK and MultiPhen were compared by O'Reilly *et al.* and van der Sluis *et al.*, which showed that both methods result in the same power when restricting the analysis to normally distributed traits [12,15], corroborating our findings. TATES was compared to MANOVA (which is similar to MV-PLINK) and shown to be only outperformed by MANOVA in the particular condition that the genetic variant affects only one of multiple strongly correlated traits [15]. In contrast, we observed that MV-PLINK outperformed TATES in almost all scenarios.

In addition to power (and type I errors), there are other characteristics that are important to take into account when deciding upon the appropriate multivariate GWAS analysis. MV-PLINK output results contain trait loadings, which indicate how much each trait contributed to the multivariate association result [7]. MV-BIMBAM outputs marginal posterior probabilities for each trait being unaffected, directly affected or indirectly affected by the QTL, conditional on an overall association with at least one trait [22]. PCHAT gives the weights for each of the traits included in the analysis which were used to construct the optimal linear combination of the traits to detect an association with the QTL [4]. MultiPhen output contains the betas and p-values for the association of each trait with the QTL based on the joint model including all traits [12]. This additional information, which is not provided by MV-SNPTEST and TATES, can be used to obtain insight into underlying biology and facilitates the discrimination between independent and pleiotropic QTL effects. Furthermore, MV-PLINK, MultiPhen, TATES and UV-MA allow analysis of a combination of quantitative and binary (case-control) traits [7,12,15]. MV-BIMBAM and UV-MA can be applied to summary data, without access to raw phenotype and genotype data [22]. Also, MV-SNPTEST, MultiPhen, TATES, UV-PCA and UV-MA are able to handle genotype probabilities as obtained by imputation while the other methods are not [12,15,20]. Finally, our study showed large differences in run time between the methods.

Our simulations are not exhaustive. Data were simulated for three traits according to an additive model, and analyzed accordingly. We did not simulate and analyze other, non-additive genetic models and/or (higher-order) interactions, nor did we study scenarios with more than three traits. In addition, priors for MV-SNPTEST and MV-BIMBAM and input parameters (*e.g.* number of (bagging) subsets) for PCHAT were not varied. Also, we did not study the effect of missing data. This was explored by Klei *et al.* for PCHAT who concluded that dropping individuals with missing data had a substantial diminishing effect on power of the test [4]. In addition, Van der Sluis *et al.* [15] reported that 10% missingness completely at random hardly affected the power to detect QTLs when the QTL affected all traits, but that it resulted in a higher power drop for MANOVA compared to TATES when the QTL was only associated to one of the traits. Finally, we did not simulate trait outliers in our data. O'Reilly showed that this could result in substantial inflation of the statistics for CCA for low frequency variants [12]. However, in our opinion outliers should be handled appropriately prior to association analyses.

Taken together, our study showed substantial differences in power between the methods, dependent on the simulation scenario. For some of the simulation scenarios, a large increase in power of multivariate compared to univariate analyses was observed, which suggests that the multivariate methods might be able to identify genetic variants that are currently not identifiable by standard univariate analysis. Overall, MV-PLINK, MV-SNPTEST, MultiPhen and MV-BIMBAM performed best for the majority of the tested scenarios, with a remarkable increase in power for scenarios with an opposite sign of genetic and residual correlation. As a consequence, results of these methods will be biased towards QTLs that cause a genetic correlation that is opposite in sign to the residual correlation. PCHAT and TATES showed a robust performance over all simulation scenarios and are therefore recommended to use if one aims to obtain a reflection of the underlying genetic architecture of the traits.

References

1 Willer CJ, Sanna S, Jackson AU, et al. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat Genet 2008;40:161-9.

2 Zhu W, Zhang H. Why Do We Test Multiple Traits in Genetic Association Studies? J Korean Stat Soc 2009;38:1-10.

3 Allison DB, Thiel B, St Jean P, et al. Multiple phenotype modeling in gene-mapping studies of quantitative traits: power advantages. Am J Hum Genet 1998;63:1190-1201.

4 Klei L, Luca D, Devlin B, Roeder K. Pleiotropy and principal components of heritability combine to increase power for association analysis. Genet Epidemiol 2008;32:9-19.

5 Chavali S, Barrenas F, Kanduri K, Benson M. Network properties of human disease genes with pleiotropic effects. BMC Syst Biol 2010;4:78.

6 Cotsapas C, Voight BF, Rossin E, et al. Pervasive sharing of genetic effects in autoimmune disease. PLoS Genet 2011;7:e1002254.

7 Ferreira MA, Purcell SM. A multivariate test of association. Bioinformatics 2009;25:132-3.

8 Huang J, Johnson AD, O'Donnell CJ. PRIMe: a method for characterization and evaluation of pleiotropic regions from multiple genome-wide association studies. Bioinformatics 2011;27:1201-6.

9 Liu J, Pei Y, Papasian CJ, Deng HW. Bivariate association analyses for the mixture of continuous and binary traits with the use of extended generalized estimating equations. Genet Epidemiol 2009;33:217-27.

10 Liu YZ, Pei YF, Liu JF, et al. Powerful bivariate genome-wide association analyses suggest the SOX6 gene influencing both obesity and osteoporosis phenotypes in males. PLoS One 2009;4:e6827.

11 Medland SE, Neale MC. An integrated phenomic approach to multivariate allelic association. Eur J Hum Genet 2010;18:233-9.

12 O'Reilly PF, Hoggart CJ, Pomyen Y, et al. MultiPhen: joint model of multiple phenotypes can increase discovery in GWAS. PLoS One 2012;7:e34861.

13 Ried JS, Doring A, Oexle K, et al. PSEA: Phenotype Set Enrichment Analysis--a new method for analysis of multiple phenotypes. Genet Epidemiol 2012;36:244-52.

14 Saint-Pierre A, Kaufman JM, Ostertag A, et al. Bivariate association analysis in selected samples: application to a GWAS of two bone mineral density phenotypes in males with high or low BMD. Eur J Hum Genet 2011;19:710-6.

15 van der Sluis S, Posthuma D, Dolan CV. TATES: efficient multivariate genotype-phenotype analysis for genome-wide association studies. PLoS Genet 2013;9:e1003235.

16 Irvin MR, Shrestha S, Chen YD, et al. Genes linked to energy metabolism and immunoregulatory mechanisms are associated with subcutaneous adipose tissue distribution in HIV-infected men. Pharmacogenet Genomics 2011;21:798-807.

17 Yang F, Tang Z, Deng H. Bivariate association analysis for quantitative traits using generalized estimation equation. J Genet Genomics 2009;36:733-43.

18 Yang Q, Wu H, Guo CY, Fox CS. Analyze multivariate phenotypes in genetic association studies by combining univariate association tests. Genet Epidemiol 2010;34:444-54.
19 Zheng G, Wu CO, Kwak M, et al. Joint analysis of binary and quantitative traits with data sharing and outcome-dependent sampling. Genet Epidemiol 2012;36:263-73.
20 Marchini J, Howie B, Myers S, et al. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat Genet 2007;39:906-13.
21 Guan Y, Stephens M. Practical issues in imputation-based association mapping. PLoS Genet 2008;4:e1000279.
22 Stephens M. A unified framework for association analysis with multiple related phenotypes. PLoS One 2013;8:e65245.
23 Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.
24 Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 2010;26:2190-1.
25 Cirulli ET, Goldstein DB. Uncovering the roles of rare variants in common disease through whole-genome sequencing. Nat Rev Genet 2010;11:415-25.
26 Evans DM. The power of multivariate quantitative-trait loci linkage analysis is influenced by the correlation between variables. Am J Hum Genet 2002;70:1599-1602.
27 Fisher RA. Statistical Methods for Research Workers. 1925. Oliver and Boyd, Edinburgh.
28 Stephens M, Balding DJ. Bayesian statistical methods for genetic association studies. Nat Rev Genet 2009;10:681-90.

Supplemental material

Supplemental Tables 5.1-5.5 can be found online:
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0095923

PART II

Effects on atherosclerosis

CHAPTER 6

Serum hepcidin is associated with presence of plaque in postmenopausal women of a general population

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ABSTRACT

Iron and the iron regulatory hormone hepcidin, major determinant of body iron distribution, are hypothesized to play a role in cardiovascular disease. Here, we assess the associations of hepcidin as well as ferritin, iron, total iron binding capacity and transferrin saturation (*i.e.* iron parameters) with non-invasive measurements of atherosclerosis (NIMA) in males and females of a population-based cohort. We included 766 participants of the Nijmegen Biomedical Study aged 46–67 years for whom serum measurements of hepcidin, iron parameters, and NIMA were available. NIMA were presence of plaque, ankle-brachial index (ABI) and intima media thickness (IMT). We performed multivariable logistic and linear regression analyses using quartiles of hepcidin and iron parameters. Analyses were stratified by gender and adjusted for several demographic, clinical and biochemical determinants, including traditional risk factors of cardiovascular disease based on the Framingham risk score. Hepcidin and the ratio hepcidin/ferritin, reflecting hepcidin expression relative to iron stores, were significantly associated with the presence of plaque in females [adjusted odds ratios for quartile 4 vs quartile 1 (95% CIs) of 3.07 (1.36; 6.90) and 2.31 (1.03; 5.18), respectively]. The hepcidin/ferritin ratio was significantly and negatively associated with ABI at rest in males and females [adjusted betas for quartile 4 vs quartile 1 (95% CIs) of -0.03 (-0.07; 0.00) and -0.04 (-0.06; -0.01), respectively]. Our results suggest that the body iron distribution as determined by hepcidin affects the development of atherosclerosis in females.

Introduction

A few decennia ago, Sullivan proposed the 'iron hypothesis', which suggests that iron deficiency plays a protective role against heart disease [1]. With this hypothesis he aimed to explain the sex difference in cardiovascular disease (CVD) risk and the increase in CVD incidence among women after menopause. According to the hypothesis, loss of iron with menstruation is responsible for the lower risk of heart disease in premenopausal women compared to men and postmenopausal women. The original hypothesis was formulated without specification of a mechanism. It is proposed that stored iron, which is known to be redox-active [2], catalyzes the formation of reactive oxygen species, which oxidize low-density lipoprotein (LDL) cholesterol. This has been shown to induce the formation of foam cells and ultimately atherosclerosis [3,4].

Until now, several epidemiological studies have investigated the associations between body iron stores and CVD, most of them using blood donation history as a proxy for body iron levels, but they remain inconclusive. Some studies confirmed the iron hypothesis [5-8], whereas Ascherio *et al*. demonstrated that the number of lifelong blood donations among 38,244 men was strongly associated with lower plasma ferritin levels, but not with the risk of myocardial infarction or fatal coronary heart disease over 4 years of follow-up [9]. Some studies reported on the associations between blood donation and (sub)clinical measures of atherosclerosis instead of on cardiovascular events and/or deaths [10-12]. Zheng *et al*. found decreased body iron stores, decreased oxidative stress and enhanced vascular function, as measured by flow-mediated dilation, in high-frequency blood donors compared to low-frequency donors [10]. Engberink and colleagues concluded that the intima media thickness (IMT) of the common carotid artery was slightly reduced in frequent donors as compared to low-frequency donors, although not statistically significant [11]. Finally, Peffer and colleagues showed that serum ferritin levels were lower in high-frequency donors compared to low-frequency donors, but they did not observe clear beneficial effects on measures of subclinical atherosclerosis as assessed by IMT, pulse-wave velocity and ankle-brachial index (ABI) [12].

In 2001, a peptide with antimicrobial properties was discovered, named hepcidin [13]. This small 25 amino-acid peptide hormone plays a key role in systemic regulation of iron homeostasis [14]. Hepcidin is synthesized by various cell-types, but predominantly by hepatocytes. It is upregulated by elevated body iron status and inflammatory cytokines, and decreased in iron deficiency and low oxygen tension. Hepcidin binds and subsequently degrades the cellular iron exporter ferroportin in the membrane of macrophages, leading to changes in body iron distribution by leaving iron trapped inside these iron recycling cells [15]. Hepcidin is of additional value when assessing iron status, because of its central role in iron regulation. Besides, the ratios of hepcidin to ferritin and hepcidin to transferrin saturation (TS) are informative for assessing iron status, given the dependence of hepcidin expression on stored iron and circulating iron, respectively [16,17].

It has been hypothesized that increased hepcidin concentrations may increase cardiovascular risk, by slowing or preventing the mobilization of iron from macrophages. This iron trapping in macrophages subsequently activates these cells to become more atherogenic [18,19]. Indeed, there is some evidence for a role of hepcidin in the development of atherosclerosis from *in vitro* studies and studies in patients on hemodialysis [20,24]. However, the association of serum hepcidin with atherosclerosis has not been investigated in the general population. Here, we studied the associations of serum hepcidin and its ratios to ferritin and TS, as well as serum ferritin, serum iron, total iron binding capacity (TIBC; a proxy for transferrin), and TS (*i.e.*, iron parameters) with non-invasive measures of atherosclerosis: presence of plaque, ABI at rest and after exercise and IMT. We used a well-phenotyped subsample of 766 participants aged 46-67 years from the Nijmegen Biomedical Study (NBS) and stratified analyses by gender in order to account for differences in iron status and CVD risk between males and females during life.

Methods

Study population

This study was performed in a subset of participants from the Nijmegen Biomedical Study (NBS). Details of the NBS have been described before [25]. Briefly, the NBS is a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboud university medical center, Nijmegen, The Netherlands. 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. Between 2005 and 2008, the NIMA (non-invasive measurements of atherosclerosis) substudy was performed by the Department of Internal Medicine. In the NBS-NIMA, all NBS participants aged 50-70 years at that time were re-invited to fill out an additional QN, donate a fasting blood sample, and undergo anthropometric measurements and non-invasive measurements of atherosclerosis. A total of 1491 subjects participated in NBS-NIMA (response 71%). Approval to conduct the NBS and NBS-NIMA study was obtained from the Radboud university medical center Institutional Review Board. All participants gave written informed consent for participation in the NBS.

Our study is based on 1491 subjects who participated both in the NBS in 2002 ('baseline') and the NBS-NIMA in 2005 ('follow-up'). The following data as measured in the NBS in 2002 were extracted: hepcidin, ferritin, iron, TIBC, TS, creatinine, C-reactive protein (CRP), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol and liver enzyme alanine aminotransferase (ALT) as measured in non-fasting serum; age at inclusion, length, weight [used

to derive body mass index (BMI)], the report of a cerebrovascular accident or myocardial infarction, use of antihypertensives, lipid lowering medication or anticoagulants, presence of diabetes, smoking, presence of a regular menstruation (used to determine menopausal state), use of hormone replacement therapy (HRT), presence of anemia and the report of a cerebrovascular accident or myocardial infarction for parents, children, brothers or sisters as measured via the QN. The following data were extracted from the NBS-NIMA study: high sensitive CRP and folate as measured in fasting serum; systolic blood pressure (SBP) measurements and NIMA (see below); and use of antihypertensives, lipid lowering medication or anticoagulants as measured via the QN.

For this study, we excluded subjects without hepcidin measurement (n=329). We also excluded premenopausal women (n=157) because of the differences in hepcidin level, iron status and CVD risk between pre- and postmenopausal women [26]. Furthermore, we excluded participants with a history of CVD at baseline (2002) (n=52) and persons who used antihypertensives, lipid lowering medication or medication for CVD at baseline (n=187) as a proxy for presence of atherosclerosis to obtain an 'at-risk population', resulting in a total of 766 eligible participants.

Laboratory methods and clinical measurements

The iron parameters, creatinine, CRP and ALT were measured as described recently [26]. Creatinine was used to calculate the estimated glomerular filtration rate (eGFR) as described before [26]. Serum hepcidin was measured with an in-house developed and validated competitive enzyme-linked immunosorbent assay as described before [26,27]. High sensitive CRP was determined by ELISA (Dako, Glastrup, Denmark). Folate was determined by a microbiological assay using a colistin-sulfate resistant strain of *Lactobacillus leichmanniias* as described before [28]. Measurements of SBP, TC, HDL cholesterol and NIMA measurements were performed as described before [29]. NIMA measurements used for this study comprise the carotid intima media thickness (IMT), presence of plaque and the ankle-brachial index (ABI). In short, IMT was measured at the most distal centimeter of both common carotid arteries before the bifurcation into the internal and external carotid arteries. Presence of plaque was defined as a focal thickening of the arterial wall of at least 1.5 times the mean IMT, as defined by the Mannheim Intima-media thickness consensus [30]. Both carotid arteries (as well as the common carotid, internal carotid as the external carotid arteries) were scanned from most proximal to most distal to check for focal thickenings. ABI was determined by dividing the lowest of two ankle pressures by the highest of two arm pressures, which were measured with a hand-held Doppler device. The ABI was measured at rest and after exercise. A lower ABI indicates obstruction in the peripheral arteries and thus atherosclerosis.

Statistical analysis

Statistical analyses were performed with SPSS for Windows, release 20 (IBM Corporation, Armonk, NY).

Distributions of serum hepcidin, serum ferritin, the ratios hepcidin/ferritin and hepcidin/TS, high sensitive CRP, ALT and folate were skewed towards higher values and therefore transformed with the natural logarithm to normalize their distributions. Outliers, defined as values that differed more than three times the standard deviation (SD) from the mean of continuous variables, were reduced to mean ± 3 SD (maximal number of outliers per trait: 13). Blood sampling time and CRP were categorized into 3 groups based on clinically relevant cutoffs. For blood sampling time, these were before 12 PM, 12 PM to 5 PM, and later than 5 PM, according to Dutch routine and in line with previously reported hepcidin concentration patterns throughout the day [26,31,32]. For CRP, cutoffs of less than 5 mg/L, 5 to 20 mg/L, and more than 20 mg/L were used [26].

Serum hepcidin, the hepcidin ratios and the iron parameters were transformed into gender-specific quartiles (Q). Parameters that varied over the day, *i.e.* serum hepcidin, hepcidin/ferritin, hepcidin/TS, serum iron and TS were first adjusted for time of blood sampling categories using linear regression analyses.

To optimize the information on atherosclerosis in a single proxy for the hard endpoint CVD, we also constructed one dichotomous measure of atherosclerosis based on measurements of presence of plaque, IMT and ABI, using cutoffs from clinical guidelines [33]. The combined measure of atherosclerosis was defined as IMT >0.9 mm OR ABI <0.9 OR a difference between ABI in rest and exercise >0.15 OR presence of plaque.

Characteristics of the study population were described per quartile of serum hepcidin. For all continuous variables, median and 5th and 95th percentiles (P5 and P95, respectively) were calculated from original untransformed values. Categorical variables were expressed in numbers and corresponding percentages.

Multivariable logistic regression analyses were used to study the associations of presence of plaque and the combined measure of atherosclerosis with quartiles of hepcidin and iron parameters. Multivariable linear regression analyses were used to study the associations of IMT and ABI at rest and after exercise with quartiles of hepcidin and iron parameters. We adjusted for the following potential confounding factors: time of blood sampling, CRP, high sensitive CRP, BMI, eGFR, ALT, folate, presence of anemia, time between baseline and follow-up and traditional risk factors based on the Framingham risk score [34]: age, systolic blood pressure, smoking, diabetes, total cholesterol, HDL cholesterol and reported use of antihypertensive treatment, lipid lowering medication or anticoagulants in NBS-NIMA. For females, we additionally adjusted for the use of HRT. Resulting odds ratios (OR) of logistic models express the increase or decrease in odds for presence of plaque relative to Q1. Resulting betas of linear models express the increase or decrease in IMT or ABI using Q1 as a reference, thus Q2 vs Q1, Q3 vs Q1 and Q4 vs Q1. Explained variances (adjusted r^2 for linear models and Nagelkerke r^2 for logistic models) were obtained to indicate the amount of variance in

IMT and ABI and in the risk of presence of plaque that was explained by the determinants in addition to the variance explained by the potential confounding factors. Note that the Nagelkerke r^2 is an approximation of explained variance and that comparison of its value for different models is only valid if applied to the same set of samples.

All analyses were stratified by gender.

Results

Characteristics of the study population

Characteristics of the subjects included in the study, stratified by gender and hepcidin quartiles, are presented in Table 6.1. A total of 420 participants (55%) was male. Median age at inclusion of the male participants was 57 years; that of the females was 56 years. Serum hepcidin quartiles were constructed separately for males and females, but the resulting concentration ranges per quartile were approximately the same. Serum ferritin concentration was lower in females than in males, whereas the ratios of hepcidin to ferritin and hepcidin to TS were higher in females per hepcidin quartile. Both serum ferritin and the ratios increased with quartiles of hepcidin. Median iron concentration, TIBC and TS were comparable over the quartiles and between males and females. Use of antihypertensives, lipid lowering medication and anticoagulants in NBS-NIMA, was higher in males than in females. High sensitive CRP increased with quartiles of hepcidin whereas SBP, BMI, TC and HDL cholesterol were comparable over hepcidin quartiles for both males and females.

Association with presence of plaque, IMT and ABI at rest and after exercise

Table 6.2 shows the results of multivariable logistic regression analyses for presence of plaque stratified by gender. In females, hepcidin and the ratios hepcidin/ferritin and hepcidin/TS were positively associated with the presence of plaque. The strongest associations were found for Q4 vs Q1 of hepcidin, hepcidin/ferritin and hepcidin/TS, with adjusted ORs (95% CIs) of 3.07 (1.36; 6.90), 2.31 (1.03; 5.18) and 2.22 (0.99; 4.95) and a p for trend of 0.04, 0.03 and 0.04, respectively. In males, hepcidin showed a positive association with the presence of plaque, but this association was weaker than in females [OR (95% CI) Q4 vs Q1 1.89 (0.94; 3.77), p for trend 0.05]. The ratios hepcidin/ferritin and hepcidin/TS showed some indication for positive association with presence of plaque, but ORs did not consistently increase over the quartiles.

TABLE 6.1 Descriptives of the study population (N=766) stratified by gender and hepcidin quartiles.

	Males (N=420)*			
Variable#	Q1 (N=104)	Q2 (N=105)	Q3 (N=105)	Q4 (N=104)
Age at inclusion, years	56 (47 - 65)	56 (47 - 66)	56 (48 - 66)	59 (47 - 65)
Serum hepcidin, nmoles/L	2.4 (0.3 - 4.4)	6.1 (3.3 - 9.1)	10.5 (5.5 - 13.9)	16.9 (9.0 - 32.7)
Serum ferritin, µg/L	52.9 (11.3 - 161.5)	129.7 (46.7 - 322.3)	212.7 (62.6 - 545.5)	261.0 (126.2 - 626.5)
Hepcidin/ferritin, µmoles/µg	40.9 (14.1 - 98.9)	46.0 (18.6 - 119.9)	48.9 (19.3 - 145.8)	66.9 (22.8 - 157.1)
Hepcidin/TS, µmoles/L/%	76.7 (18.2 - 170.7)	189.1 (91.4 - 396.5)	342.5 (123.7 - 620.7)	548.8 (224.7 - 1560.5)
Serum iron, µmoles/L	18.0 (7.3 - 29.8)	18.0 (10.0 - 31.0)	18.0 (10.0 - 30.4)	18.0 (8.0 - 30.5)
TIBC, µmoles/L	62.0 (50.0 - 82.0)	58.0 (49.0 - 70.7)	57.0 (46.0 - 69.7)	57.0 (42.3 - 69.5)
TS, %	28.7 (10.3 - 47.9)	31.5 (17.9 - 51.2)	29.6 (17.3 - 55.4)	30.6 (14.3 - 48.9)
eGFR, mL/min/1.73 m^2	87.1 (63.5 - 117.5)	88.4 (66.0 - 117.9)	83.1 (67.3 - 113.2)	83.3 (64.0 - 113.5)
TC, mmoles/L	5.7 (4.2 - 7.0)	6.0 (4.6 - 7.5)	6.0 (4.5 - 7.7)	6.0 (4.4 - 7.7)
HDL cholesterol, mmoles/L	1.2 (0.9 - 1.8)	1.3 (0.9 - 1.9)	1.2 (0.8 - 1.8)	1.2 (0.8 - 1.9)
SBP, mmHg	127 (107 - 147)	130 (108 - 156)	129 (108 - 147)	130 (109 - 153)
BMI, kg/m^2	25.4 (21.1 - 32)	24.6 (20.2 - 32.7)	25.5 (21.7 - 31.9)	25.5 (21.5 - 32.3)
High sensitive CRP, mg/L	1.1 (0.2 - 12.3)	1.1 (0.2 - 10.6)	1.2 (0.3 - 9.6)	1.5 (0.2 - 10.1)
ALT, U/L	13.0 (6.0 - 29.1)	13.0 (6.0 - 30.6)	15.0 (7.0 - 30.0)	14.0 (7.1 - 31.0)
Folate, nmoles/L	11.8 (4.4 - 42.8)	11.6 (4.5 - 47.2)	11.1 (4.6 - 32.4)	11.2 (4.1 - 39.3)
IMT, mm	0.85 (0.67 - 1.04)	0.84 (0.68 - 1.05)	0.86 (0.71 - 1.02)	0.84 (0.72 - 1.03)
ABI at rest	1.14 (1.00 - 1.30)	1.13 (0.96 - 1.30)	1.13 (1.00 - 1.29)	1.13 (0.98 - 1.29)
ABI after exercise	1.15 (1.00 - 1.38)	1.14 (0.74 - 1.36)	1.13 (0.88 - 1.35)	1.11 (0.87 - 1.30)
CRP				
<5 mg/L	98 (94%)	95 (90%)	94 (90%)	81 (78%)
5-20 mg/L	5 (5%)	10 (10%)	9 (9%)	18 (17%)
>20 mg/L	1 (1%)	0 (0%)	0 (0%)	5 (5%)
Time of blood sampling				
Before 12 PM	23 (22%)	21 (20%)	20 (19%)	26 (25%)
Between 12 PM and 5 PM	53 (51%)	51 (49%)	55 (52%)	50 (48%)
After 5 PM	28 (27%)	33 (31%)	30 (29%)	28 (27%)
Diabetes	3 (3%)	3 (3%)	0 (0%)	1 (1%)
Anemia	3 (3%)	1 (1%)	4 (4%)	3 (3%)
Use of antihypertensives	11 (11%)	10 (10%)	13 (12%)	12 (12%)
Use of lipid lowering medication	9 (9%)	8 (8%)	8 (8%)	12 (12%)
Use of anticoagulants	9 (9%)	3 (3%)	6 (6%)	9 (9%)
Smoking				
Current	22 (21%)	30 (29%)	20 (19%)	23 (22%)
Former	61 (59%)	58 (55%)	62 (59%)	53 (51%)
Never	21 (20%)	17 (16%)	23 (22%)	28 (27%)
Family history of CVD	47 (45%)	62 (59%)	48 (46%)	55 (53%)
Presence of plaque	35 (34%)	41 (39%)	43 (41%)	52 (50%)
Time between baseline and follow-up	4.0 (2.9 - 5.1)	3.8 (2.8 - 5.1)	3.8 (2.8 - 5.0)	3.9 (2.9 - 5.2)

Continuous variables are presented as median (P5-P95). Categorical variables are presented as N (%).

* Numbers per hepcidin quartile do not add up to 766 but 761 due to 5 missing values on the variable 'time of blood sampling', which was used to construct the hepcidin quartiles.

\# Hepcidin, ferritin, hepcidin/ferritin, hepcidin/TS, iron, TIBC, TS, eGFR, TC, HDL, BMI, CRP, ALT, time of blood sampling, diabetes, anemia, smoking, use of hormone replacement therapy and family history of CVD are measured in the NBS in 2002 and SBP, high sensitive CRP, folate, use of antihypertensives, lipid lowering medication and anticoagulants, presence of plaque, IMT and ABI at rest and after exercise are measured in the NBS-NIMA study between 2005 and 2008.

N indicates number; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; eGFR, estimated glomerular filtration rate; TC, total cholesterol; HDL, high-density lipoprotein; SBP, systolic blood pressure; BMI, body-mass index; CRP, C-reactive protein; CVD, cardiovascular disease; HRT, hormone replacement therapy; IMT, intima media thickness; ABI, ankle-brachial index.

TABLE 6.1 (CONTINUED) Descriptives of the study population (N=766) stratified by gender and hepcidin quartiles.

Variable#	Females (N=346)*			
	Q1 (N=85)	Q2 (N=86)	Q3 (N=86)	Q4 (N=86)
Age at inclusion, years	55 (48 - 65)	56 (47 - 66)	57 (48 - 66)	58 (48 - 64)
Serum hepcidin, nmoles/L	3.1 (0.2 - 5.0)	6.7 (3.3 - 9.1)	10.4 (6.0 - 13.1)	17 (9.6 - 27.0)
Serum ferritin, µg/L	41.7 (8.8 - 159.8)	77.5 (46.7 - 322.3)	115.9 (42.0 - 266.7)	149.1 (54.8 - 392.5)
Hepcidin/ferritin, µmoles/µg	62.3 (11.7 - 161.2)	78.3 (18.6 - 119.9)	84.8 (39.9 - 230.4)	121.4 (45.5 - 323.1)
Hepcidin/TS, µmoles/L/%	109.2 (13.6 - 256.2)	234.0 (91.4 - 396.5)	355.2 (161.2 - 734.4)	658.2 (272.0 - 1394.1)
Serum iron, µmoles/L	16.0 (5.5 - 26.0)	17.0 (10.0 - 31.0)	17.0 (9.4 - 25.0)	16.0 (8.0 - 26.0)
TIBC, µmoles/L	61.0 (46.2 - 81.0)	57.5 (49.0 - 70.7)	58 (44.4 - 72.0)	59.5 (43.7 - 74.3)
TS, %	25.6 (9.7 - 46.0)	28.6 (17.9 - 51.2)	30.4 (15.3 - 47.5)	28.2 (14.0 - 47.0)
eGFR, mL/min/1.73 m^2	83.7 (57.9 - 105.4)	81.9 (66.0 - 117.9)	80.8 (57.1 - 107.2)	79.1 (60.5 - 112.3)
TC, mmoles/L	6.4 (4.8 - 8.7)	6.2 (4.6 - 7.5)	6.1 (4.7 - 8.1)	6.4 (5.2 - 8.1)
HDL cholesterol, mmoles/L	1.5 (1.2 - 2.1)	1.6 (0.9 - 1.9)	1.5 (1.0 - 2.1)	1.5 (0.9 - 2.1)
SBP, mmHg	123 (102 - 156)	125 (108 - 156)	125 (107 - 155)	127 (107 - 154)
BMI, kg/m^2	23.8 (19.6 - 29.7)	24.1 (20.2 - 32.7)	25 (21.1 - 32.6)	24.9 (19.9 - 32)
High sensitive CRP, mg/L	1.0 (0.3 - 11.5)	1.2 (0.3 - 17.3)	1.8 (0.2 - 25.7)	2.0 (0.2 - 16.2)
ALT, U/L	11.0 (6.0 - 34.0)	11.0 (5.0 - 27.6)	11.0 (6.0 - 25.0)	12.0 (6.0 - 34.0)
Folate, nmoles/L	17.1 (4.3 - 52.7)	14.1 (5.0 - 68.0)	14.7 (4.8 - 52.9)	15.5 (5.6 - 51.0)
IMT, mm	0.79 (0.65 - 1.01)	0.81 (0.70 - 1.01)	0.82 (0.67 - 1.03)	0.82 (0.67 - 1.02)
ABI at rest	1.11 (1.00 - 1.28)	1.10 (0.99 - 1.24)	1.10 (0.97 - 1.27)	1.08 (0.98 - 1.21)
ABI after exercise	1.12 (1.00 - 1.31)	1.11 (0.90 - 1.36)	1.12 (0.84 - 1.32)	1.13 (0.90 - 1.36)
CRP				
<5 mg/L	70 (82%)	77 (90%)	62 (72%)	66 (77%)
5-20 mg/L	11 (13%)	8 (9%)	24 (28%)	17 (20%)
>20 mg/L	1 (1%)	1 (1%)	0 (0%)	3 (3%)
Time of blood sampling				
Before 12 PM	17 (20%)	13 (15%)	21 (24%)	16 (19%)
Between 12 PM and 5 PM	52 (61%)	54 (63%)	56 (65%)	50 (58%)
After 5 PM	16 (19%)	19 (22%)	9 (10%)	20 (23%)
Diabetes	0 (0%)	3 (3%)	1 (1%)	1 (1%)
Anemia	25 (29%)	23 (27%)	15 (17%)	19 (22%)
Use of antihypertensives	4 (5%)	4 (5%)	5 (6%)	16 (19%)
Use of lipid lowering medication	4 (5%)	4 (5%)	5 (6%)	6 (7%)
Use of anticoagulants	2 (2%)	4 (5%)	3 (3%)	5 (6%)
Smoking				
Current	8 (9%)	16 (19%)	16 (19%)	26 (30%)
Former	48 (56%)	36 (42%)	34 (40%)	26 (30%)
Never	29 (34%)	34 (40%)	36 (42%)	34 (40%)
Use of HRT	9 (11%)	9 (11%)	9 (11%)	11 (13%)
Family history of CVD	54 (64%)	40 (47%)	53 (62%)	46 (53%)
Presence of plaque	19 (22%)	28 (33%)	27 (31%)	41 (48%)
Time between baseline and follow-up	3.8 (3.0 - 5.2)	3.8 (2.9 - 5.2)	3.9 (2.9 - 5.1)	3.8 (2.9 - 5.1)

Continuous variables are presented as median (P5-P95). Categorical variables are presented as N (%).

* Numbers per hepcidin quartile do not add up to 766 but 761 due to 5 missing values on the variable 'time of blood sampling', which was used to construct the hepcidin quartiles.

\# Hepcidin, ferritin, hepcidin/ferritin, hepcidin/TS, iron, TIBC, TS, eGFR, TC, HDL, BMI, CRP, ALT, time of blood sampling, diabetes, anemia, smoking, use of hormone replacement therapy and family history of CVD are measured in the NBS in 2002 and SBP, high sensitive CRP, folate, use of antihypertensives, lipid lowering medication and anticoagulants, presence of plaque, IMT and ABI at rest and after exercise are measured in the NBS-NIMA study between 2005 and 2008.

N indicates number; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; eGFR, estimated glomerular filtration rate; TC, total cholesterol; HDL, high-density lipoprotein; SBP, systolic blood pressure; BMI, body-mass index; CRP, C-reactive protein; CVD, cardiovascular disease; HRT, hormone replacement therapy; IMT, intima media thickness; ABI, ankle-brachial index.

Results of multivariable linear regression analyses for IMT, ABI at rest and ABI after exercise are presented in Tables 6.3-6.5. In males, hepcidin showed an association with IMT [beta (95% CI) Q4 vs Q1: -0.03 (-0.06; -0.01), p for trend 0.05]. In females, Q2 and Q3 vs Q1 of iron and TS showed a significant and positive association with IMT [betas (95% CIs) Q2 vs Q1: 0.05 (0.01; 0.08) and 0.04 (0.00; 0.07), respectively; betas (95% CIs) Q3 vs Q1: 0.04 (0.01; 0.08) and 0.04 (0.01; 0.08, respectively], but not for Q4 vs Q1. IMT was not associated with iron and TS in males.

ABI at rest (Table 6.4) showed a negative and significant association with the ratio hepcidin/ferritin, with increasing strength over quartiles of the hepcidin/ferritin ratio: adjusted betas (95% CIs) for Q2 vs Q1: -0.01 (-0.04; 0.01), Q3 vs Q1: -0.03 (-0.05; 0.00), Q4 vs Q1: -0.04 (-0.06; -0.01), p for trend 0.003. The same trend was observed in males, with a significant association for Q4 vs Q1 [beta (95% CI) -0.03 (-0.07; 0.00), p for trend 0.03]. The association results of hepcidin/ferritin ratio with ABI after exercise (Table 6.5) were less consistent, but also showed some indication for a negative association.

The combined dichotomous measure of the NIMA showed positive and significant associations with hepcidin and the ratios hepcidin/ferritin and hepcidin/TS in females [adjusted ORs Q4 vs Q1 (95% CIs) 2.20 (1.04; 4.67), 2.60 (1.20; 5.63) and 2.27 (1.06; 4.85), respectively] (Supplemental Table 6.1).

The regression models including the potential confounders only resulted in explained variances (Nagelkerke r^2) of 18% and 24% for presence of plaque in males and females, respectively. In males, an increase to 20% was seen for inclusion of hepcidin, ferritin, the ratio hepcidin/ferritin and iron. In females, inclusion of hepcidin, hepcidin ratios, iron or TS led to an increase in explained variance to a maximum of 27%. This increase of maximal 3% is relatively large, as the 20 potential confounders together explained 24% of the variance. Inclusion of the ratio hepcidin/ferritin in the models for ABI at rest and ABI after exercise increased the explained variance with 2% in females. Models for IMT for both genders and models for ABI at rest and ABI after exercise in males resulted in a maximum of 1% increase in explained variance.

TABLE 6.2 Associations of quartiles of serum hepcidin and the iron parameters with presence of plaque stratified by gender.*

	Males (N=358)			Females (N=309)		
		95% CI			95% CI	
Variable	Odds Ratio	Lower limit	Upper limit	Odds Ratio	Lower limit	Upper limit
Hepcidin, nmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.10	0.56	2.17	2.00	0.89	4.48
Q3	1.50	0.77	2.93	1.57	0.70	3.56
Q4	1.89	0.94	3.77	**3.07**	**1.36**	**6.90**
Ferritin, µg/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.92	0.46	1.82	0.95	0.43	2.11
Q3	0.87	0.45	1.69	0.90	0.42	1.95
Q4	1.54	0.78	3.05	0.95	0.42	2.19
Hepcidin/ferritin, µmoles/µg – Q1	REF	REF	REF	REF	REF	REF
Q2	1.32	0.68	2.56	1.15	0.51	2.57
Q3	0.85	0.43	1.66	1.50	0.67	3.34
Q4	1.90	0.96	3.77	**2.31**	**1.03**	**5.18**
Hepcidin/TS, µmoles/L/% – Q1	REF	REF	REF	REF	REF	REF
Q2	1.18	0.60	2.31	1.06	0.47	2.38
Q3	1.66	0.86	3.23	1.23	0.53	2.81
Q4	1.29	0.65	2.57	2.22	0.99	4.95
Iron, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.21	0.61	2.40	1.35	0.65	2.83
Q3	1.83	0.93	3.60	0.47	0.20	1.08
Q4	1.07	0.51	2.23	0.85	0.37	1.94
TIBC, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.10	0.57	2.12	0.74	0.35	1.57
Q3	1.18	0.59	2.38	1.09	0.50	2.37
Q4	1.12	0.57	2.17	0.94	0.41	2.12
TS, % – Q1	REF	REF	REF	REF	REF	REF
Q2	0.86	0.43	1.71	1.36	0.63	2.94
Q3	1.08	0.55	2.13	0.56	0.25	1.24
Q4	1.50	0.75	2.97	1.12	0.50	2.49

Presence of plaque was defined as a focal thickening of the arterial wall of at least 1.5 times the mean IMT.

Associations were tested using multivariable logistic regression analyses with quartiles of serum hepcidin or the iron parameters as determinants and presence of plaque as dichotomous outcome. Resulting odds ratios express the increase or decrease in odds for presence of plaque for each quartile relative to quartile 1, thus Q2 vs. Q1, Q3 vs. Q1 and Q4 vs. Q1. Significant associations are indicated in bold.

* Results are adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate, smoking, systolic blood pressure, total cholesterol, high-density-lipoprotein cholesterol, diabetes, anemia and alanine aminotransferase as measured in the NBS in 2002 and use of antihypertensives, lipid lowering medication and anticoagulants, high sensitive C-reactive protein and folate as measured in the NBS-NIMA study between 2005 and 2008 and time between baseline and follow-up. Results for females are additionally adjusted for use of hormone replacement therapy as measured in the NBS in 2002.

N indicates number; 95% CI, 95% confidence interval; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; REF, reference category; NA, not applicable.

TABLE 6.3 Associations of quartiles of serum hepcidin and the iron parameters with intima media thickness (IMT) stratified by gender.*

	Males (N=358)			Females (N=309)		
		95% CI			95% CI	
Variable	Odds Ratio	Lower limit	Upper limit	Odds Ratio	Lower limit	Upper limit
Hepcidin, nmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.01	-0.03	0.02	0.03	-0.01	0.06
Q3	0.00	-0.03	0.03	0.00	-0.04	0.03
Q4	**-0.03**	**-0.06**	**-0.01**	-0.01	-0.04	0.03
Ferritin, µg/L – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.01	-0.04	0.02	0.01	-0.03	0.04
Q3	-0.03	-0.06	0.00	0.00	-0.04	0.04
Q4	-0.02	-0.04	0.01	-0.01	-0.05	0.02
Hepcidin/ferritin, µmoles/µg – Q1	REF	REF	REF	REF	REF	REF
Q2	0.01	-0.02	0.04	0.02	-0.01	0.06
Q3	0.02	-0.01	0.05	0.03	-0.01	0.07
Q4	0.00	-0.03	0.03	0.01	-0.03	0.04
Hepcidin/TS, µmoles/L/% – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.01	-0.04	0.02	0.02	-0.02	0.05
Q3	0.00	-0.02	0.03	-0.01	-0.05	0.03
Q4	-0.03	-0.06	0.00	0.00	-0.04	0.03
Iron, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.00	-0.03	0.02	**0.05**	**0.01**	**0.08**
Q3	0.00	-0.03	0.03	**0.04**	**0.01**	**0.08**
Q4	0.00	-0.03	0.03	0.02	-0.01	0.06
TIBC, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.01	-0.02	0.04	-0.03	-0.06	0.00
Q3	0.00	-0.03	0.03	-0.02	-0.05	0.02
Q4	0.03	0.00	0.06	-0.02	-0.06	0.01
TS, % – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.01	-0.03	0.02	**0.04**	**0.00**	**0.07**
Q3	0.00	-0.03	0.03	**0.04**	**0.01**	**0.08**
Q4	-0.01	-0.04	0.02	0.03	-0.01	0.07

IMT was measured at the most distal centimeter of both common carotid arteries before the bifurcation into the internal and external carotid arteries. Associations were tested using multivariable linear regression analyses with quartiles of serum hepcidin or the iron parameters as determinants and IMT as continuous outcome. Resulting betas express the change in IMT for each quartile relative to quartile 1, thus Q2 vs. Q1, Q3 vs. Q1 and Q4 vs. Q1. Significant associations are indicated in bold.

* Results are adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate, smoking, systolic blood pressure, total cholesterol, high-density-lipoprotein cholesterol, diabetes, anemia and alanine aminotransferase as measured in the NBS in 2002 and use of antihypertensives, lipid lowering medication and anticoagulants, high sensitive C-reactive protein and folate as measured in the NBS-NIMA study between 2005 and 2008 and time between baseline and follow-up. Results for females are additionally adjusted for use of hormone replacement therapy as measured in the NBS in 2002.

N indicates number; 95% CI, 95% confidence interval; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; REF, reference category; NA, not applicable.

TABLE 6.4 Associations of quartiles of serum hepcidin and the iron parameters with ankle-brachial index (ABI) at rest stratified by gender.*

	Males (N=358)			Females (N=309)		
		95% CI			95% CI	
Variable	Odds Ratio	Lower limit	Upper limit	Odds Ratio	Lower limit	Upper limit
Hepcidin, nmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.01	-0.02	-0.05	0.01
Q3	-0.01	-0.04	0.03	-0.02	-0.04	0.01
Q4	0.00	-0.03	0.04	-0.02	-0.05	0.01
Ferritin, µg/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.00	-0.03	0.03	-0.01	-0.04	0.02
Q3	0.02	-0.02	0.05	-0.02	-0.05	0.01
Q4	0.00	-0.03	0.03	0.00	-0.03	0.03
Hepcidin/ferritin, µmoles/µg – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.01	-0.01	-0.04	0.01
Q3	-0.03	-0.06	0.00	**-0.03**	**-0.05**	**0.00**
Q4	**-0.03**	**-0.07**	**0.00**	**-0.04**	**-0.06**	**-0.01**
Hepcidin/TS, µmoles/L/% – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.02	-0.01	-0.04	0.01
Q3	0.00	-0.04	0.03	-0.01	-0.04	0.02
Q4	-0.01	-0.04	0.02	-0.02	-0.05	0.01
Iron, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.02	-0.01	0.05	0.00	-0.03	0.02
Q3	0.03	-0.01	0.06	0.01	-0.02	0.04
Q4	0.01	-0.02	0.04	-0.02	-0.04	0.01
TIBC, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	**-0.03**	**-0.06**	**0.00**	**-0.03**	**-0.05**	**0.00**
Q3	0.01	-0.03	0.04	-0.02	-0.05	0.00
Q4	-0.03	-0.06	0.01	-0.01	-0.04	0.01
TS, % – Q1	REF	REF	REF	REF	REF	REF
Q2	0.00	-0.03	0.04	0.00	-0.02	0.03
Q3	0.02	-0.02	0.05	-0.01	-0.03	0.02
Q4	0.01	-0.02	0.04	-0.01	-0.04	0.02

ABI was determined by dividing the lowest of two ankle pressures by the highest of two arm pressures, which were measured with a hand-held Doppler device. Associations were tested using multivariable linear regression analyses with quartiles of serum hepcidin or the iron parameters as determinants and ABI at rest as continuous outcome. Resulting betas express the change in ABI at rest for each quartile relative to quartile 1, thus Q2 vs. Q1, Q3 vs. Q1 and Q4 vs. Q1. Significant associations are indicated in bold.

* Results are adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate, smoking, systolic blood pressure, total cholesterol, high-density-lipoprotein cholesterol, diabetes, anemia and alanine aminotransferase as measured in the NBS in 2002 and use of antihypertensives, lipid lowering medication and anticoagulants, high sensitive C-reactive protein and folate as measured in the NBS-NIMA study between 2005 and 2008 and time between baseline and follow-up. Results for females are additionally adjusted for use of hormone replacement therapy as measured in the NBS in 2002.

N indicates number; 95% CI, 95% confidence interval; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; REF, reference category; NA, not applicable.

TABLE 6.5 Associations of quartiles of serum hepcidin and the iron parameters with ankle-brachial index (ABI) after exercise stratified by gender.*

	Males (N=358)			Females (N=309)		
		95% CI			95% CI	
Variable	Odds Ratio	Lower limit	Upper limit	Odds Ratio	Lower limit	Upper limit
Hepcidin, nmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.01	-0.02	-0.05	0.01
Q3	-0.01	-0.04	0.03	-0.02	-0.04	0.01
Q4	0.00	-0.03	0.04	-0.02	-0.05	0.01
Ferritin, µg/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.00	-0.03	0.03	-0.01	-0.04	0.02
Q3	0.02	-0.02	0.05	-0.02	-0.05	0.01
Q4	0.00	-0.03	0.03	0.00	-0.03	0.03
Hepcidin/ferritin, µmoles/µg – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.01	-0.01	-0.04	0.01
Q3	-0.03	-0.06	0.00	**-0.03**	**-0.05**	**0.00**
Q4	**-0.03**	**-0.07**	**0.00**	**-0.04**	**-0.06**	**-0.01**
Hepcidin/TS, µmoles/L/% – Q1	REF	REF	REF	REF	REF	REF
Q2	-0.02	-0.05	0.02	-0.01	-0.04	0.01
Q3	0.00	-0.04	0.03	-0.01	-0.04	0.02
Q4	-0.01	-0.04	0.02	-0.02	-0.05	0.01
Iron, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.02	-0.01	0.05	0.00	-0.03	0.02
Q3	0.03	-0.01	0.06	0.01	-0.02	0.04
Q4	0.01	-0.02	0.04	-0.02	-0.04	0.01
TIBC, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	**-0.03**	**-0.06**	**0.00**	**-0.03**	**-0.05**	**0.00**
Q3	0.01	-0.03	0.04	-0.02	-0.05	0.00
Q4	-0.03	-0.06	0.01	-0.01	-0.04	0.01
TS, % – Q1	REF	REF	REF	REF	REF	REF
Q2	0.05	0.00	0.10	-0.03	-0.07	0.02
Q3	0.07	0.02	0.12	-0.02	-0.07	0.02
Q4	0.05	-0.01	0.10	-0.01	-0.05	0.04

ABI was determined by dividing the lowest of two ankle pressures by the highest of two arm pressures, which were measured with a hand-held Doppler device. Associations were tested using multivariable linear regression analyses with quartiles of serum hepcidin or the iron parameters as determinants and ABI after exercise as continuous outcome. Resulting betas express the change in ABI after exercise for each quartile relative to quartile 1, thus Q2 vs. Q1, Q3 vs. Q1 and Q4 vs. Q1. Significant associations are indicated in bold.

* Results are adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate, smoking, systolic blood pressure, total cholesterol, high-density-lipoprotein cholesterol, diabetes, anemia and alanine aminotransferase as measured in the NBS in 2002 and use of antihypertensives, lipid lowering medication and anticoagulants, high sensitive C-reactive protein and folate as measured in the NBS-NIMA study between 2005 and 2008 and time between baseline and follow-up. Results for females are additionally adjusted for use of hormone replacement therapy as measured in the NBS in 2002.

N indicates number; 95% CI, 95% confidence interval; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; REF, reference category; NA, not applicable.

Discussion

To our knowledge, this is the first study that investigated the associations between hepcidin, iron and non-invasive measurements of atherosclerosis in a general population. After adjustment for several demographic, clinical and biochemical determinants, we demonstrated that serum hepcidin and the ratio hepcidin/ferritin were significantly and positively associated with the presence of plaque in females. Suggestive evidence for a positive association with presence of plaque was observed for hepcidin and the ratio hepcidin/ferritin in males and the ratio hepcidin/TS in females. Furthermore, the hepcidin/ferritin ratio was significantly and negatively associated with ABI at rest in males and females. Iron and TS showed evidence for a positive association with IMT in females, but the effects did not consistently increase over the quartiles of iron and TS.

In our study, we used three measures that reflect the presence of atherosclerosis. Presence of plaque has been shown to be a better predictor of myocardial infarction risk and the extent of underlying coronary artery disease than IMT [35]. In addition, ABI is the only of the three NIMA that is routinely applied in clinical practice. As hepcidin and the ratio hepcidin/ferritin showed consistent associations with presence of plaque and ABI, the two most evident markers of atherosclerosis, our results suggest that hepcidin plays a role in the development of atherosclerosis in females.

Our results confirm several previous studies that investigated serum hepcidin concentration in relation to cardiovascular or metabolic diseases in patient populations. In patients on maintenance hemodialysis, hepcidin was shown to be associated with arterial stiffness and fatal and non-fatal cardiovascular events [20,21]. Valenti *et al*. showed that hepcidin correlates with monocyte chemoattractant protein-1 levels in patients with metabolic alterations, and that these levels are an independent predictor of the presence of carotid plaques [36]. In response to this paper, Kroot *et al*. demonstrated that total hepcidin and hepcidin-25 levels were significantly higher in subjects with metabolic syndrome than in age-matched controls in a nested case-control study in the NBS [37]. In another paper, Valenti *et al*. reported that hepcidin-25 was independently associated with carotid plaques in patients with non-alcoholic fatty liver disease [38]. In addition, hepcidin was reported to increase significantly and linearly with increasing number of metabolic syndrome features, paralleling the trend of serum ferritin [39]. Finally, increased hepcidin production was observed in patients with (low-grade) inflammation, obesity and other metabolic syndrome alterations, which are traditionally associated with CVD [40-42].

Our findings are in agreement with the hypothesis that hepcidin increases iron deposition in macrophages within atherosclerotic plaques with subsequent increased lipid peroxidation and progression to foam cells [18], resulting in an increased risk of atherosclerosis. There

is additional evidence for this hypothesis from recent *in vitro* and mice studies. Saeed *et al*. demonstrated that suppression of hepatic hepcidin production resulted in reduced macrophage intracellular iron content, which increased the efflux capacity of cholesterol and thereby decreased the formation of foam cells and atherosclerosis [23]. Using human monocytes derived from atherosclerotic plaques, Finn *et al*. showed that hepcidin increased the intracellular iron content, resulting in increased ROS and decreased cholesterol efflux [24]. These observations are consistent with those obtained in a mouse model of atherosclerosis and in macrophage cell lines, suggesting that the interaction of (locally produced) hepcidin, trapped iron, and accumulated lipids is critical for pro-atherosclerotic activation of macrophages, contributing to their destabilization [22]. Our results for the hepcidin/ferritin ratio, reflecting the amount of hepcidin relative to ferritin as a measure of intracellular iron, match with the latter findings, since the ratio hepcidin/ferritin showed significant association with the presence of plaque and ABI. This underscores the role of hepcidin in determining the body iron distribution and thus the interplay of hepcidin and ferritin levels in the development of atherosclerosis.

Our results might explain the inconsistent results of previous epidemiological studies on the association between body iron stores and CVD risk [5-12]. Hepcidin levels were not measured in these studies, except for the study by Peffer *et al*. [12] in which a subset (n=269) of the NBS-NIMA population was used, but the associations between hepcidin and NIMA were not investigated in that study. Furthermore, our results might explain the paradox of no obvious increase in atherosclerosis in patients with hemochromatosis gene (*HFE*)-related hereditary hemochromatosis despite their sometimes significant iron overload: hepcidin levels are relatively low, resulting in decreased retention of iron by macrophages and therefore a more rapid clearance of iron from arterial lesions [18,19,43]. Interestingly, the p.Cys282Tyr variant (rs1800562) of the *HFE* gene, that is associated with a decrease in the hepcidin/ferritin ratio [44,45], has also been associated with decreased levels of LDL and total cholesterol levels [46]. Finally, a lower incidence of coronary artery disease in patients with β-thalassemia major and intermedia [47,48] might not only be due to increased cholesterol requirements associated with erythroid hyperplasia and therefore relatively low serum cholesterol levels [47], but also to relatively low hepcidin levels which might play a role in the protection against CVD as well [49,50].

Interestingly, the associations of serum hepcidin and the ratio hepcidin/ferritin with presence of plaque and ABI were far stronger in females compared to males. It is known that some of the traditional cardiovascular risk factors have a different impact on males and females [51], but the reason for this remains unexplained. Hormones might be responsible for gender-related differences, but this is unlikely in our study as we focused on postmenopausal women, who are more similar to men regarding hormonal status, and adjusted for the use of hormone replacement therapy.

Our study has some limitations. First, we performed several association tests in our male and female population separately; replication of the results in other populations will have to confirm that our positive findings are not due to chance effects and a relatively small sample size. Secondly, it is difficult to draw conclusions about causality based on the results of our study. The fact that hepcidin and iron parameters were measured in a non-CVD population 3 to 5 years prior to the NIMA does however reduce the likeliness of a reverse mechanism, where atherosclerosis affects hepcidin levels. Also, NIMA measurements were not performed in 2002; we used presence of CVD or use of medication for CVD as a proxy for presence of atherosclerosis in order to obtain an at-risk population. In addition, our analyses rely on one measurement of serum hepcidin, which we used as a proxy for the hepcidin concentration in the years before and after this measurement. Hepcidin and TC were measured in serum collected during the day and not in fasting state, which makes the measurement less precise due to the known circadian rhythm of hepcidin and the influence of food intake on hepcidin and cholesterol levels [32,52]. We adjusted our analyses for time of blood sampling to reduce the noise caused by this measurement error, but the precision of the reported associations might still be decreased. Moreover, we used serum concentrations of hepcidin, ferritin and iron, whereas the associations of interest might be cell type or tissue specific. Finally, the number of CVD events in our population was too small to analyze the association of hepcidin, the hepcidin ratios and the iron parameters with the hard endpoint of CVD. Nevertheless, presence of plaque, IMT and ABI have shown to predict CVD, both in patients and in the general population [35,53,54].

In conclusion, this epidemiological study is the first to describe associations between hepcidin and presence of plaque, ABI and IMT. Our results indicate that hepcidin and the hepcidin/ferritin ratio are associated with atherosclerosis, as measured by the presence of plaque and ABI, in postmenopausal women in a general population. Our results suggest that it is the body iron distribution as determined by serum hepcidin and its ratio to ferritin instead of the total body iron load that plays a role in the development of atherosclerosis. We warrant replication of our results in an independent population using hard cardiovascular endpoints and the set-up of functional studies to disentangle causal relationships, in addition to studies that use hard cardiovascular endpoints. Eventually, hepcidin could be a novel biomarker and therapeutic target for CVD.

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References

1 Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1:1293-4.
2 Tuomainen TP, Loft S, Nyyssonen K, et al. Body iron is a contributor to oxidative damage of DNA. Free Radic Res 2007;41:324-8.
3 Kraml PJ, Klein RL, Huang Y, et al. Iron loading increases cholesterol accumulation and macrophage scavenger receptor I expression in THP-1 mononuclear phagocytes. Metabolism 2005;54:453-9.
4 Lapenna D, Pierdomenico SD, Ciofani G, et al. Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques. Free Radic Biol Med 2007;42:492-8.
5 Kiechl S, Willeit J, Egger G, et al. Body iron stores and the risk of carotid atherosclerosis: Prospective results from the bruneck study. Circulation 1997;96:3300-7.
6 Salonen JT, Tuomainen TP, Salonen R, et al. Donation of blood is associated with reduced risk of myocardial infarction. The Kuopio ischaemic heart disease risk factor study. Am J Epidemiol 1998;148:445-51.
7 Meyers DG, Jensen KC, Menitove JE. A historical cohort study of the effect of lowering body iron through blood donation on incident cardiac events. Transfusion 2002;42:1135-9.
8 Meyers DG, Strickland D, Maloley PA, et al. Possible association of a reduction in cardiovascular events with blood donation. Heart 1997;78:188-93.
9 Ascherio A, Rimm EB, Giovannucci E, et al. Blood donations and risk of coronary heart disease in men. Circulation 2001;103:52-7.
10 Zheng H, Cable R, Spencer B, et al. Iron stores and vascular function in voluntary blood donors. Arterioscler Thromb Vasc Biol 2005;25:1577-83.
11 Engberink MF, Geleijnse JM, Durga J, et al. Blood donation, body iron status and carotid intima-media thickness. Atherosclerosis 2008;196:856-62.
12 Peffer K, den Heijer M, Holewijn S, et al. The effect of frequent whole blood donation on ferritin, hepcidin, and subclinical atherosclerosis. Transfusion 2013;53:1468-74.
13 Ganz T. Hepcidin and iron regulation, 10 years later. Blood 2011;117:4425-33.
14 Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: Diagnostic implications. Clin Chem 2011;57:1650-69.
15 Ganz T, Nemeth E. Hepcidin and iron homeostasis. Biochim Biophys Acta 2012;1823:1434-43.
16 Fleming RE, Ponka P. Iron overload in human disease. N Engl J Med 2012;366:348-59.
17 Hentze MW, Muckenthaler MU, et al. Two to tango: Regulation of mammalian iron metabolism. Cell 2010;142:24-38.
18 Sullivan JL. Macrophage iron, hepcidin, and atherosclerotic plaque stability. Exp Biol Med 2007;232:1014-20.
19 Sullivan JL. Iron in arterial plaque: Modifiable risk factor for atherosclerosis. Biochim Biophys Acta 2009;1790:718-23.

20 Kuragano T, Itoh K, Shimonaka Y, et al. Hepcidin as well as TNF-alpha are significant predictors of arterial stiffness in patients on maintenance hemodialysis. Nephrol Dial Transplant 2011;26:2663-7.
21 Van der Weerd NC, Grooteman MP, Bots ML, et al. Hepcidin-25 is related to cardiovascular events in chronic haemodialysis patients. Nephrol Dial Transplant 2012;0:1-8.
22 Li JJ, Meng X, Si HP, et al. Hepcidin destabilizes atherosclerotic plaque via overactivating macrophages after erythrophagocytosis. Arterioscler Thromb Vasc Biol 2012;32:1158-66.
23 Saeed O, Otsuka F, Polavarapu R, et al. Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler Thromb Vasc Biol 2012;32:299-307.
24 Finn AV, Nakano M, Polavarapu R, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 2012;59:166-77.
25 Hoogendoorn EH, Hermus AR, de Vegt F, et al. 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.
26 Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: Reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.
27 Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570-9.
28 Jung AY, Botma A, Lute C, et al. Plasma B vitamins and LINE-1 DNA methylation in leukocytes of patients with a history of colorectal adenomas. Mol Nutr Food Res 2013;57:698–708.
29 Holewijn S, den Heijer M, Swinkels DW, et al. The metabolic syndrome and its traits as risk factors for subclinical atherosclerosis. J Clin Endocrinol Metab 2009;94:2893-9.
30 Touboul PJ, Hennerici MG, Meairs S, et al. Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). Cerebrovasc Dis 2012;34:290-6.
31 Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. Blood 2008;112:4292-7.
32 Kroot JJ, Hendriks JC, Laarakkers CM, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: Implications for clinical studies. Anal Biochem 2009;389:124-9.
33 Holewijn S, den Heijer M, Kiemeney LA, et al. Combining risk markers improves cardiovascular risk prediction in women. Clin Sci 2014;126:139-46.
34 D'Agostino RB Sr., Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: The framingham heart study. Circulation 2008;117:743-53.
35 Rundek T, Brook RD, Spence JD. Letter by rundek et al regarding article, "prediction of clinical cardiovascular events with carotid intima-media thickness: A systematic review and meta-analysis". Circulation 2007;116:e317;author reply e318.

36 Valenti L, Dongiovanni P, Motta BM, et al. Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations. Arterioscler Thromb Vasc Biol 2011;31:683-90.
37 Kroot JJ, van Dijk BAC, Laarakkers CMM, et al. Serum hepcidin levels are elevated in the metabolic syndrome. Arterioscler Thromb Vasc Biol Published online April 29, 2011.
38 Valenti L, Swinkels DW, Burdick L, et al. Serum ferritin levels are associated with vascular damage in patients with nonalcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2011;21:568-75.
39 Martinelli N, Traglia M, Campostrini N, et al. Increased serum hepcidin levels in subjects with the metabolic syndrome: A population study. PloS one 2012;7:e48250.
40 Aigner E, Theurl I, Theurl M, et al. Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. Am J Clin Nutr 2008;87:1374-83.
41 Swinkels DW, Drenth JP. Hepcidin in the management of patients with mild non-hemochromatotic iron overload: Fact or fiction? J Hepatol 2008;49:680-5.
42 Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and nash. Gastroenterology 2006;131:788-96.
43 Sullivan JL. Do hemochromatosis mutations protect against iron-mediated atherogenesis? Circ Cardiovasc Genet 2009;2:652-7.
44 Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. 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 2013;50:593-8.
45 Traglia M, Girelli D, Biino G, et al. 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.
46 Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 2010;466:707-13.
47 Shalev H, Kapelushnik J, Moser A, Knobler H, Tamary H. Hypocholesterolemia in chronic anemias with increased erythropoietic activity. Am J Hematol. 2007;82:199-202.
48 Crowley JP, Sheth S, Capone RJ, Schilling RF. A paucity of thalassemia trait in italian men with myocardial infarction. Acta Haematol. 1987;78:249-251.
49 Nemeth E. Hepcidin in beta-thalassemia. Ann N Y Acad Sci. 2010;1202:31-35.
50 Origa R, Galanello R, Ganz T, Giagu N, Maccioni L, Faa G, Nemeth E. Liver iron concentrations and urinary hepcidin in beta-thalassemia. Haematologica. 2007;92:583-588.
51 Roeters van Lennep JE, Westerveld HT, Erkelens DW, van der Wall EE. Risk factors for coronary heart disease: Implications of gender. Cardiovasc Res. 2002;53:538-549.
52 Schaap CC, Hendriks JC, Kortman GA, Klaver SM, Kroot JJ, Laarakkers CM, Wiegerinck ET, Tjalsma H, Janssen MC, Swinkels DW. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin Chem. 2013;59:527-535.

53 Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: A systematic review and meta-analysis. Circulation. 2007;115:459-467.
54 Ankle Brachial Index Consortium, Fowkes FG, Murray GD, et al. Ankle brachial index combined with framingham risk score to predict cardiovascular events and mortality: A meta-analysis. JAMA 2008;300:197-208.

SUPPLEMENTAL TABLE 6.1 Associations of serum hepcidin and the iron parameters with atherosclerosis as a combined measure of presence of plaque, IMT and ABI at rest and after exercise stratified by gender.*

	Males (N=358)			Females (N=309)		
		95% CI			95% CI	
Variable	Odds Ratio	Lower limit	Upper limit	Odds Ratio	Lower limit	Upper limit
Hepcidin, nmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.12	0.58	2.16	**2.18**	**1.04**	**4.56**
Q3	1.32	0.68	2.55	1.54	0.73	3.24
Q4	0.99	0.49	2.01	**2.20**	**1.04**	**4.67**
Ferritin, µg/L – Q1	REF	REF	REF	REF	REF	REF
Q2	0.94	0.47	1.87	1.30	0.62	2.73
Q3	0.74	0.38	1.43	1.02	0.49	2.12
Q4	0.84	0.42	1.69	0.80	0.36	1.79
Hepcidin/ferritin, µmoles/µg – Q1	REF	REF	REF	REF	REF	REF
Q2	1.46	0.75	2.84	1.51	0.71	3.22
Q3	1.76	0.90	3.47	**2.61**	**1.22**	**5.58**
Q4	1.67	0.83	3.36	**2.60**	**1.20**	**5.63**
Hepcidin/TS, µmoles/L/% – Q1	REF	REF	REF	REF	REF	REF
Q2	1.18	0.61	2.29	1.31	0.62	2.75
Q3	1.56	0.80	3.07	1.36	0.63	2.93
Q4	0.82	0.41	1.63	**2.27**	**1.06**	**4.85**
Iron, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.01	0.50	2.03	1.21	0.59	2.47
Q3	1.18	0.59	2.34	0.82	0.39	1.73
Q4	0.93	0.44	1.97	0.71	0.32	1.57
TIBC, µmoles/L – Q1	REF	REF	REF	REF	REF	REF
Q2	1.45	0.74	2.83	0.79	0.39	1.60
Q3	1.05	0.51	2.13	1.11	0.53	2.32
Q4	1.59	0.81	3.14	0.74	0.34	1.60
TS, % – Q1	REF	REF	REF	REF	REF	REF
Q2	0.79	0.40	1.59	1.83	0.88	3.82
Q3	0.88	0.44	1.77	0.64	0.30	1.36
Q4	0.94	0.47	1.91	1.09	0.51	2.32

Atherosclerosis was defined as IMT >0.9 mm OR ABI <0.9 OR a difference between ABI in rest and exercise >0.15 OR presence of plaque.
Associations were tested using multivariable logistic regression analyses with quartiles of serum hepcidin or the iron parameters as determinants and atherosclerosis as dichotomous outcome. Resulting odds ratios express the increase or decrease in odds for atherosclerosis for each quartile relative to quartile 1, thus Q2 vs. Q1, Q3 vs. Q1 and Q4 vs. Q1. Significant associations are indicated in bold.

* Results are adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate, smoking, systolic blood pressure, total cholesterol, high-density-lipoprotein cholesterol, diabetes, anemia and alanine aminotransferase as measured in the NBS in 2002 and use of antihypertensives, lipid lowering medication and anticoagulants, high sensitive C-reactive protein and folate as measured in the NBS-NIMA study between 2005 and 2008 and time between baseline and follow-up. Results for females are additionally adjusted for use of hormone replacement therapy as measured in the NBS in 2002.

N indicates number; 95% CI, 95% confidence interval; Q1-4, quartile 1-4; TIBC, total iron binding capacity; TS, transferrin saturation; REF, reference category; NA, not applicable.

CHAPTER 7

Iron and hepcidin as risk factors in atherosclerosis: what do the genes say?

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ABSTRACT

Previous reports suggested a role for iron and hepcidin in atherosclerosis. Here, we evaluated the causality of these associations from a genetic perspective via (i) a Mendelian randomization (MR) approach, (ii) study of association of atherosclerosis-related single nucleotide polymorphisms (SNPs) with iron and hepcidin, and (iii) estimation of genomic correlations between hepcidin, iron and atherosclerosis. Analyses were performed in a general population sample. Iron parameters (serum iron, serum ferritin, total iron-binding capacity and transferrin saturation), serum hepcidin and genome-wide SNP data were available for N=1,819; non-invasive measurements of atherosclerosis (NIMA), *i.e.* presence of plaque, intima media thickness and ankle-brachial index (ABI), for N=549. For the MR, we used 12 iron-related SNPs that were previously identified in a genome-wide association meta-analysis on iron status, and assessed associations with NIMA. Other than for rs651007, associated with decreased ferritin concentration and decreased atherosclerosis risk, we observed no SNP associations that fit the hypothesized directions of effect between iron and NIMA. Two of six NIMA-related SNPs showed association with the ratio hepcidin/ferritin, suggesting that an increased hepcidin/ferritin ratio increases atherosclerosis risk. Genomic correlations were close to zero, except for hepcidin and ferritin with ABI at rest [-0.27 (SE 0.34) and -0.22 (SE 0.35), respectively] and ABI after exercise [-0.29 (SE 0.34) and -0.30 (0.35), respectively]. The negative sign indicates an increased atherosclerosis risk with increased hepcidin and ferritin concentrations. Our results suggest a potential causal role for hepcidin and ferritin in atherosclerosis, but not for the other iron parameters.

Introduction

In 1981, the 'iron hypothesis' was proposed, stating that iron depletion protects against heart disease [1]. According to this hypothesis, premenopausal women have a lower risk of heart disease compared to men and postmenopausal women due to loss of iron with menstruation. The hypothesis was specified without a mechanism, but it is proposed that high levels of body iron stores promote cardiovascular disease (CVD) by catalyzing low-density lipoprotein (LDL) cholesterol oxidation and thereby atherosclerosis [2,3]. Since the proposal of the 'iron hypothesis', several epidemiological studies have investigated the associations between body iron stores and CVD or (sub)clinical measures of atherosclerosis, but they remain inconclusive [4-11]. The inconsistent results might be explained by the fact that it is not the total body iron load, but the body iron *distribution* as determined by serum hepcidin that drives the association with atherosclerosis and CVD risk. Hepcidin, key regulator of systemic iron homeostasis, has been hypothesized to increase CVD risk by slowing or preventing the mobilization of iron from macrophages [12], promoting transformation of these cells into foam cells and ultimately atherosclerosis [3,12]. In a recent epidemiological study we demonstrated that serum hepcidin and the ratio of hepcidin to ferritin, *i.e.* hepcidin expression relative to body iron stores, are associated with atherosclerosis in the general population, especially in postmenopausal women [13]. We did not observe associations of the iron parameters, *i.e.* serum ferritin, serum iron, total-iron binding capacity (TIBC) and transferrin saturation (TS), with atherosclerosis [13]. However, disentangling the specific causal roles of hepcidin and iron parameters in atherosclerosis and CVD in observational population studies is fraught with difficulties due to potential for residual confounding, reverse causation, and the existing phenotypic correlations between iron parameters and hepcidin.

In this study, we aimed to investigate the causal roles of hepcidin, the ratios hepcidin/ferritin and hepcidin/TS, and the iron parameters in atherosclerosis, as measured by non-invasive measurements of atherosclerosis (NIMA), by focusing on their underlying genetics. More specifically, we 1) applied a Mendelian randomization (MR) approach, 2) evaluated associations of genetic determinants of NIMA with hepcidin and iron parameters, and 3) estimated the genomic correlations of hepcidin and the iron parameters with NIMA based on genome-wide chip data.

In the MR approach, genetic determinants of the risk factor(s) of interest, in this case iron status and hepcidin, are used to estimate the causal effect of the risk factor on a disease outcome, in this case NIMA [14]. As genetic variants are randomly distributed in the population, this observational design mimics the randomization in a clinical trial and hence allows for assessment of causality. The second step allowed us to evaluate whether published NIMA-related genetic variants show cross-trait association with hepcidin and the iron parameters. This might indicate presence of pleiotropy, where a single genetic variant affects multiple traits independently. It can also indicate a causal relationship between two

correlated traits, where a single genetic variant indirectly affects a second trait (*i.e.* NIMA) due to a causal association with a first, intermediate trait (*i.e.* iron and/or hepcidin). Third, the estimation of genomic correlations allowed us to evaluate the extent to which the same genetic variants, captured via a genome-wide chip, impact on hepcidin or iron parameters and NIMA. Existence of a genomic correlation between two traits can indicate pleiotropy or causality, as for cross-trait associations. A positive genomic correlation indicates that the same genetic variants influence two traits in the same direction, while a negative genomic correlation indicates an opposite direction of effect.

The boost in the identification of genetic variants for complex traits via genome-wide association studies (GWAS) has facilitated the design of MR studies in recent years. For the iron parameters, several GWAS have been published [15-20]. Recently, a large meta-analysis of GWAS on biochemical markers for iron status was completed by the Genetics of Iron Status (GIS) Consortium. The study included 23,986 subjects from eleven population-based studies in the discovery phase and up to 24,986 subjects in the replication phase (Benyamin *et al*., in press). This meta-analysis led to the identification of 12 single nucleotide polymorphisms (SNPs) statistically significantly associated with at least one of the iron parameters at a genome-wide level (Supplemental Table 7.1), which we used for the current study in the MR analysis.

The complex genetic etiology of hepcidin is relatively unexplored. Traglia *et al*. published a GWAS on serum hepcidin in the genetic isolate Val Borbera, in which no statistically significantly associated loci were found [21]. In addition, studies into the SNPs C282Y (rs1800562) in the hereditary hemochromatosis gene (*HFE*) and A736V (rs855791) in the transmembrane serine protease 6 gene (*TMPRSS6*), which have repeatedly been associated with the iron parameters, only associated with the ratios hepcidin/ferritin and hepcidin/TS and not with serum hepcidin [22]. Thus, no genetic determinants of hepcidin are currently available.
There have also been numerous GWAS into genetic determinants of NIMA. Sixteen GWAS were combined in a meta-analysis for common carotid intima media thickness (IMT) and the presence of carotid plaque (total N=42,484), which revealed three (nearest genes *ZHX2*, *APOC1* and *PINX1*) and two (nearest genes *PIK3CG* and *EDNRA*) different SNPs that were statistically significantly associated with IMT and plaque, respectively [23]. A meta-analysis of 21 GWAS for ankle-brachial index (ABI) (total N=58,409) identified one genome-wide significant association with nearest gene *CDKN2B* [24]. We used these six SNPs to study cross-trait associations with iron parameters and hepcidin (Supplemental Table 7.2).
Here, we studied the roles of hepcidin, the ratios hepcidin/ferritin and hepcidin/TS, and the iron parameters in NIMA by an MR approach, cross-trait associations, and genomic correlations. We used a subsample of 1819 participants aged 42–76 years from the Nijmegen Biomedical Study (NBS), a well-phenotyped sample of the general population.

Methods

Study population

Details of the NBS have been described before [25]. Briefly, the NBS is a population-based survey conducted by the Radboud university medical center, Nijmegen, The Netherlands. 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 (NBS-1). A total of 9350 (42%) persons filled out the QN, of which 6468 (69%) donated blood samples. In 2005 the second phase of NBS was started (NBS-2), for which all participants of the first phase were re-invited to fill out a second questionnaire. Those who participated in NBS-2 and were aged 50-70 years at that time were asked to also participate in the NIMA (non-invasive measurements of atherosclerosis) substudy performed by the Department of Internal Medicine. For the NBS-2-NIMA study, participants had to fill out an additional QN, donate a fasting blood sample, and undergo anthropometric measurements and non-invasive measurements of atherosclerosis. A total of 1491 subjects participated in NBS-2-NIMA (response 71%). Approval to conduct the NBS and NBS-2-NIMA study was obtained from the Radboud university medical center Institutional Review Board. All participants gave written informed consent.

Genotype data (Illumina HumanHapCNV370-Duo BeadChip) were available for those 1980 NBS participants that were selected to serve as controls in GWAS [26]. A total of 1819 samples passed quality control [sample yield ≥96% (after exclusion of intensity-only markers (n=23,573)), Caucasian ancestry ≥90% (based on Structure analysis), SNP yield ≥96%]. Measurements of hepcidin and the iron parameters (iron, ferritin, TS and TIBC) were available for all these samples; NIMA for a subset of 549 participants.

Laboratory methods and clinical measurements

Total serum iron, TIBC, TS and ferritin were measured as described before [27]. Serum hepcidin was measured with an in-house developed and validated competitive enzyme-linked immunosorbent assay [27,28]. We used the following NIMA measurements for this study: IMT, presence of plaque and the ankle-brachial index at rest (ABIR) and after exercise (ABIEX). The NIMA and total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and triglycerides (TGC) were measured as described before [13,29].

Selection and measurement of genetic variants

Genome-wide SNP data were used to estimate genomic correlations and were available for 1819 NBS participants, as indicated above. SNP quality control [minor allele frequency (MAF) ≥1%, and Hardy-Weinberg equilibrium (HWE) p-value >10-6] resulted in availability of 323,414 SNPs. Density was increased by imputation, which was performed with 1000-genomes phase1 integrated version 3 as a reference sample using IMPUTE v2 software [30].

We used in total 18 SNPs for the MR approach and cross-trait associations: 12 iron-related SNPs (Benyamin *et al*., in press) (Supplemental Table 7.1) and six NIMA-related SNPs [23,24] (Supplemental Table 7.2). Five of these SNPs were directly measured (rs1800562, rs855791, rs744653, rs9990333, and rs6486121) and 13 SNPs were imputed, all of them with a quality of imputation of >0.99 as measured using the SNPtest info measure.

Statistical analysis

SNP associations

For the MR approach and cross-trait associations, genotype probabilities (P) of the 12 iron-related and 6-NIMA related SNPs were transformed to dosages, *i.e.* dosage = P_{AA}*0 + P_{AB}*1 +P_{BB}*2; allele B was the effect allele as presented in Supplemental Tables 7.1 and 7.2. Hepcidin, the hepcidin ratios and ferritin were log-transformed to normalize their distributions. For these log-transformed variables and for the other iron parameters, standardized residuals adjusted for age, squared age and time of blood sampling were derived. This was done separately for men and women to adjust for gender. Outliers that differed more than four times the SD from the mean were excluded. SNP associations of the iron-related SNPs with NIMA and the NIMA-related SNPs with iron parameters, hepcidin and hepcidin ratios were studied using logistic (presence of plaque) and linear (IMT, ABI, hepcidin, hepcidin ratios, iron parameters) regression models. Resulting odds ratios (OR) of logistic regression models express the effect of an additional effect allele on risk of presence of plaque, *i.e.* AB vs AA and BB vs AB. Resulting betas of linear models express the effect of an additional effect allele on measures of IMT, ABI, and on standardized residuals of iron, TS, TIBC, log-hepcidin, log-hepcidin/ferritin, log-hepcidin/TS, and log-ferritin. We also studied the associations between iron-related SNPs and NIMA with adjustment for the potential confounding factors TC, LDL, HDL and TGC, and stratified by gender.

Estimation of genomic correlations and genome-wide SNP explained variances

We estimated genomic correlations (rG) based on only directly measured autosomal SNPs using the software packages GCTA [31] and Bayz [32]. The use of GCTA to estimate rG using genome- or chromosome-wide SNPs for complex traits has been described [33] and applied in other studies before (*e.g.* [34]). However, recent results of Visscher *et al*. showed that the statistical power to detect statistically significant rG was limited with a sample size of N=1000 [35]. As our sample size for estimation of rG was smaller than 1000, we also estimated rG with a Bayesian multivariate model as implemented in the software package Bayz; this Bayesian analysis handles large sets of traits in a simultaneous analysis, and therefore we expected Bayz to produce more precise estimates compared to GCTA, which is limited to two-trait analysis.

In GCTA, rG are calculated for pairs of traits using the genetic relationship matrix (GRM) and a bivariate restricted maximum likelihood analysis (GREML) [33]. For the estimation of the GRM for pairs of individuals we only retained individuals with a pairwise relationship

<0.025 to remove cryptic relatedness from the data, as recommended by Yang *et al.*[36]. This resulted in 423 to 431 individuals for the NIMA and 1456 to 1481 individuals for hepcidin and the iron parameters.

In software package Bayz, Bayesian multivariate models including up to 8 traits simultaneously were used to estimate rG[32,37]. The genomic model from Janss *et al.*[32] is based on use of an eigenvector decomposition of the GRM in a random regression model; the GRM used is the same one as used in GCTA GREML analyses. The multi-trait implementation makes use of latent variables to model the covariance[37]. To determine the required number of latent variables, the Bayesian Deviance Information Criterion (DIC)[38] was computed. The Bayesian model uses uniform priors for variances to express a-priori ignorance about the model parameters. From the Bayesian model, the marginal posterior means for rG are reported. The marginal posterior standard deviation expresses uncertainty on the estimate, and, with uninformative prior distributions, should be similar to the frequentist SE. For our study, four different multivariate models were constructed to prevent simultaneous inclusion of highly dependent and correlated variables. Thus, hepcidin and ferritin were not included in the same model as the ratio hepcidin/ferritin, as also holds for serum iron and TIBC with TS, *i.e.* the ratio of serum iron over TIBC. Model 1 included hepcidin, ferritin, iron, TIBC, presence of plaque, IMT, ABIR and ABIEX and was used to determine rG for all included variables. Model 2 included the ratio hepcidin/ferritin, iron, TIBC, presence of plaque, IMT, ABIR and ABIEX, model 3 included the ratio hepcidin/TS, ferritin, presence of plaque, IMT, ABIR and ABIEX, and model 4 included hepcidin, ferritin, TS, presence of plaque, IMT, ABIR and ABIEX; these latter three models were used to estimate rG for the ratio hepcidin/ferritin, the ratio hepcidin/TS, and TS, respectively.

Prior to GCTA and Bayz analyses, the following steps were taken: 1) pairwise LD pruning to remove highly correlated SNPs (window size 100, step 5 and r^2 0.98) using the software package PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/)[39], resulting in 297,574 SNPs for analysis; 2) log-transformation of the variables hepcidin, ferritin and the ratios hepcidin/ferritin and hepcidin/TS to normalize their distributions; 3) transformation of hepcidin, the iron parameters and NIMA to sex-specific residuals using age, squared age and for hepcidin and the iron parameters also time of blood sampling as determinants in regression models; 4) reduction of outliers to mean ± 4SD (maximal number of outliers per trait was six); and 5) standardization of the traits to zero mean and unit variance.

Results

Characteristics of the subjects included in our study are presented in Table 7.1. The percentage of males of the total study population was 49% and median age at inclusion was 63 years. NIMA were available for a subset of 549 participants (49% male) with a median age of 59 years.

TABLE 7.1 Characteristics of the study population.

Variable†	Total N	Median (P5-P95) or N (%)*
Gender, males	1819	900 (49%)
Age at inclusion, years	1819	62.9 (42.2 - 76.0)
Time of blood sampling	1811	
Before 12:00 pm		374 (21%)
Between 12:00 pm and 5:00 pm		1165 (64%)
After 5:00 pm		272 (15%)
Serum hepcidin, nmoles/L	1810	7.5 (0.9 - 22.5)
Serum ferritin, µg/L	1817	120.7 (17.8 - 421.4)
Hepcidin/ferritin, µmoles/µg	1808	60.3 (20.9 - 170.3)
Hepcidin/TS, µmoles/L/%	1791	0.25 (0.04 - 0.96)
Serum iron, µmoles/L	1800	17.0 (9.0 - 27.0)
TIBC, µmoles/L	1800	58.0 (46.0 - 75.0)
TS, %	1800	28.8 (14.1 - 48.1)
IMT, mm	549	0.85 (0.70 - 1.05)
ABI at rest	549	1.10 (0.96 - 1.27)
ABI after exercise	542	1.11 (0.76 - 1.34)
Presence of plaque	549	232 (42%)

ABI indicates ankle-brachial index; IMT, intima media thickness; N, number; P5, fifth percentile; P95, 95th percentile; TIBC, total iron-binding capacity; and TS, transferrin saturation.

* Continuous variables are presented as median (P5-P95). Categorical variables are presented as N (%).

† Hepcidin, ferritin, hepcidin/ferritin, hepcidin/TS, iron, TIBC, TS and time of blood sampling were measured in 2002 and presence of plaque, IMT, and ABI at rest and after exercise were measured between 2005 and 2008.

MR results: associations of iron-related SNPs with IMT, presence of plaque and ABI

Figure 7.1 visualizes the effects of the SNPs on the iron parameters based on the iron meta-GWAS of the GIS Consortium. The SNPs are classified into their hypothesized effects on the risk of atherosclerosis according to the 'iron hypothesis': SNPs that increase serum iron, TS and/or ferritin are hypothesized to increase the risk of atherosclerosis, and vice versa. The hypothesized effect of rs8177240 is classified as unknown, because the T allele of this SNP decreases iron and transferrin and increases TS. The hypothesized effects of rs4921915, rs6486121 and rs174577 are also classified as unknown, because these SNPs are only associated with transferrin.

Results of the single SNP association analyses are presented in Table 7.2. Only the T allele of rs651007, associated with decreased ferritin, showed effects that were consistent with

the hypothesized directions of effects for *all* NIMA, with nominal significant effects on IMT [beta -0.021 (95% CI -0.038; -0.004)] and ABI after exercise [beta 0.034 (95% CI 0.004; 0.063)]. SNP rs9990333 showed a nominally significant association with presence of plaque that was directionally consistent [OR 1.32 (95% CI 1.03; 1.68)], but this SNP showed inconsistent, nonsignificant effects on other NIMA. Two other nominally significant effects were found for rs8177240 and rs4921915, but their hypothesized directions of effect were unknown. Adjustment of the associations for TC, LDL, HDL and TGC revealed similar results (Supplemental Table 7.3).

Stratification by gender revealed that effects of rs651007 were consistent with the hypothesized directions of effects for all NIMA in men and for *all* NIMA except for ABI at rest in women (Supplemental Table 7.4). The effect of rs651007 on IMT was stronger in women, whereas the effect of rs651007 on presence of plaque and ABI after exercise was stronger in men. In addition to rs651007, rs855791 showed consistent associations with all NIMA in men, whereas rs1799945 and rs7385804 showed consistent associations with all NIMA in women. Effect estimates were not systematically higher or lower in men or women. Associations of the gender-specific results remained similar after adjustment for TC, LDL, HDL, and TGC (Supplemental Table 7.5).

FIGURE 7.1 Twelve SNPs identified in meta-GWAS for iron parameters and included in the Mendelian randomization analysis. Betas ± standard error for serum iron, transferrin, transferrin saturation (TS), and ferritin (log) based on the meta-analyses of genome-wide association studies on iron status performed by the Genetics of Iron Status Consortium (discovery and replication combined (N=48,972)). (A) SNPs that are hypothesized to increase the risk of atherosclerosis according to the 'iron hypothesis'; (B) SNPs that are hypothesized to decrease the risk of atherosclerosis according to the 'iron hypothesis'; (C) SNPs for which the hypothesized effect on atherosclerosis risk is unknown.

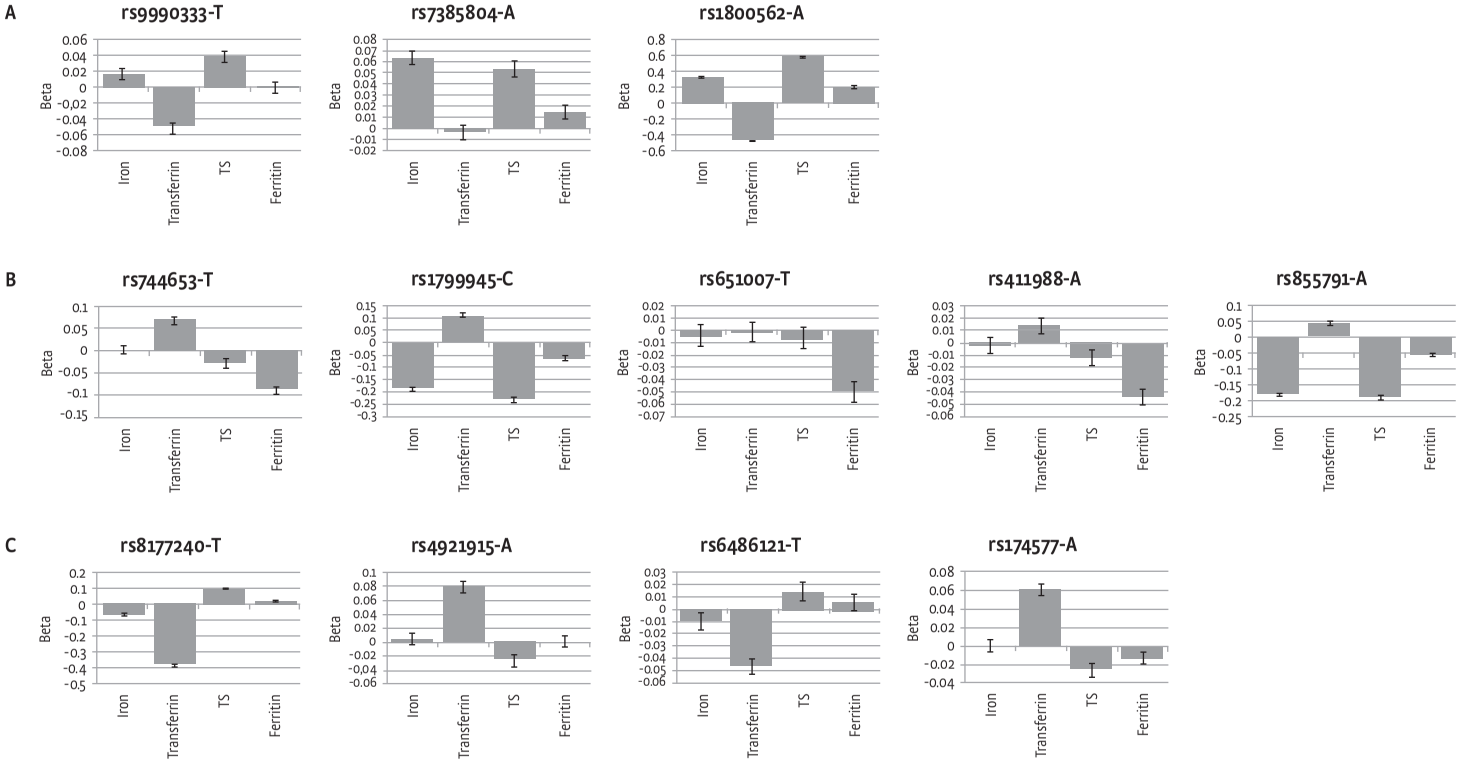

TABLE 7.2 Association of the iron-related SNPs with non-invasive measurements of atherosclerosis.

	Presence of plaque			IMT			ABI at rest			ABI after exercise		
SNP – Tested allele	H*	OR	95% CI	H*	Beta	95% CI	H*	Beta	95% CI	H*	Beta	95% CI
rs744653 – T	<1	1.10	0.78; 1.55	-	-0.006	-0.025; 0.014	+	-0.013	-0.031; 0.005	+	-0.019	-0.052; 0.014
rs8177240 – T[#]	?	1.13	0.87; 1.46	?	-0.010	-0.025; 0.004	?	**0.014**	**0.000; 0.027**	?	**0.030**	**0.005; 0.055**
rs9990333 – T	>1	**1.32**	**1.03; 1.68**	+	0.012	-0.002; 0.025	-	0.006	-0.006; 0.019	-	0.016	-0.007; 0.040
rs1800562 – A	>1	1.03	0.62; 1.69	+	-0.017	-0.045; 0.011	-	-0.006	-0.032; 0.020	-	-0.005	-0.054; 0.043
rs1799945 – C	<1	1.19	0.86; 1.64	-	0.011	-0.007; 0.029	+	0.003	-0.014; 0.019	+	-0.006	-0.037; 0.024
rs7385804 – A	>1	0.92	0.72; 1.18	+	-0.006	-0.020; 0.008	-	0.000	-0.013; 0.013	-	0.018	-0.006; 0.042
rs4921915 – A[†]	?	**1.42**	**1.04; 1.92**	?	-0.006	-0.023; 0.010	?	0.002	-0.014; 0.017	?	0.005	-0.023; 0.034
rs651007 – T	<1	0.84	0.62; 1.15	-	**-0.021**	**-0.038; -0.004**	+	0.003	-0.013; 0.019	+	**0.034**	**0.004; 0.063**
rs6486121 – T[†]	?	0.94	0.73; 1.20	?	0.012	-0.002; 0.026	?	-0.004	-0.017; 0.009	?	-0.007	-0.031; 0.018
rs174577 – A[†]	?	1.10	0.85; 1.42	?	0.001	-0.013; 0.015	?	0.004	-0.009; 0.017	?	0.015	-0.009; 0.040
rs411988 – A	<1	0.92	0.73; 1.17	-	-0.001	-0.015; 0.012	+	0.004	-0.008; 0.017	+	-0.010	-0.033; 0.013
rs855791 – A	<1	1.20	0.94; 1.54	-	-0.010	-0.023; 0.004	+	0.005	-0.008; 0.018	+	0.010	-0.013; 0.034

Associations were tested using logistic (presence of plaque) and linear regression (IMT and ABI at rest and after exercise). Resulting odds ratios (OR) of logistic models express the effect of each extra tested allele on odds for presence of plaque. Resulting betas of linear models express the effect of each extra tested allele on IMT or ABI.

ABI indicates ankle-brachial index; CI, confidence interval; H, hypothesized effect; IMT, intima media thickness; OR, odds ratio; SNP, single nucleotide polymorphism.

* Hypothesized effect on the NIMA according to the 'iron hypothesis' (see Figure 7.1). Presence of plaque, a higher IMT and a lower ABI indicate presence of atherosclerosis.

[#] This SNP decreases iron and transferrin and increases TS, so the hypothesized effect on atherosclerosis is unknown.

[†] These SNPs only show association with transferrin, so the hypothesized effect on atherosclerosis is unknown.

Association of NIMA-related SNPs with the iron parameters and hepcidin

Associations of top SNPs for IMT, plaque and ABI with hepcidin, the ratios hepcidin/ferritin and hepcidin/TS, and the iron parameters are shown in Supplemental Table 7.6. Three nominally significant associations were observed. The IMT-associated SNP rs11781551 showed association with TIBC, but the observed direction of effect was opposite to the hypothesized direction. This SNP also showed association with the ratio hepcidin/ferritin, in addition to the ABI-associated SNP rs10757269, and observed directions of effect were consistent with hypothesized directions for both SNPs.

Genomic correlations

Table 7.3 shows the genomic correlations of hepcidin, the ratios hepcidin/ferritin and hepcidin/TS, and the iron parameters with NIMA as obtained by GCTA (Table 7.3A) and Bayz (Table 7.3B). Point estimates of genomic correlations resulting from the two methods were mostly dissimilar. A substantial part of the estimates by GCTA resulted in values of 0, -1 or 1, indicating convergence of the models to extremes, as expected due to our relatively small sample size.

Bayz estimates of genomic correlations with NIMA were close to 0, except for the genomic correlation of hepcidin and ferritin with ABI at rest [-0.27 (SE 0.34) and -0.22 (SE 0.35), respectively] and ABI after exercise [-0.29 (SE 0.34) and -0.30 (0.35), respectively].

Evidence for and against a role of hepcidin and the iron parameters in atherosclerosis based on the results of the current study is summarized in Table 7.4.

TABLE 7.3 A Genomic correlations (SE) estimated with GCTA.

	Presence of plaque	IMT	ABI at rest	ABI after exercise
Hepcidin	0.18 (1.48)	0.11 (1.49)	0.01 (1.29)	0.02 (1.40)
Ferritin	0.15 (1.49)	-1.00 (4.74)	1.00 (38.1)	0.01 (1.45)
Hepcidin/ferritin	-1.00 (4.35)	0.09 (4.35)	-1.00 (>4E5)	0.25 (2.39)
Hepcidin/TS	-1.00 (15.38)	-1.00 (4.87)	0.02 (1.37)	0.01 (1.24)
Iron	0.05 (1.53)	1.00 (4.18)	0.08 (1.05)	-1.00 (38.1)
TIBC	1.00 (17.3)	0.04 (1.16)	1.00 (55.5)	-1.00 (113.7)
TS	0.06 (1.36)	1.00 (147.7)	0.06 (0.96)	1.00 (>1E5)

ABI indicates ankle-brachial index; IMT, intima media thickness; SE, standard error; TIBC, total iron-binding capacity; and TS, transferrin saturation.

TABLE 7.3 B Genomic correlations (SE) estimated with Bayz.

	Presence of plaque	IMT	ABI at rest	ABI after exercise
Hepcidin*	0.01 (0.27)	-0.01 (0.31)	-0.27 (0.34)	-0.29 (0.34)
Ferritin*	-0.03 (0.28)	0.01 (0.32)	-0.22 (0.35)	-0.30 (0.35)
Hepcidin/ferritin#	0.06 (0.21)	-0.02 (0.24)	-0.10 (0.27)	-0.01 (0.28)
Hepcidin/TS$	0.12 (0.19)	0.10 (0.21)	-0.07 (0.27)	0.03 (0.29)
Iron*	-0.04 (0.20)	0.06 (0.21)	0.04 (0.25)	0.09 (0.26)
TIBC*	-0.01 (0.14)	0.04 (0.15)	-0.08 (0.16)	-0.07 (0.17)
TS†	-0.01 (0.19)	0.00 (0.19)	0.05 (0.21)	0.04 (0.22)

ABI indicates ankle-brachial index; IMT, intima media thickness; SE, standard error; TIBC, total iron-binding capacity; and TS, transferrin saturation.

* Correlations in these rows come from an 8-trait analysis including hepcidin, ferritin, iron, TIBC, presence of plaque, IMT, ABI at rest and ABI after exercise.

\# Correlations in this row come from a 7-trait analysis including the ratio hepcidin/ferritin, iron, TIBC, presence of plaque, IMT, ABI at rest and ABI after exercise.

$ Correlations in this row come from a 6-trait analysis including the ratio hepcidin/TS, ferritin, presence of plaque, IMT, ABI at rest and ABI after exercise.

† Correlations in this row come from a 7-trait analysis including hepcidin, ferritin, TS, presence of plaque, IMT, ABI at rest and ABI after exercise.

TABLE 7.4 Summary of evidence for and against a role of hepcidin and the iron parameters in atherosclerosis resulting from this study.

Trait	Evidence for causal role	Evidence against causal role
Hepcidin	Weak genomic correlations with ABI at rest and ABI after exercise	- No nominally significant associations with all NIMA-related SNPs - Genomic correlations with presence of plaque and IMT ~0
Ferritin	- Directionally consistent associations of rs651007 with NIMA - Weak genomic correlations with ABI at rest and ABI after exercise	- No directionally consistent associations of other variants (notably rs411988) with NIMA - No nominally significant associations with all NIMA-related SNPs - Genomic correlations with presence of plaque and IMT ~0
Hepcidin/ferritin	Nominally significant associations with two NIMA-related SNPs	- No nominally significant associations with four NIMA-related SNPs - Genomic correlations with all NIMA ~0
Hepcidin/TS		- No nominally significant associations with all NIMA-related SNPs - Genomic correlations with all NIMA ~0
Iron		- No directionally consistent associations of iron-related SNPs with NIMA - No nominally significant associations with all NIMA-related SNPs - Genomic correlations with all NIMA ~0
TIBC		- No directionally consistent associations of iron-related SNPs with NIMA - No nominally significant associations with all NIMA-related SNPs - Genomic correlations with all NIMA ~0
TS		- No directionally consistent associations of iron-related SNPs with NIMA - No nominally significant associations with all NIMA-related SNPs - Genomic correlations with all NIMA ~0

ABI indicates ankle-brachial index; IMT, intima media thickness; NIMA, non-invasive measurement of atherosclerosis; TIBC, total iron-binding capacity; and TS, transferrin saturation.

Discussion

In this study, we investigated relationships of iron parameters and hepcidin with NIMA in a sample of the general population by performing an MR approach, assessing associations of NIMA-related SNPs with iron parameters and hepcidin, and studying genomic correlations of iron parameters, hepcidin, and hepcidin ratios with NIMA. Overall, the results suggest a potential role for ferritin and hepcidin in atherosclerosis, but do not provide evidence for causal effects of the other iron parameters on NIMA (Table 7.4).

The lack of evidence for a causal effect of iron, TIBC and TS on NIMA is in contradiction with the 'iron hypothesis', but confirms results from our previous observational study in which we did not find evidence for an association between iron status and NIMA as well [13]. Results of that study indicated that the iron *distribution*, as determined by serum hepcidin and the ratio hepcidin/ferritin, plays a role in the development of atherosclerosis.

The three different approaches that we used provided weak evidence for a potential causal role for hepcidin, ferritin and the ratio hepcidin/ferritin in atherosclerosis, but resulted in a lack of evidence for a causal role of iron, TIBC and TS in NIMA. First, iron-related top SNPs were not associated with the NIMA. For all but one of the 12 SNPs, directions of effect were inconsistent over the four NIMA and/or inconsistent with the hypothesized direction of effect according to the 'iron hypothesis'. The exception was rs651007, which is uniquely associated with a decrease in ferritin concentration and which showed a decreased risk of atherosclerosis. However, rs411988, which is also uniquely associated with ferritin with a similar effect estimate as for rs651007, showed far weaker or even no associations with the NIMA. One would expect similar results for these two SNPs [40], thus the MR approach provides contradicting evidence for a causal role of ferritin in atherosclerosis. The causal effects of hepcidin on atherosclerosis could not be studied by an MR approach, as there have been no SNPs identified yet that have been validated for association with hepcidin.

Secondly, the reverse approach of studying associations of NIMA-associated top SNPs with the iron parameters, as was previously done for coronary artery disease and IMT [41], provided additional evidence against a role of iron, TIBC and TS in atherosclerosis. It did also not provide evidence for a causal role of ferritin in atherosclerosis. We did not observe any significant and consistent associations, thus indicating no intermediate or pleiotropic effects of these SNPs on all four iron parameters. Furthermore, the nearest genes for NIMA-associated top SNPs are currently not known to be involved in iron metabolism. In contrast, two NIMA-related SNPs showed nominally significant associations with the ratio hepcidin/ferritin, with consistency in observed and hypothesized directions of effect, indicating that the ratio hepcidin/ferritin and thus the body iron distribution might be involved in atherosclerosis. Notably, two previous studies reported the SNPs rs12091564 and rs10218795 in the hemochromatosis type 2 gene (*HFE2*) to be associated with coronary artery disease (CAD)

based on a two-marker association test and haplotype analysis [42,43]. Defects in *HFE2* lead to a form of juvenile hemochromatosis, which is characterized by a severe iron overload (high serum ferritin, high TS, low hepcidin/ferritin ratio) occurring typically before the age of 30, and thus this finding might indicate a link between iron or iron distribution and atherosclerosis. However, the *HFE2* gene is also expressed in heart and skeletal muscle, thus the SNPs in *HFE2* do not necessarily associate with CAD via iron.

Third, genomic correlations of the iron parameters with the NIMA were close to zero, thus indicating that there is no overlap in genetic etiology of the traits. Exception was the modest negative genomic correlation of ferritin with ABI at rest and ABI after exercise, indicating that genetic variants that increase ferritin cause a decrease in ABI. However, these genomic correlations were far from statistically significant and ferritin did not show genomic correlations with other NIMA. In addition, we observed a negative genomic correlation of hepcidin with ABI at rest and after exercise. This confirms our previous observational study, in which we found the ratio hepcidin/ferritin to be negatively associated with ABI at rest and after exercise in postmenopausal women [13]. On the contrary, we found a significant association of hepcidin and hepcidin/ferritin with presence of plaque in our previous observational study, but genomic correlations between these variables in the current study were very weak.

There are some aspects that hampered our study. First of all, the size of the study population was limited, resulting in imprecise estimates of genomic correlations, suboptimal power of our MR analysis and low power to identify SNP associations with statistical significance. Consequently, we focused on directions of observed effect estimates and their consistency with the hypothesized direction of effect. In addition, the limited sample size decreased the power of our gender-stratified MR analyses, which we performed based on our previous gender-specific findings [13]. Secondly, our estimation of genomic correlations provided us with indirect evidence of a potential causal relation between these traits; strong genomic correlations can be the result of pleiotropy and are not evidence for causality per se. Also, weak genomic correlations do not exclude causal relationships, as causality can also be the result of (shared) environmental factors only. In addition, the genomic correlations that we reported were based on measured autosomal and common SNPs only. Finally, causal inference was limited by the fact that most of the 12 SNPs that we included in our MR approach influence more than one of the iron parameters. Furthermore, the SNPs rs1800562 in *HFE* and rs855791 in *TMPRSS6* also showed association with the ratios hepcidin/ferritin and hepcidin/TS [21,22], and with red blood cell traits (*e.g.* [44,45]). In addition, four of the loci that were found in the iron status meta-GWAS have been reported as lipid loci by the Global Lipids Genetics Consortium [46], *i.e. HFE*, *NAT2*, *ABO* and *FADS2*. However, performing the MR analyses using TC, LDL, HDL and TGC as covariates did not change our conclusions. Still, the overlap in iron and lipid loci is substantial and is therefore unlikely to be based on chance only. It might indicate causal relationships between iron status and lipid levels and therefore also atherosclerosis, although the results of our study do not support this.

In conclusion, this study is the first to evaluate the 'iron hypothesis' from a genetic point of view. Our results do not provide evidence for a role of iron, TIBC and TS in the development of atherosclerosis, which confirms the findings from our previous observational study [13]. Our results are suggestive for a potential causal role of hepcidin and ferritin in atherosclerosis. Thus, our study is in line with the hypothesis that it is the body iron *distribution* as determined by hepcidin and its ratio to ferritin instead of the absolute amount of body iron that is involved in the development of atherosclerosis. We warrant future studies to exploit any new genetic variants that are found to be associated with serum hepcidin in an MR approach. In addition, we emphasize follow-up of the current study in a larger series including multiple study populations.

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References

1 Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1:1293-4.
2 Lapenna D, Pierdomenico SD, Ciofani G, et al. Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques. Free Radic Biol Med 2007;42:492-8.
3 Kraml PJ, Klein RL, Huang Y, et al. Iron loading increases cholesterol accumulation and macrophage scavenger receptor I expression in THP-1 mononuclear phagocytes. Metabolism 2005;54:453-9.
4 Kiechl S, Willeit J, Egger G, et al. Body iron stores and the risk of carotid atherosclerosis: prospective results from the Bruneck study. Circulation 1997;96:3300-7.
5 Salonen JT, Tuomainen TP, Salonen R, et al. Donation of blood is associated with reduced risk of myocardial infarction. The Kuopio Ischaemic Heart Disease Risk Factor Study. Am J Epidemiol 1998;148:445-51.
6 Meyers DG, Jensen KC, Menitove JE. A historical cohort study of the effect of lowering body iron through blood donation on incident cardiac events. Transfusion 2002;42:1135-9.
7 Meyers DG, Strickland D, Maloley PA, et al. Possible association of a reduction in cardiovascular events with blood donation. Heart 1997;78:188-93.
8 Ascherio A, Rimm EB, Giovannucci E, et al. Blood donations and risk of coronary heart disease in men. Circulation 2001;103:52-7.
9 Zheng H, Cable R, Spencer B, et al. Iron stores and vascular function in voluntary blood donors. Arterioscler Thromb Vasc Biol 2005;25:1577-83.
10 Engberink MF, Geleijnse JM, Durga J, et al. Blood donation, body iron status and carotid intima-media thickness. Atherosclerosis 2008;196:856-62.
11 Peffer K, den Heijer M, Holewijn S, et al. The effect of frequent whole blood donation on ferritin, hepcidin, and subclinical atherosclerosis. Transfusion 2012;53:1468-74.
12 Sullivan JL. Macrophage iron, hepcidin, and atherosclerotic plaque stability. Exp Biol Med (Maywood) 2007;232:1014-20.
13 Galesloot TE, Holewijn S, Kiemeney LA, et al. Serum hepcidin is associated with presence of plaque in postmenopausal women of a general population. Arterioscler Thromb Vasc Biol 2014;34:446-56.
14 Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;32:1-22.
15 Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009;41:1173-5.
16 Benyamin B, McRae AF, Zhu G, et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60-5.
17 Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. Blood 2010;115:94-6.

18 Pichler I, Minelli C, Sanna S, et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum Mol Genet 2011;20:1232-40.
19 McLaren CE, Garner CP, Constantine CC, et al. Genome-wide association study identifies genetic loci associated with iron deficiency. PLoS One 2011;6:e17390.
20 Oexle K, Ried JS, Hicks AA, et al. Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferrin receptor (sTfR) levels. Hum Mol Genet 2011;20:1042-7.
21 Traglia M, Girelli D, Biino G, et al. 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.
22 Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. 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 2013;50:593-8.
23 Bis JC, Kavousi M, Franceschini N, et al. Meta-analysis of genome-wide association studies from the CHARGE consortium identifies common variants associated with carotid intima media thickness and plaque. Nat Genet 2011;43:940-7.
24 Murabito JM, White CC, Kavousi M, et al. Association between chromosome 9p21 variants and the ankle-brachial index identified by a meta-analysis of 21 genome-wide association studies. Circ Cardiovasc Genet 2012;5:100-12.
25 Hoogendoorn EH, Hermus AR, de Vegt F, et al. 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.
26 Kiemeney LA, Thorlacius S, Sulem P, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. Nat Genet 2008;40:1307-12.
27 Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.
28 Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570-9.
29 Holewijn S, den Heijer M, Swinkels DW, et al. The metabolic syndrome and its traits as risk factors for subclinical atherosclerosis. J Clin Endocrinol Metab 2009;94:2893-9.
30 Howie BN, Donnelly P, Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Genet 2009;5:e1000529.
31 Yang J, Lee SH, Goddard ME, et al. GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet 2011;88:76-82.
32 Janss L, de Los Campos G, Sheehan N, et al. Inferences from genomic models in stratified populations. Genetics 2012;192:693-704.
33 Lee SH, Yang J, Goddard ME, et al. Estimation of pleiotropy between complex diseases using single-nucleotide polymorphism-derived genomic relationships and restricted maximum likelihood. Bioinformatics 2012;28:2540-2.

34 Cross-Disorder Group of the Psychiatric Genomics C, Lee SH, Ripke S, et al. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. Nat Genet 2013;45:984-94.
35 Visscher PM, Hemani G, Vinkhuyzen AA, et al. Statistical power to detect genetic (co)variance of complex traits using SNP data in unrelated samples. PLoS Genet 2014;10(4):e1004269.
36 Yang J, Benyamin B, McEvoy BP, et al. Common SNPs explain a large proportion of the heritability for human height. Nat Genet 2010;42:565-9.
37 Bouwman AC, Valente BD, Janss LL, et al. Exploring causal networks of bovine milk fatty acids in a multivariate mixed model context. Genet Sel Evol 2014;46:2.
38 Spiegelhalter DJ, Best NG, Carlin BP, Van der Linde A. Bayesian measures of model complexity and fit. J R Statist Soc B 2002;64:583–639.
39 Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.
40 Palmer TM, Lawlor DA, Harbord RM, et al. Using multiple genetic variants as instrumental variables for modifiable risk factors. Stat Methods Med Res 2012;21:223-42.
41 Conde L, Bevan S, Sitzer M, et al. Novel associations for coronary artery disease derived from genome wide association studies are not associated with increased carotid intima-media thickness, suggesting they do not act via early atherosclerosis or vessel remodeling. Atherosclerosis 2011;219:684-9.
42 Slavin TP, Feng T, Schnell A, et al. Two-marker association tests yield new disease associations for coronary artery disease and hypertension. Hum Genet 2011;130:725-33.
43 Zhu X, Feng T, Li Y, et al. Detecting rare variants for complex traits using family and unrelated data. Genet Epidemiol 2010;34:171-87.
44 Soranzo N, Sanna S, Wheeler E, et al. Common variants at 10 genomic loci influence hemoglobin A(1)(C) levels via glycemic and nonglycemic pathways. Diabetes 2010;59:3229-39.
45 Kullo IJ, Ding K, Jouni H, et al. A genome-wide association study of red blood cell traits using the electronic medical record. PLoS One 2010;5:e13011.
46 Global Lipids Genetics Consortium, Willer CJ, Schmidt EM, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet 2013;45:1274-83.

SUPPLEMENTAL TABLE 7.1 Twelve SNPs identified in meta-GWAS for iron parameters and included in the Mendelian randomization analysis.

					Iron			Transferrin			TS			Ferritin (log)		
SNP	Nearest gene(s)	Effect allele	Freq*	HWE p*	Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p
rs744653	*WDR75-SLC40A1*	T	0.86	1.1E-01	0.004	0.010	7.0E-01	**0.068**	**0.010**	**1.4E-11**	-0.028	0.011	8.4E-03	**-0.089**	**0.010**	**8.4E-19**
rs8177240	*TF*	T	0.34	3.7E-01	**-0.066**	**0.007**	**6.7E-20**	**-0.380**	**0.007**	**<1E-340**	**0.100**	**0.008**	**7.2E-38**	0.021	0.007	3.9E-03
rs9990333	*TFRC*	T	0.47	7.1E-01	0.017	0.007	1.4E-02	**-0.051**	**0.007**	**2.0E-13**	0.039	0.007	7.3E-08	0.001	0.007	8.8E-01
rs1800562	*HFE*	A	0.06	3.1E-01	**0.328**	**0.016**	**2.7E-97**	**-0.479**	**0.016**	**8.9E-196**	**0.577**	**0.016**	**2.2E-270**	**0.204**	**0.016**	**1.5E-38**
rs1799945	*HFE*	C	0.16	2.1E-02	**-0.189**	**0.010**	**1.10E-81**	**0.114**	**0.010**	**9.4E-30**	**-0.231**	**0.010**	**5.1E-109**	**-0.065**	**0.010**	**1.7E-10**
rs7385804	*TFR2*	A	0.63	2.4E-01	**0.064**	**0.007**	**1.4E-18**	-0.003	0.007	7.3E-01	**0.054**	**0.008**	**6.1E-12**	0.015	0.007	3.9E-02
rs4921915	*NAT2*	A	0.78	4.9E-01	0.004	0.009	6.3E-01	**0.079**	**0.009**	**7.1E-19**	-0.026	0.009	3.6E-03	0.001	0.009	8.9E-01
rs651007	*ABO*	T	0.21	5.6E-01	-0.004	0.009	6.1E-01	-0.001	0.009	9.2E-01	-0.006	0.009	5.0E-01	**-0.050**	**0.009**	**1.3E-08**
rs6486121	*ARNTL*	T	0.64	8.2E-02	-0.009	0.007	2.0E-01	**-0.046**	**0.007**	**3.9E-10**	0.015	0.008	4.8E-02	0.006	0.007	4.2E-01
rs174577	*FADS2*	A	0.33	4.2E-02	0.001	0.007	8.8E-01	**0.062**	**0.007**	**2.3E-17**	-0.025	0.008	1.6E-03	-0.012	0.007	9.8E-02
rs411988	*TEX14*	A	0.58	3.3E-01	-0.002	0.007	7.7E-01	0.014	0.007	5.2E-02	-0.012	0.007	1.2E-01	**-0.044**	**0.007**	**1.6E-10**
rs855791	*TMPRSS6*	A	0.54	2.7E-01	**-0.181**	**0.007**	**1.3E-139**	**0.044**	**0.007**	**2.0E-09**	**-0.190**	**0.008**	**6.4E-137**	**-0.055**	**0.007**	**1.4E-14**

Betas, SE and p-values for iron, transferrin, TS and ferritin based on the findings of the Genetics of Iron Status (GIS) Consortium iron parameter meta-GWAS (discovery and replication combined) (N=48,972; including Nijmegen Biomedical Study) (Benyamin *et al.*, in press).

Freq indicates frequency; HWE, Hardy-Weinberg equilibrium; SE, standard error; SNP, single nucleotide polymorphism; TS, transferrin saturation.

* Effect allele frequency and HWE p-value in Nijmegen Biomedical Study.

SUPPLEMENTAL TABLE 7.2 SNPs identified by published meta-GWAS for IMT, presence of plaque or ABI.

Trait	SNP	Nearest gene	Effect allele	Freq*	HWE p*	Beta / OR#	SE / 95% CI#	p
IMT	rs11781551	*ZHX2*	A	0.46	0.17	-0.0078	0.0012	2.4E-11
	rs445925	*APOC1*	A	0.11	1	-0.0156	0.0028	1.7E-08
	rs6601530	*PINX1*	G	0.56	0.54	0.0078	0.0014	1.7E-08
Presence of plaque	rs17398575	*PIK3CG*	A	0.23	0.12	1.18	1.12; 1.23	2.3E-12
	rs1878406	*EDNRA*	T	0.14	0.54	1.22	1.15; 1.29	6.9E-12
ABI	rs10757269	*CDKN2B*	G	0.46	0.60	-0.0049	0.0008	2.65E-09

Betas, 95% and p-values for SNPs associated with IMT or presence of plaque are based on a meta-analysis of GWAS by Bis *et al*., 2011 [1]. Betas, 95% and p-values for the SNP associated with ABI are based on a meta-analysis of GWAS by Murabito *et al*., 2011 [2].

ABI indicates ankle-brachial index; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; IMT, intima media thickness; SE, standard error; SNP, single nucleotide polymorphism; TS, transferrin saturation.

* Effect allele frequency and HWE p-value in NBS.

\# Beta and SE for SNPs associated with IMT and ABI; OR and 95% CI for SNPs associated with presence of plaque.

SUPPLEMENTAL TABLE 7.3

Association of the iron-related SNPs with non-invasive measurements of atherosclerosis adjusted for total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides.

	Presence of plaque			IMT			ABI at rest			ABI after exercise		
SNP – Tested allele	H*	OR	95% CI	H*	Beta	95% CI	H*	Beta	95% CI	H*	Beta	95% CI
rs744653 – T	<1	1.15	0.80; 1.63	-	-0.004	-0.022; 0.014	+	-0.012	-0.030; 0.006	+	-0.013	-0.046; 0.019
rs8177240 – T[#]	?	1.10	0.85; 1.44	?	-0.011	-0.024; 0.002	?	0.013	-0.001; 0.026	?	**0.031**	**0.007; 0.056**
rs9990333 – T	>1	**1.33**	**1.04; 1.70**	+	0.010	-0.003; 0.022	-	0.007	-0.005; 0.020	-	0.016	-0.007; 0.039
rs1800562 – A	>1	1.11	0.66; 1.85	+	-0.008	-0.034; 0.018	-	-0.008	-0.034; 0.018	-	-0.007	-0.055; 0.041
rs1799945 – C	<1	1.17	0.85; 1.62	-	0.009	-0.007; 0.025	+	0.002	-0.014; 0.019	+	-0.009	-0.039; 0.021
rs7385804 – A	>1	0.92	0.71; 1.18	+	-0.007	-0.020; 0.006	-	0.000	-0.013; 0.013	-	0.019	-0.004; 0.043
rs4921915 – A[†]	?	1.35	0.99; 1.85	?	-0.011	-0.027; 0.004	?	0.004	-0.012; 0.019	?	0.008	-0.020; 0.037
rs651007 – T	<1	0.88	0.64; 1.21	-	**-0.016**	**-0.032; 0.000**	+	0.004	-0.013; 0.020	+	**0.031**	**0.001; 0.060**
rs6486121 – T[†]	?	0.93	0.72; 1.20	?	0.012	-0.001; 0.025	?	-0.003	-0.016; 0.011	?	-0.006	-0.031; 0.018
rs174577 – A[†]	?	1.14	0.88; 1.48	?	0.003	-0.010; 0.016	?	0.003	-0.010; 0.017	?	0.014	-0.010; 0.039
rs411988 – A	<1	0.91	0.72; 1.16	-	-0.001	-0.014; 0.011	+	0.004	-0.008; 0.017	+	-0.009	-0.032; 0.013
rs855791 – A	<1	1.21	0.94; 1.56	-	-0.007	-0.020; 0.005	+	0.005	-0.007; 0.018	+	0.010	-0.014; 0.033

Associations were tested using logistic (presence of plaque) and linear regression (IMT and ABI at rest and after exercise). Resulting odds ratios (OR) of logistic models express the effect of each extra tested allele on odds for presence of plaque. Resulting betas of linear models express the effect of each extra tested allele on IMT or ABI.

ABI indicates ankle-brachial index; CI, confidence interval; H, hypothesized effect; IMT, intima media thickness; OR, odds ratio; SNP, single nucleotide polymorphism.

* Hypothesized effect on the NIMA according to the 'iron hypothesis' (see Figure 7.1). Presence of plaque, a higher IMT and a lower ABI indicate presence of atherosclerosis.

[#] This SNP decreases iron and transferrin and increases TS, so the hypothesized effect on atherosclerosis is unknown.

[†] These SNPs only show association with transferrin, so the hypothesized effect on atherosclerosis is unknown.

SUPPLEMENTAL TABLE 7.4 Association of the iron-related SNPs with non-invasive measurements of atherosclerosis stratified by gender.

		Presence of plaque			IMT			ABI at rest			ABI after exercise		
Gender	SNP – Tested allele	H*	OR	95% CI	H*	Beta	95% CI	H*	Beta	95% CI	H*	Beta	95% CI
Men	rs744653 – T	<1	1.15	0.72; 1.85	-	0.015	-0.012; 0.042	+	-0.015	-0.045; 0.015	+	-0.023	-0.078; 0.032
	rs8177240 – T[#]	?	1.21	0.84; 1.76	?	**-0.022**	**-0.043; -0.001**	?	0.020	-0.003; 0.043	?	**0.046**	**0.004; 0.089**
	rs9990333 – T	>1	1.07	0.75; 1.52	+	0.006	-0.014; 0.026	-	0.013	-0.009; 0.036	-	0.037	-0.003; 0.078
	rs1800562 – A	>1	1.04	0.48; 2.26	+	-0.006	-0.050; 0.038	-	-0.024	-0.073; 0.025	-	-0.005	-0.096; 0.085
	rs1799945 – C	<1	**1.64**	**1.04; 2.58**	-	**0.026**	**0.001; 0.051**	+	-0.001	-0.029; 0.027	+	-0.019	-0.071; 0.033
	rs7385804 – A	>1	0.72	0.51; 1.03	+	-0.016	-0.036; 0.004	-	0.011	-0.011; 0.033	-	0.039	-0.001; 0.079
	rs4921915 – A[†]	?	1.18	0.76; 1.82	?	-0.027	-0.051; -0.002	?	0.005	-0.023; 0.032	?	0.012	-0.038; 0.063
	rs651007 – T	<1	0.78	0.50; 1.22	-	-0.013	-0.038; 0.011	+	0.009	-0.019; 0.037	+	**0.063**	**0.013; 0.114**
	rs6486121 – T[†]	?	0.91	0.64; 1.29	?	0.015	-0.004; 0.035	?	-0.012	-0.034; 0.010	?	-0.019	-0.059; 0.021
	rs174577 – A[†]	?	1.22	0.85; 1.73	?	-0.008	-0.028; 0.012	?	0.008	-0.014; 0.031	?	-0.001	-0.042; 0.040
	rs411988 – A	<1	0.93	0.66; 1.30	-	-0.008	-0.027; 0.011	+	0.004	-0.017; 0.026	+	-0.018	-0.057; 0.022
	rs855791 – A	<1	0.86	0.60; 1.23	-	-0.006	-0.026; 0.014	+	0.010	-0.012; 0.033	+	0.019	-0.023; 0.061
Women	rs744653 – T	<1	1.07	0.63; 1.80	-	**-0.025**	**-0.049; -0.001**	+	-0.011	-0.031; 0.009	+	-0.015	-0.052; 0.021
	rs8177240 – T[#]	?	1.00	0.69; 1.46	?	-0.004	-0.021; 0.014	?	0.007	-0.007; 0.021	?	0.016	-0.011; 0.042
	rs9990333 – T	>1	1.37	0.96; 1.96	+	0.002	-0.015; 0.019	-	-0.003	-0.017; 0.011	-	0.002	-0.024; 0.027
	rs1800562 – A	>1	1.26	0.64; 2.47	+	-0.009	-0.042; 0.024	-	0.010	-0.016; 0.037	-	-0.009	-0.058; 0.040
	rs1799945 – C	<1	0.84	0.53; 1.33	-	-0.004	-0.026; 0.018	+	0.006	-0.012; 0.024	+	0.006	-0.027; 0.039
	rs7385804 – A	>1	1.19	0.82; 1.73	+	0.004	-0.014; 0.021	-	-0.010	-0.025; 0.004	-	-0.003	-0.029; 0.023
	rs4921915 – A[†]	?	**1.76**	**1.10; 2.82**	?	0.010	-0.010; 0.030	?	-0.001	-0.017; 0.015	?	0.000	-0.030; 0.030
	rs651007 – T	<1	0.94	0.60; 1.48	-	**-0.025**	**-0.046; -0.004**	+	-0.001	-0.018; 0.016	+	0.006	-0.026; 0.038
	rs6486121 – T[†]	?	0.93	0.64; 1.37	?	0.006	-0.012; 0.024	?	0.004	-0.011; 0.019	?	0.007	-0.020; 0.035
	rs174577 – A[†]	?	0.93	0.63; 1.37	?	0.006	-0.012; 0.024	?	-0.002	-0.017; 0.013	?	**0.033**	**0.006; 0.060**
	rs411988 – A	<1	0.83	0.58; 1.18	-	-0.002	-0.019; 0.015	+	0.003	-0.011; 0.016	+	-0.002	-0.027; 0.023
	rs855791 – A	<1	**1.79**	**1.24; 2.58**	-	-0.010	-0.027; 0.007	+	0.001	-0.012; 0.015	+	0.002	-0.023; 0.027

Associations were tested using logistic (presence of plaque) and linear regression (IMT and ABI at rest and after exercise). Resulting odds ratios (OR) of logistic models express the effect of each extra tested allele on odds for presence of plaque. Resulting betas of linear models express the effect of each extra tested allele on IMT or ABI.

ABI indicates ankle-brachial index; CI, confidence interval; H, hypothesized effect; IMT, intima media thickness; OR, odds ratio; SNP, single nucleotide polymorphism.

* Hypothesized effect on the NIMA according to the 'iron hypothesis' (see Figure 7.1). Presence of plaque, a higher IMT and a lower ABI indicate presence of atherosclerosis.

[#] This SNP decreases iron and transferrin and increases TS, so the hypothesized effect on atherosclerosis is unknown.

[†] These SNPs only show association with transferrin, so the hypothesized effect on atherosclerosis is unknown.

SUPPLEMENTAL TABLE 7.5 Association of the iron-related SNPs with non-invasive measurements of atherosclerosis adjusted for total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides, and stratified by gender.

		Presence of plaque			IMT			ABI at rest			ABI after exercise		
Gender	SNP – Tested allele	H*	OR	95% CI	H*	Beta	95% CI	H*	Beta	95% CI	H*	Beta	95% CI
Men	rs744653 – T	<1	1.13	0.69; 1.85	-	0.013	-0.012; 0.039	+	-0.009	-0.039; 0.021	+	-0.008	-0.063; 0.047
	rs8177240 – T[#]	?	1.20	0.82; 1.76	?	**-0.020**	**-0.040; 0.000**	?	0.016	-0.008; 0.039	?	**0.044**	**0.002; 0.087**
	rs9990333 – T	>1	1.06	0.73; 1.53	+	0.006	-0.014; 0.025	-	0.013	-0.009; 0.035	-	0.035	-0.005; 0.076
	rs1800562 – A	>1	1.04	0.47; 2.32	+	-0.007	-0.049; 0.035	-	-0.020	-0.068; 0.029	-	0.003	-0.086; 0.092
	rs1799945 – C	<1	**1.60**	**1.00; 2.57**	-	0.020	-0.004; 0.044	+	0.001	-0.027; 0.029	+	-0.016	-0.067; 0.035
	rs7385804 – A	>1	0.72	0.50; 1.03	+	-0.016	-0.035; 0.003	-	0.011	-0.011; 0.033	-	0.038	-0.002; 0.077
	rs4921915 – A[†]	?	1.17	0.74; 1.83	?	**-0.024**	**-0.047; 0.000**	?	0.005	-0.023; 0.032	?	0.013	-0.037; 0.063
	rs651007 – T	<1	0.82	0.52; 1.29	-	-0.009	-0.033; 0.014	+	0.009	-0.018; 0.037	+	**0.061**	**0.011; 0.111**
	rs6486121 – T[†]	?	0.89	0.62; 1.28	?	0.016	-0.003; 0.035	?	-0.009	-0.031; 0.013	?	-0.016	-0.056; 0.024
	rs174577 – A[†]	?	1.26	0.88; 1.82	?	-0.009	-0.028; 0.010	?	0.009	-0.013; 0.031	?	0.001	-0.039; 0.042
	rs411988 – A	<1	0.91	0.64; 1.30	-	-0.005	-0.023; 0.014	+	0.003	-0.019; 0.024	+	-0.019	-0.057; 0.020
	rs855791 – A	<1	0.88	0.60; 1.27	-	-0.001	-0.020; 0.019	+	0.011	-0.012; 0.034	+	0.020	-0.021; 0.061
Women	rs744653 – T	<1	1.12	0.65; 1.90	-	-0.022	-0.046; 0.002	+	-0.013	-0.033; 0.007	+	-0.014	-0.051; 0.023
	rs8177240 – T[#]	?	0.93	0.67; 1.44	?	-0.006	-0.023; 0.012	?	0.007	-0.007; 0.021	?	0.016	-0.010; 0.043
	rs9990333 – T	>1	1.40	0.98; 2.02	+	0.004	-0.012; 0.020	-	-0.003	-0.017; 0.010	-	0.001	-0.024; 0.026
	rs1800562 – A	>1	1.30	0.65; 2.58	+	-0.003	-0.035; 0.029	-	0.007	-0.020; 0.034	-	-0.008	-0.057; 0.042
	rs1799945 – C	<1	0.81	0.51; 1.29	-	-0.003	-0.025; 0.018	+	0.007	-0.011; 0.025	+	0.003	-0.030; 0.036
	rs7385804 – A	>1	1.21	0.83; 1.78	+	0.004	-0.013; 0.021	-	-0.011	-0.026; 0.003	-	-0.001	-0.027; 0.025
	rs4921915 – A[†]	?	**1.74**	**1.08; 2.81**	?	0.002	-0.018; 0.022	?	0.002	-0.015; 0.018	?	-0.001	-0.032; 0.030
	rs651007 – T	<1	0.94	0.59; 1.48	-	**-0.022**	**-0.043; -0.002**	+	-0.001	-0.018; 0.017	+	0.004	-0.028; 0.036
	rs6486121 – T[†]	?	0.91	0.62; 1.35	?	0.006	-0.011; 0.024	?	0.005	-0.010; 0.019	?	0.006	-0.022; 0.033
	rs174577 – A[†]	?	0.91	0.61; 1.35	?	0.009	-0.009; 0.026	?	-0.004	-0.019; 0.011	?	**0.033**	**0.006; 0.061**
	rs411988 – A	<1	0.83	0.58; 1.19	-	-0.002	-0.018; 0.014	+	0.003	-0.011; 0.016	+	-0.002	-0.027; 0.023
	rs855791 – A	<1	**1.75**	**1.20; 2.53**	-	-0.013	-0.030; 0.003	+	0.001	-0.013; 0.015	+	0.001	-0.024; 0.026

Associations were tested using logistic (presence of plaque) and linear regression (IMT and ABI at rest and after exercise). Resulting odds ratios (OR) of logistic models express the effect of each extra tested allele on odds for presence of plaque. Resulting betas of linear models express the effect of each extra tested allele on IMT or ABI.

ABI indicates ankle-brachial index; CI, confidence interval; H, hypothesized effect; IMT, intima media thickness; OR, odds ratio; SNP, single nucleotide polymorphism.

* Hypothesized effect on the NIMA according to the 'iron hypothesis' (see Figure 7.1). Presence of plaque, a higher IMT and a lower ABI indicate presence of atherosclerosis.

[#] This SNP decreases iron and transferrin and increases TS, so the hypothesized effect on atherosclerosis is unknown.

[†] These SNPs only show association with transferrin, so the hypothesized effect on atherosclerosis is unknown.

SUPPLEMENTAL TABLE 7.6 Associations of NIMA-related SNPs with hepcidin and iron parameters. Nominally significant associations are indicated in bold.

Source	Trait	SNP	Nearest gene	Effect (beta or OR) [SE or 95% CI]	P-value	Trait	Tested allele	H*	Effect	SE	P	N
Bis *et al.*, 2011	Carotid IMT	rs11781551-A	*ZHX2*	-0.0078 [0.0012]	2.4E-11	hepcidin	A	-	-0.03	0.03	0.33	1800
						hepcidin/ferritin	**A**	**-**	**-0.06**	**0.03**	**0.03**	**1794**
						hepcidin/TS	A	-	-0.02	0.03	0.46	1780
						iron	A	-	0.02	0.03	0.60	1790
						TIBC	A	+	-0.02	0.03	0.59	1787
						TS	A	-	0.04	0.03	0.29	1786
						ferritin	A	-	0.00	0.03	1.00	1809
	Carotid IMT	rs445925-A	*APOC1*	-0.0156 [0.0028]	1.7E-08	hepcidin	G	+	0.02	0.06	0.69	1800
						hepcidin/ferritin	G	+	0.11	0.06	0.06	1794
						hepcidin/TS	G	+	0.02	0.06	0.78	1780
						iron	G	+	-0.02	0.06	0.78	1790
						TIBC	G	-	0.04	0.06	0.46	1787
						TS	G	+	-0.05	0.06	0.37	1786
						ferritin	G	+	0.03	0.06	0.58	1809
	Carotid IMT	rs6601530-G	*PINX1*	0.0078 [0.0014]	1.7E-08	hepcidin	G	+	-0.02	0.03	0.50	1800
						hepcidin/ferritin	G	+	-0.01	0.03	0.77	1794
						hepcidin/TS	G	+	-0.02	0.03	0.48	1780
						iron	G	+	0.02	0.03	0.66	1790
						TIBC	G	-	0.03	0.03	0.43	1787
						TS	G	+	0.02	0.03	0.63	1786
						ferritin	G	+	0.02	0.03	0.64	1809
	Presence of plaque	rs17398575-A	*PIK3CG*	1.18 [1.12-1.23]	2.3E-12	hepcidin	A	+	0.03	0.04	0.52	1800
						hepcidin/ferritin	A	+	0.03	0.04	0.47	1794
						hepcidin/TS	A	+	0.01	0.04	0.71	1780
						iron	A	+	-0.01	0.04	0.80	1790
						TIBC	A	-	-0.01	0.04	0.85	1787
						TS	A	+	0.00	0.04	0.92	1786
						ferritin	A	+	0.00	0.04	0.99	1809
	Presence of plaque	rs1878406-T	*EDNRA*	1.22 [1.15-1.29]	6.9E-12	hepcidin	T	+	0.03	0.05	0.60	1800
						hepcidin/ferritin	T	+	0.03	0.05	0.58	1794
						hepcidin/TS	T	+	0.03	0.05	0.58	1780
						iron	T	+	0.08	0.05	0.09	1790
						TIBC	T	-	0.01	0.05	0.91	1787
						TS	T	+	0.05	0.05	0.35	1786
						ferritin	T	+	0.01	0.05	0.88	1809
Murabito *et al.*, 2012	ABI	rs10757269-G	*CDKN2B*	-0.0049 [0.0008]	2.7E-09	hepcidin	G	+	0.01	0.03	0.70	1800
						hepcidin/ferritin	**G**	**+**	**0.07**	**0.03**	**0.03**	**1794**
						hepcidin/TS	G	+	0.01	0.03	0.67	1780
						iron	G	+	0.02	0.03	0.56	1790
						TIBC	G	-	0.02	0.03	0.81	1787
						TS	G	+	0.03	0.03	0.46	1786
						ferritin	G	+	0.05	0.03	0.15	1809

Associations were tested using linear regression. Resulting betas express the effect of each extra tested allele standardized residuals of hepcidin, hepcidin ratios and the iron parameters.

ABI indicates ankle-brachial index; CI, confidence interval; H, hypothesized effect; IMT, intima media thickness; OR, odds ratio; SE, standard error; SNP, single nucleotide polymorphism.

* Hypothesized effect on hepcidin, hepcidin ratios and the iron parameters according to the 'iron hypothesis'. Presence of plaque, a higher IMT and a lower ABI indicate presence of atherosclerosis.

Supplemental references

1 Bis JC, Kavousi M, Franceschini N, et al. Meta-analysis of genome-wide association studies from the CHARGE consortium identifies common variants associated with carotid intima media thickness and plaque. Nat Genet 2011;43:940-7.

2 Murabito JM, White CC, Kavousi M, et al. Association between chromosome 9p21 variants and the ankle-brachial index identified by a meta-analysis of 21 genome-wide association studies. Circ Cardiovasc Genet 2012;5:100-12.

CHAPTER 8

General discussion

This research project started almost a decade after the discovery of hepcidin [1,2], when knowledge on hepcidin function and regulation was mainly based on *in vitro* and animal experiments. There were only a few studies on hepcidin in humans; these were based on relatively small groups of healthy subjects or patients, the latter mostly in the context of iron disorders [3]. In this thesis, we presented population-based studies on serum hepcidin concentrations. We 1) aimed to identify biochemical correlates and genetic determinants of serum hepcidin, and 2) studied the effects of hepcidin on atherosclerosis as determined by non-invasive measurements of atherosclerosis (NIMA). In this chapter, we describe the inferences from our studies together with directions for future research.

Serum hepcidin concentrations should be evaluated in the context of serum ferritin

Based on our study of associations of serum hepcidin with a selection of (biochemical) parameters (**chapter 2**), serum ferritin appeared to be the strongest correlate of serum hepcidin concentration. This association was robust to adjustment for other iron parameters and biochemical variables that we tested, *e.g.* C-reactive protein (CRP), body-mass index (BMI), and age. The other iron parameters showed far less strong associations with serum hepcidin.

The strong association that we found between hepcidin and ferritin was reported before; correlation estimates based on studies in smaller populations and without adjustment for other biochemical variables varied between 0.63 and 0.76 [4-6]. The correlation was later also confirmed in the Italian Val Borbera population, although less strong with estimates of 0.32 and 0.53 for men and women, respectively [7]. The question remains whether the relation between serum hepcidin and ferritin is causal, and if so, whether it reflects a response of hepcidin to body iron stores [8,9] or vice versa, the response of ferritin to ferroportin degradation by hepcidin [10].

Several observations indicate that it is the *balance* between these processes, reflected by the hepcidin/ferritin ratio, that may be of utmost importance in view of human health and disease. For example, in patients with hereditary hemochromatosis (HH) due to homozygosity for C282Y, hepcidin concentrations are only slightly lower than in controls [11-13], but the ratio

hepcidin/ferritin is clearly decreased compared to controls [12,13]. Also, the ratio hepcidin/ferritin was demonstrated to be a potential biomarker for cirrhosis [14]; it was significantly lower in chronic liver disease patients compared with healthy controls, whereas serum hepcidin was not. Furthermore, the ratio hepcidin/ferritin declined progressively with increasing fibrosis in chronic liver disease patients, and was shown to be a significant and independent predictor of cirrhosis [14]. In addition, the ratio hepcidin/ferritin was suggested to be helpful in distinguishing dysmetabolic hyperferritinemia/dysmetabolic iron overload syndrome from other iron overload disorders, in which hepcidin is inappropriately low [15]. Finally, evidence for the importance of evaluation of serum hepcidin concentrations relative to other (iron) parameters is also provided by an increased value for the ratio of hepcidin to TS in the diagnosis of iron-refractory iron deficiency anemia (IRIDA) due to defects in the *TMPRSS6* gene in the absence of inflammation [16].

The importance of the ratio hepcidin/ferritin is also supported by our findings in **chapters 6 and 7**. The ratio hepcidin/ferritin showed statistically significant associations with presence of plaque in women, the ankle-brachial index (ABI) at rest in men and women, and ABI after exercise in women, whereas hepcidin was only statistically significantly associated with presence of plaque in women (**chapter 6**). Finally, the ratio hepcidin/ferritin was the only variable that showed nominally significant associations with NIMA-related SNPs (**chapter 7**).

In view of the increased insights into the potential importance of the ratio hepcidin/ferritin, we provided reference ranges for the ratio hepcidin/ferritin in men and pre- and postmenopausal women on the website www.hepcidinanalysis.com, in addition to the reference ranges for serum hepcidin that we provided in **chapter 2**. It is important to note that these reference ranges are assay-specific. A recent effort to harmonize the different hepcidin assays resulted in the construction of hepcidin consensus (HEPCON1) values, which allow cross-method comparison of absolute hepcidin values [17]. Furthermore, we included the ratio hepcidin/ferritin in subsequent association studies (**chapter 3 and 4**) and would advise future studies on iron parameters and serum hepcidin concentration to do the same. If the ratio hepcidin/ferritin proves to be of importance in future studies on human health and disease, we recommend to investigate which factors influence the ratio, like we did for serum hepcidin in **chapter 2**.

Neither a candidate gene approach nor a meta-analysis of GWAS based on all cohorts available worldwide resulted in identification of common genetic variants associated with serum hepcidin yet: the search should continue

We used two approaches to search for common variants associated with serum hepcidin. In a candidate-gene study, we focused on the most important genetic variants of two very strong candidate genes, *i.e.* rs1800562 in *HFE* and rs855791 in *TMPRSS6*. SNP rs855791 was previously found to be strongly associated with serum iron, transferrin, and transferrin saturation (TS); rs1800562 with these three parameters and serum ferritin [18-22]. It was common-

ly thought that these associations could be explained by an intermediate effect on serum hepcidin based on knowledge from knockout mice models and mutations in patients [13, 23-25]. However, both Traglia *et al.* [7] and our group unexpectedly did not observe associations of these variants with serum hepcidin, but only with the ratio hepcidin/ferritin and to a lesser extent with the ratio hepcidin/TS. Adjustment for hepcidin or the ratio hepcidin/ferritin did not decrease the strength of the SNP–iron parameter associations. This indicates that rs1800562 and rs855791 influence iron uptake and/or export via an unknown mechanism, which could potentially involve a different, and currently unidentified, intermediate factor than hepcidin. The fact that ferroportin is found in vertebrates and hepcidin is not [26,27], provides support for a hepcidin-independent mechanism. Notably, the new insights that our study and that of Traglia provided, in addition to the knowledge based on laboratory experiments and patient series, underscores the added value of findings in (replicated) population-based studies. It also emphasizes the importance of candidate-gene studies to learn more about mechanisms behind SNP associations. Hence, we recommend to zoom in on associations of iron-related genes with hepcidin and the ratio hepcidin/ferritin in future candidate-gene approaches.

In addition to the candidate-gene study, we performed a meta-analysis of genome-wide association studies (meta-GWAS) to search for genetic determinants of serum hepcidin and its ratios in a hypothesis-free manner. Our meta-GWAS included all currently available cohorts worldwide that have both hepcidin measurements, iron parameters and genotype data for their samples. This resulted in a sample of over 6,000 individuals. It provided us with 80% power to discover variants that explain 0.62% and 0.78% of hepcidin variation, assuming respectively perfect LD between the SNP marker and the causal variant (best case scenario) and an imperfect LD of r^2=0.8 [28]. Still, our meta-GWAS yielded only one potential locus that affects serum hepcidin concentrations. This suggests that the heritability of hepcidin cannot be explained by common genetic variants with a moderate to large effect, but rather by common variants with small effects and/or rare variants with large or small effects. Also, we did not find novel genome-wide statistically significant loci for the ratio hepcidin/ferritin. Replication of the most significant novel top findings for the ratio hepcidin/ferritin, as we initiated for serum hepcidin, should be considered for future studies.

As discovery of common genetic variants for serum hepcidin and its ratio to ferritin is relevant for elucidation of biology and identification of biomarkers and therapeutic targets, performance of more powerful GWAS is valuable. There are several possible approaches to increase the power to identify these variants via GWAS. The first option is to get more out of the currently available data by application of a multivariate (MV-) GWAS on serum hepcidin and (a selection of) the iron parameters. We invested in a methodological study comparing a selection of MV-GWAS methods (**chapter 5**), which showed that MV-GWAS can provide additional power compared to univariate analyses, even when the genetic correlations between traits are weak. The iron parameters are measured in all three cohorts that are in-

cluded in the current meta-GWAS on serum hepcidin. It is thus relatively easy and cheap to perform a MV-GWAS in the three cohorts and combine results in a meta-analysis, as we did for the univariate GWAS. Interpretation of these multivariate association results, though, is not straightforward: a multivariate association can be driven by one of the traits, a subset of the traits, or even by all traits. New signals from a MV analysis should therefore be studied for association with the individual traits, and interesting signals should be replicated in independent samples using both multivariate and univariate analysis.

The second option would be to increase the sample size of the current meta-analysis. The development of an affordable and precise high-throughput method for serum hepcidin measurement, as offered by our recently developed ELISA mimetic assay [29], will facilitate the addition of a substantial number of extra samples.

Also, power could be gained by reducing non-genetic sources of variation of serum hepcidin concentration. Pre-analytical variation could be decreased by reducing the time between blood sampling and freezing of the sample, and by minimizing the number of freeze-thaw steps and pipetting steps. Day-to-day differences within individuals are large and were shown to range from 3.9% to 15.9% [30]. To reduce this biological variation, it could be considered to perform multiple hepcidin measurements a few days in a row for every participant. As hepcidin has a circadian rhythm (**chapter 2** and [31,32]), these measurements should be performed approximately at the same time every day. Finally, variation in hepcidin concentration could be reduced by adjustment for non-genetic determinants of serum hepcidin or performance of analyses in homogeneous subsets. We included age, gender and time of blood sampling in our analyses (**chapters 3, 4, 6 and 7**), and excluded participants from analyses as to reduce the influence of inflammation (C-reactive protein ≥10 mg/L) and iron deficiency (ferritin <30ng/mL) (**chapter 3 and 4**), but other factors, *e.g.* alcohol consumption [33], and pregnancy [34], could be included as well. The potential gain in power by analyzing a subset, despite the decrease in sample size, was demonstrated by the smaller p-values found for rs354202 in the subset-analysis of our meta-GWAS (**chapter 4**).

Low frequency (1-5% frequency) and rare (<1% frequency) genetic variants are not (or poorly) captured by the genome-wide SNP arrays due to their low correlation with common SNP markers present on these chips. Application of newer techniques such as exome or whole-genome sequencing will enable actual measurement of uncommon single nucleotide and structural variants and assessment of their association with serum hepcidin and iron parameters. These rare variants are expected to have relatively large effects, and might therefore be present in individuals in the tails of the hepcidin distribution, *i.e.* among individuals with the highest and lowest hepcidin concentrations. Identification of these rare variants might therefore not only be useful to increase insight into hepcidin regulatory mechanisms, but may also be useful for future selection of individuals that could profit from (preventive) hepcidin-targeting therapies. Sequencing data could also help to pinpoint the

causal variant of a region identified by GWAS, whether common or rare. However, application of sequencing techniques in large cohorts is currently still very expensive, and there are big challenges associated with data storage, quality control, and analysis [35]. To efficiently identify associations with rare variants on short term, it will be more feasible to apply so-called exome-chips, which allow the measurement of over 200k low-frequency and rare genetic variants in the human exome. Downside of this chip is that the non-coding part of the DNA is ignored, and that it may not include population-specific variants, as selection of the variants on the chip was based on their occurrence in a set of ~12,000 sequenced genomes from both the general population and case series.

Our studies indicate that the body iron distribution as determined by serum hepcidin and the ratio hepcidin/ferritin may affect the development of atherosclerosis, but further studies are needed before hepcidin or its ratio to ferritin could be considered as therapeutic target for cardiovascular disease

Our studies into the roles of hepcidin and iron parameters in atherosclerosis (**chapter 6 and 7**) are to our knowledge the first that are based on data in an unselected general population, and are complementary to the work that has been done using patient series and *in vitro* and animal models. Results of our studies suggest that it is not the absolute amount of body iron that increases the risk of atherosclerosis, which is in contrast to the original 'iron hypothesis' proposed by Sullivan in 1981 [36], but rather the body iron distribution as determined by serum hepcidin and its ratio to ferritin. This confirms a publication by Sullivan in 2007, in which he stated an additional mechanism by which iron depletion could protect against atherosclerotic lesion progression involving hepcidin [37]. It provides also an explanation for the fact that HH patients with iron overload do not show an increased incidence of atherosclerosis, as their hepcidin levels are relatively low, resulting in decreased retention of iron by macrophages [38].

Our results are in agreement with findings on the role of hepcidin in patient populations [15, 39-47] and results from *in vitro* and mice studies [48-50], but are discrepant with two studies [51,52] (summarized in Table 8.1). Notably, the reliability of some of the hepcidin assays used in these studies is doubtful (indicated in Table 8.1), as full validation reports are not available. The role of the ratio hepcidin/ferritin in atherosclerosis was not investigated before.

The first discrepant study is a case-control study (N=75) from 2006 in which serum hepcidin levels were measured by a commercially available prohepcidin ELISA kit and compared between healthy controls and patients who had previously experienced an atherosclerotic event [52]. Prohepcidin levels among both groups were similar, but potential confounding factors were not taken into account. More importantly, prohepcidin levels do not correlate with serum hepcidin, neither do they respond to relevant physiological stimuli, nor is prohepcidin able to degrade ferroportin [53-56]. Thus, this study does in fact not contribute any evidence against a role of serum hepcidin in atherosclerosis.

The second discrepant study is a recently published mouse study by Kautz *et al*., which argued against a significant role of macrophage iron in atherosclerosis progression [51]. Results of the study by Kautz *et al*. indicate that increased macrophage iron as a result of a ferroportin mutation, which replicates the effect of increased hepcidin concentration, does not promote atherosclerosis. They support their results by stating that macrophages are highly resistant to iron-induced damage, and that systemic inflammation is not an invariant feature of atherosclerosis [51]. Nevertheless, their findings do not corroborate with results from two experimental studies based on a similar mouse model by Saeed *et al*. and Li *et al*. [48,50]. Kautz *et al*. explain their discrepant findings with the study by Saeed *et al*. by suggesting that the effect of macrophage iron on atherosclerosis could be so small that contradictory conclusions might be reached due to differences in study design. Kautz *et al*. do not mention the study by Li *et al*. The latter study found that hepcidin overexpression did not change plaque size, which corroborates results by Kautz *et al*., but rather plaque composition, increasing plaque destabilization, which was not studied by Kautz *et al*.

TABLE 8.1 Summary of studies that investigated the (causal) role of hepcidin in atherosclerosis.

Type of study	Source	Evidence for a (causal) role of hepcidin in atherosclerosis	Evidence against a (causal) role of hepcidin in atherosclerosis
In vitro studies	Finn *et al.*, 2012 [49]	Hepcidin increased the intracellular iron content of human monocytes derived from macrophages, resulting in increased reactive oxygen species and decreased cholesterol efflux	
	Li *et al.*, 2012 [50]*	The interaction of hepcidin, trapped iron, and accumulated lipids was critical for proatherosclerotic activation of macrophages, contributing to plaque destabilization	
Animal studies	Saeed *et al.*, 2012 [48]	Systemic pharmacological suppression of hepcidin increased macrophage-specific expression of cholesterol efflux transporters and attenuated atherosclerosis	
	Li *et al.*, 2012 [50]*	- Hepcidin was upregulated in carotid plaques in ApoE$^{-/-}$ mice - Hepcidin overexpression did not alter plaque size, but increased plaque destabilization by affecting plaque composition (enhanced intraplaque macrophage infiltration, and suppression of the contents of collagen and vascular smooth muscle cells), and increased oxidized LDL in intraplaque macrophages	
	Kautz *et al.*, 2013 [51]		- Hepcidin was not increased in a standard mouse model of atherosclerosis (ApoE$^{-/-}$ mice) - Increased macrophage iron, achieved by a mutation in the iron exporter ferroportin or through parenteral iron administration, did not increase size of atherosclerotic lesions
Studies in patients	Malyszko *et al.*, 2006 [46]*	Serum hepcidin levels were higher in kidney transplant recipients with versus without coronary artery disease	
	Valenti *et al.*, 2011 [41]	Serum hepcidin and macrophage iron levels correlated with monocyte chemoattractant protein-1 release and vascular damage in patients with metabolic syndrome	
	Valenti *et al.*, 2011 [42]	Hepcidin-25 was independently associated with carotid plaques in patients with nonalcoholic fatty liver disease	
	Kuragano *et al.*, 2011 [39]	Serum hepcidin levels were associated with arterial stiffness in patients on maintenance hemodialysis	
	Abdel-Khalek *et al.*, 2011 [47]*	Serum hepcidin was statistically significantly and positively correlated with coronary calcium score as marker for plaque in patients with rheumatoid arthritis, independent of inflammatory markers	No significant correlation was observed between serum hepcidin levels and lipid profile
	Van der Weerd *et al.*, 2013 [40]	Hepcidin-25 levels were associated with fatal and non-fatal cardiovascular events, even after adjustment for inflammation, in haemodialysis patients	
	Ulu *et al.*, 2014 [44]*	Serum hepcidin was associated with arterial stiffness in patients on continuous ambulatory peritoneal dialysis	
	Samouilidou *et al.*, 2014 [45]*	Serum hepcidin levels were higher in patients with chronic kidney disease on hemodialysis and peritoneal dialysis, and were significantly related to triglycerides (positive correlation) and high-density lipoprotein cholesterol (negative correlation)	
Studies in cases and controls	Oguz *et al.*, 2006 [52]#		Serum hepcidin levels were similar in 40 patients who had previously experienced an atherosclerotic event as in 19 healthy subjects
	Kroot *et al.*, 2011 [43]	Total hepcidin and hepcidin-25 levels were significantly higher in subjects with metabolic syndrome than in age-matched controls in a nested case-control study in the Nijmegen Biomedical Study	
	Martinelli *et al.*, 2012 [15]	Hepcidin increased significantly and linearly with increasing number of metabolic syndrome features	

TABLE 8.1 (CONTINUED) Summary of studies that investigated the (causal) role of hepcidin in atherosclerosis.

Type of study	Source	Evidence for a (causal) role of hepcidin in atherosclerosis	Evidence against a (causal) role of hepcidin in atherosclerosis
Studies in an unselected population	Galesloot *et al.*, 2013 and 2014 (chapter 6 and 7)	- Hepcidin and the hepcidin/ferritin ratio significantly increased the risk of presence of plaque in women - The hepcidin/ferritin ratio was significantly and negatively associated with ankle-brachial index at rest in men and women, which corresponds to an increased risk of atherosclerosis - Two of six NIMA-related SNPs were nominally statistically significantly associated with the ratio hepcidin/ferritin, corresponding to an increased risk of atherosclerosis - Hepcidin showed a moderate negative genetic correlation with ABI at rest and ABI after exercise, corresponding to an increased risk of atherosclerosis, although the correlations were insignificant	- Hepcidin and the ratio hepcidin/ferritin were not associated with IMT in men and women, not with ABI at rest and after exercise in women, and not with presence of plaque and ABI at rest and after exercise in men - Hepcidin was not associated with all NIMA-related SNPs and the ratio hepcidin/ferritin was not associated with four of six NIMA-related SNPs - Genomic correlations of hepcidin with presence of plaque and IMT were ~0; genomic correlations of the ratio hepcidin/ferritin with all NIMA were ~0

ABI indicates ankle-brachial index; IMT, intima-media thickness; NIMA, non-invasive measurements of atherosclerosis.

* It is doubtful whether the hepcidin assays used in these studies are reliable, because full validation reports are not available.

\# This study measured serum prohepcidin levels instead of bioactive hepcidin-25. Prohepcidin levels do not correlate with serum hepcidin, neither do they respond to relevant physiological stimuli, nor is prohepcidin able to degrade ferroportin [53-56].

The evidence for a (causal) role of hepcidin and the ratio hepcidin/ferritin in atherosclerosis in the general population is not very strong. Although we had the availability of a unique dataset containing data on both hepcidin concentration and NIMA and also genotypes for a subset of this group, our sample size was limited. This led to suboptimal power, resulting in wide confidence intervals for the associations, genomic correlations and SNP effects estimated in **chapter 6 and 7**. Furthermore, we observed inconsistent associations of hepcidin and the hepcidin ratios for the different NIMA (**chapter 6**), that each reflect the presence of atherosclerosis. In addition, we were unable to study causal effects of hepcidin on NIMA by an MR approach due to lack of genetic determinants for hepcidin (**chapter 4**). The 12 genetic variants associated with iron status that we used for our MR, were not perfect as proxies for the different iron parameters due to their pleiotropic character, *i.e.* they influence more than one iron parameter, or even show association with other, potentially confounding, traits. Four of the 12 SNPs have been reported for association with lipid traits, which are known to be associated with atherosclerosis, but including the lipid traits as covariates in our MR analyses did not change the conclusions based on unadjusted results. Finally, the estimates of genomic correlations and cross-trait associations of NIMA-related SNPs with hepcidin and iron parameters provided additional evidence for a role of hepcidin and ferritin in atherosclerosis, but the resulting associations and correlations do however not necessarily reflect causal relations, as they might also be a result of pleiotropy.

Taken together, our studies combined with results from previous efforts still provide only limited evidence for a causal role of hepcidin and the ratio hepcidin/ferritin in atherosclerosis (Table 8.1). To increase knowledge on the hepcidin-atherosclerosis relation in humans, we first of all call for studies that replicate our findings in independent populations with a larger sample size. These studies could be improved by relying on multiple serum hepcidin measurements over time. We also recommend to extend our MR analysis with genetic determinants for serum hepcidin and the ratio hepcidin/ferritin. This is dependent on actually finding variants and their suitability for disentangling causal effects of hepcidin and its ratio to ferritin versus effects of the iron parameters. The variants should either specifically associate with hepcidin or the ratio hepcidin/ferritin and not with the iron parameters, or they should influence these traits in an opposite direction. Application of a MV-GWAS could be used in order to find the latter type of variant, especially by using the methods MV-PLINK, MV-SNPtest, MV-BIMBAM and MultiPhen, as we found that these methods show a notable increase in power for scenarios with a positive residual and negative genetic correlation (**chapter 5**). Furthermore, future MR studies could consider application of a factorial MR (Burgess *et al*., submitted), which enables estimation of the causal effect of a specific risk factor using pleiotropic genetic variants. This could be used to estimate the causal effects of the individual iron parameters using the current set of 12 mostly pleiotropic SNPs, which we were unable to do due to our limited sample size and resulting extremely wide confidence intervals. Finally, we recommend to study the association of hepcidin and the iron parameters with the hard(er) endpoint of clinical disease, *i.e.* occurrence of a cardiovascular event or coronary heart disease.

Besides population-based studies, we warrant additional functional studies to test for causality of the role of hepcidin and the ratio hepcidin/ferritin in atherosclerosis in an experimental setting. These studies could investigate effects of hepcidin agonists, compounds that mimic the function of hepcidin or stimulate its endogenous synthesis, and hepcidin antagonists, which decrease hepcidin production or block its function [57-59]. There are currently several efforts ongoing to develop these compounds, and to test them in preclinical studies in the context of iron disorders [58,59]. Some of them have even been moved forward to phase I and II clinical trials in the context of cancer-related anemias [58]. These studies might also differentiate between systemic hepcidin levels, *i.e.* serum hepcidin levels, and local cell or tissue-specific concentrations of hepcidin, which might be of importance in the context of atherosclerotic plaques, as also mentioned by Kautz *et al.* [51].

References

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

2 Krause A, Neitz S, Magert HJ, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147-50.

3 Piperno A, Mariani R, Trombini P, et al. Hepcidin modulation in human diseases: from research to clinic. World J Gastroenterol 2009;15:538-51.

4 Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.

5 Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. Blood 2008;112:4292-7.

6 Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int 2009;75:976-81.

7 Traglia M, Girelli D, Biino G, et al. 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.

8 Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: regulation of Mammalian iron metabolism. Cell 2010;142:24-38.

9 Fleming RE, Ponka P. Iron overload in human disease. N Engl J Med 2012;366:348-59.

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

11 Bridle KR, Frazer DM, Wilkins SJ, et al. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 2003;361:669-73.

12 Piperno A, Girelli D, Nemeth E, et al. Blunted hepcidin response to oral iron challenge in HFE-related hemochromatosis. Blood 2007;110:4096-100.

13 van Dijk BA, Laarakkers CM, Klaver SM, et al. 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;142:979-85.

14 Tan TC, Crawford DH, Franklin ME, et al. The serum hepcidin:ferritin ratio is a potential biomarker for cirrhosis. Liver Int 2012;32:1391-9.

15 Martinelli N, Traglia M, Campostrini N, et al. Increased serum hepcidin levels in subjects with the metabolic syndrome: a population study. PLoS One 2012;7:e48250.

16 Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. Blood 2014;123:3873-86.

17 Kroot JJ, van Herwaarden AE, Tjalsma H, et al. Second round robin for plasma hepcidin methods: first steps toward harmonization. Am J Hematol 2012;87:977-83.

18 Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nat Genet 2009;41:1173-5.

19 Benyamin B, McRae AF, Zhu G, et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. Am J Hum Genet 2009;84:60-5.
20 Middelberg RP, Ferreira MA, Henders AK, et al. Genetic variants in LPL, OASL and TOMM40/APOE-C1-C2-C4 genes are associated with multiple cardiovascular-related traits. BMC Med Genet 2011;12:123.
21 Pichler I, Minelli C, Sanna S, et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. Hum Mol Genet 2011;20:1232-40.
22 Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. Blood 2010;115:94-6.
23 Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. Science 2008;320:1088-92.
24 Ahmad KA, Ahmann JR, Migas MC, et al. Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 2002;29:361-6.
25 Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008;40:569-71.
26 Hilton KB, Lambert LA. Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene 2008;415:40-8.
27 Ganz T. Systemic iron homeostasis. Physiol Rev 2013;93:1721-41.
28 Feng S, Wang S, Chen CC, et al. GWAPower: a statistical power calculation software for genome-wide association studies with quantitative traits. BMC Genet 2011;12:12.
29 Grebenchtchikov N, Geurts-Moespot AJ, Trentmann S, et al. Engineered Human Lipocalin as an Antibody Mimetic: Application to Analysis of the Small Peptide Hormone Hepcidin. Clin Chem 2014;60:897-9.
30 Murphy AT, Witcher DR, Luan P, et al. Quantitation of hepcidin from human and mouse serum using liquid chromatography tandem mass spectrometry. Blood 2007;110:1048-54.
31 Schaap CC, Hendriks JC, Kortman GA, et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. Clin Chem 2013;59:527-35.
32 Kemna EH, Tjalsma H, Podust VN, et al. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. Clin Chem 2007;53:620-8.
33 Bridle K, Cheung TK, Murphy T, et al. Hepcidin is down-regulated in alcoholic liver injury: implications for the pathogenesis of alcoholic liver disease. Alcohol Clin Exp Res 2006;30:106-12.
34 van Santen S, Kroot JJ, Zijderveld G, et al. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. Clin Chem Lab Med 2013;51:1395-401.
35 Bush WS, Moore JH. Chapter 11: Genome-wide association studies. PLoS Comput Biol 2012;8:e1002822.
36 Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1:1293-4.

37 Sullivan JL. Macrophage iron, hepcidin, and atherosclerotic plaque stability. Exp Biol Med (Maywood) 2007;232:1014-20.
38 Sullivan JL. Do hemochromatosis mutations protect against iron-mediated atherogenesis? Circ Cardiovasc Genet 2009;2:652-7.
39 Kuragano T, Itoh K, Shimonaka Y, et al. Hepcidin as well as TNF-alpha are significant predictors of arterial stiffness in patients on maintenance hemodialysis. Nephrol Dial Transplant 2011;26:2663-7.
40 van der Weerd NC, Grooteman MP, Bots ML, et al. Hepcidin-25 is related to cardiovascular events in chronic haemodialysis patients. Nephrol Dial Transplant 2013;28:3062-71.
41 Valenti L, Dongiovanni P, Motta BM, et al. Serum hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in patients with metabolic syndrome alterations. Arterioscler Thromb Vasc Biol 2011;31:683-90.
42 Valenti L, Swinkels DW, Burdick L, et al. Serum ferritin levels are associated with vascular damage in patients with nonalcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2011;21:568-75.
43 Kroot JJ, van Dijk BAC, Laarakkers CMM, et al. Serum hepcidin levels are elevated in the metabolic syndrome. Arterioscler Thromb Vasc Biol Published online April 29, 2011.
44 Ulu SM, Yuksel S, Altuntas A, et al. Associations between serum hepcidin level, FGF-21 level and oxidative stress with arterial stiffness in CAPD patients. Int Urol Nephrol 2014.
45 Samouilidou E, Pantelias K, Petras D, et al. Serum hepcidin levels are associated with serum triglycerides and interleukin-6 concentrations in patients with end-stage renal disease. Ther Apher Dial 2014;18:279-83.
46 Malyszko J, Malyszko JS, Pawlak K, et al. Hepcidin, an acute-phase protein and a marker of inflammation in kidney transplant recipients with and without coronary artery disease. Transplant Proc 2006;38:2895-8.
47 Abdel-Khalek MA, El-Barbary AM, Essa SA, et al. Serum hepcidin: a direct link between anemia of inflammation and coronary artery atherosclerosis in patients with rheumatoid arthritis. J Rheumatol 2011;38:2153-9.
48 Saeed O, Otsuka F, Polavarapu R, et al. Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler Thromb Vasc Biol 2012;32:299-307.
49 Finn AV, Nakano M, Polavarapu R, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 2012;59:166-77.
50 Li JJ, Meng X, Si HP, et al. Hepcidin destabilizes atherosclerotic plaque via overactivating macrophages after erythrophagocytosis. Arterioscler Thromb Vasc Biol 2012;32:1158-66.
51 Kautz L, Gabayan V, Wang X, et al. Testing the iron hypothesis in a mouse model of atherosclerosis. Cell Rep 2013;5:1436-42.
52 Oguz A, Uzunlulu M, Hekim N. Hepcidin is not a marker of chronic inflammation in atherosclerosis. Anadolu Kardiyol Derg 2006;6:239-42.

53 Kemna EHJM, Kartikasari AER, van Tits LJH, et al. Regulation of hepcidin: Insights from biochemical analyses on human serum samples. Blood Cells Mol Dis 2008;40:339-46.
54 Kemna E, Pickkers P, Nemeth E, et al. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood 2005;106:1864-6
55 Roe MA, Spinks C, Heath AL, et al. Serum prohepcidin concentration: no association with iron absorption in healthy men; and no relationship with iron status in men carrying HFE mutations, hereditary haemochromatosis patients undergoing phlebotomy treatment, or pregnant women. Br J Nutr 2007;97:544-9.
56 Gagliardo B, Kubat N, Faye A, et al. Pro-hepcidin is unable to degrade the iron exporter ferroportin unless maturated by a furin-dependent process. J Hepatol 2009;50:394-401.
57 Kroot JJ, Tjalsma H, Fleming RE, et al. Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-69.
58 Ruchala P, Nemeth E. The pathophysiology and pharmacology of hepcidin. Trends Pharmacol Sci 2014;35:155-61.
59 Fung E, Nemeth E. Manipulation of the hepcidin pathway for therapeutic purposes. Haematologica 2013;98:1667-76.

Summary

Hepcidin is the key molecule of systemic iron homeostasis. It is known to regulate the amount of circulating iron by controlling two processes: (i) the uptake of iron from the intestine, and (ii) the release of iron from macrophages that recycle iron from senescent erythrocytes. Hepcidin thus determines body iron distribution, *i.e.* an increased serum hepcidin concentration leads to a decrease in serum iron and an increase in the amount of iron trapped inside the reticulo-endothelial macrophages, and vice versa. Hepcidin is involved in various iron disorders, with hepcidin deficiency leading to iron overload and hepcidin excess resulting in low circulating iron and consequently iron-restricted anemia. Hepcidin has also been associated with iron disorders related to other diseases, *e.g.* chronic kidney disease, cancer, and the metabolic syndrome. It is also suggested to play a role in the development of atherosclerosis, finally leading to cardiovascular disease (CVD). Manipulation of hepcidin concentrations is thus potentially useful in clinical practice, and an improved understanding of hepcidin regulation will contribute to the potential of using hepcidin as a therapeutic target.

Although hepcidin was discovered more than 10 years ago, at the beginning of the 21st century, our understanding of regulation of hepcidin production is still incomplete. Most studies focused on *in vitro* or animal models, and only a few explored hepcidin concentrations in patient groups or small series of healthy individuals. In this thesis, we focused on serum hepcidin concentrations in the general population and aimed to identify biochemical correlates and genetic determinants of serum hepcidin using epidemiological studies (Part I). Secondly, we investigated hepcidin as a potential determinant of atherosclerosis and aimed to elucidate its effect on non-invasive measurements of atherosclerosis (NIMA) (Part II).

Chapter 1 provides a general introduction on this thesis. It includes an overview of the current knowledge on hepcidin regulation and of the suggested relation between iron, hepcidin and atherosclerosis. The objectives and outline of this thesis are given as well as a description of the Nijmegen Biomedical Study (NBS), since all studies in this thesis are based on data from this unselected sample of the inhabitants of the municipality of Nijmegen.

PART I: Determinants of serum hepcidin

Chapter 2 describes a study in which we constructed reference ranges for serum hepcidin and investigated (biochemical) correlates of serum hepcidin in almost 3,000 participants of the general population. Reference ranges were assessed in a subset from which we excluded persons with characteristics evidently influencing serum hepcidin concentration. We demonstrated that serum hepcidin concentrations are lower for premenopausal than for postmenopausal women but are constant over age in men. Furthermore, we observed a trend of increasing hepcidin concentration during the day. We applied regression analyses to study associations of hepcidin with the iron parameters [serum ferritin, serum iron, total iron-binding capacity (TIBC), transferrin saturation (TS)], body-mass index (BMI), alanine aminotransferase (ALT) as a marker of liver damage, estimated glomerular filtration rate (eGFR) as a marker of kidney function, and the inflammatory marker C-reactive protein (CRP). This revealed that serum ferritin is the strongest correlate of serum hepcidin in both men and women, explaining approximately 60% of serum hepcidin variance. Additional significant, though less strong, associations were observed for CRP and TIBC in men and for TIBC, ALT, and eGFR in women.

In **chapter 3**, we performed a candidate-gene association study focusing on *HFE* and *TMPRSS6*. The single nucleotide polymorphisms (SNPs) rs1800562 in *HFE* (p.Cys282Tyr) and rs855791 in *TMPRSS6* had previously been associated with iron, and it was commonly believed that these associations could be explained by the intermediate effect on hepcidin concentration. However, the results of an Italian study did not support an intermediate role for hepcidin in the SNP–iron parameter associations. We replicated this finding in the NBS. We showed that rs1800562 and rs855791 are not associated with serum hepcidin, but did find evidence for associations of these SNPs with the ratios hepcidin/ferritin and hepcidin/TS. The ratios hepcidin/ferritin and hepcidin/TS reflect the amount of hepcidin relative to iron stores and circulating iron, respectively. Adjustment for hepcidin or the ratio hepcidin/ferritin did not decrease the strength of associations of the SNPs with iron, suggesting that these SNPs exert a pleiotropic effect on the iron parameters and hepcidin ratios. This unexpected finding in the Italian as well as our Dutch study indicates that there are hepcidin-independent mechanisms that are responsible for the associations of rs1800562 and rs855791 with iron.

The aim of **chapter 4** was to identify genetic determinants of serum hepcidin to obtain better insights in its regulation. We performed a genome-wide association study (GWAS) on serum hepcidin in the NBS and combined the results with the GWAS results from two other cohorts in a meta-analysis. These three cohorts are the only three cohorts worldwide that have both genome-wide single nucleotide polymorphism (SNP) data and serum hepcidin measurements. In total, we included 6,096 samples. We replicated six genetic variants in up to 3,826 additional independent samples from two of the three cohorts. Our study revealed

one interesting locus (linkage disequilibrium region from *EML6* to *SPTBN1* (alias *ELF*), lead SNP rs354202) potentially associated with serum hepcidin concentration. 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 effect estimates and p-values of the associations of rs354202 with the ratios hepcidin/ferritin and hepcidin/TS were not genome-wide significant. The common variants rs1800562 (p.Cys282Tyr) in *HFE* and rs855791 (p.Ala736Val) in *TMPRSS6* showed strong associations with the ratio hepcidin/ferritin but not with hepcidin.

In conclusion, we warrant follow-up of rs354202 in additional association studies and recommend to start functional studies if the association of rs354202 with serum hepcidin has been clearly established.

In **chapter 5**, we presented the results of a comparison study of a selection of multivariate (MV) GWAS methods. In a MV-GWAS, multiple traits are analyzed for their association with genetic variants jointly. The goal of this study was to guide researchers in their choice of an appropriate MV-GWA method with respect to their research question and data, aiming to increase the appropriate use of MV-GWAS. We focused on a selection of six methods that are readily available as free software packages. We used simulated data to compare the methods, and we determined empirical significance thresholds to fix type I errors at α=5% and allow for a fair power comparison. We simulated data for 1000 participants, three quantitative traits and one quantitative trait locus (QTL). Our results showed that all methods can increase power compared to a univariate analysis that has appropriately been adjusted for testing multiple traits, even when only one of the traits is associated with the QTL. Four of the methods showed similar performance across all simulation scenarios: MV-PLINK, MV-SNPTEST, MV-BIMBAM and MultiPhen. These methods performed best over almost all of the simulation scenarios, with a sharp increase in power for scenarios with a genetic correlation that was opposite in sign compared to the residual correlation. The two other methods, TATES and PCHAT, showed a more constant performance over the simulation scenarios, but always resulted in lower power than the other four methods. In conclusion, we recommend to apply MV-GWAS methods, because it increases power to identify genetic determinants of the trait(s) of interest, even when genetic correlations between the traits are weak.

PART II: Effects on atherosclerosis

In **chapter 6**, we assessed the associations of hepcidin and the iron parameters with non-invasive measurements of atherosclerosis (NIMA) in men and women separately. NIMA were presence of plaque, intima-media thickness, and ankle-brachial index at rest and after exercise. We included 766 NBS participants for which we had measurements of

hepcidin, iron parameters, NIMA and potential confounding factors. Associations were studied using multivariable logistic and linear regression analyses using quartiles of hepcidin and iron parameters. We adjusted for the following potential confounding factors: time of blood sampling, CRP, high sensitive CRP, BMI, eGFR, ALT, folate, presence of anemia, time between baseline and follow-up and traditional risk factors based on the Framingham risk score, *i.e.* age, systolic blood pressure, smoking, diabetes, total cholesterol, HDL cholesterol and reported use of antihypertensive treatment, lipid lowering medication or anticoagulants. For females, we additionally adjusted for the use of hormone replacement therapy. Hepcidin and the hepcidin/ferritin ratio were significantly associated with the presence of plaque in women. The hepcidin/ferritin ratio was significantly and negatively associated with ankle-brachial index at rest in men and women. Our results indicate that the body iron distribution as determined by hepcidin and the ratio hepcidin/ferritin plays a role in atherosclerosis in women and to a lesser extent in men.

In **chapter 7**, we studied the associations of hepcidin and the iron parameters with NIMA from a genetic perspective. We aimed to shed light on causality of the associations that we identified in **chapter 6** by use of a Mendelian randomization (MR) approach. For this, we used 12 genetic variants that were among the top results of a recent meta-analysis of GWAS on iron status. We could not perform a MR analysis for hepcidin due to the lack of established genetic determinants for serum hepcidin. We also studied associations of atherosclerosis-related SNPs with iron and hepcidin. Finally, we estimated genomic correlations of hepcidin and the iron parameters with atherosclerosis. Other than for rs651007, associated with decreased ferritin concentration and decreased atherosclerosis risk, we observed no SNP associations that fit the hypothesized directions of effect between iron and NIMA. Associations of the six NIMA-related SNPs with hepcidin and iron parameters revealed two nominally statistically significant and directionally consistent associations with the ratio hepcidin/ferritin. The associations of these two SNPs with NIMA corroborate with an increased risk of atherosclerosis, and they associated positively with hepcidin/ferritin. The estimated genomic correlations were all close to zero , except for hepcidin and ferritin with ABI at rest and ABI after exercise for which we found non-significant negative correlations. These negative correlations indicate that increased hepcidin and ferritin concentrations are associated with decreased ABI, which corresponds to an increased risk of atherosclerosis. Taken together, our results indicate a potential causal role for hepcidin and ferritin in atherosclerosis, but not for the other iron parameters.

In conclusion, our population-based studies have led to new insights into biochemical and genetic determinants of serum hepcidin and its effects on atherosclerosis in humans. We revealed that ferritin is the most important correlate of serum hepcidin, and that the other iron parameters showed a less strong association with serum hepcidin. The importance of the balance between hepcidin and ferritin is demonstrated by studies in which the ratio hepcidin/ferritin was reported to be a biomarker for disease, as was shown for hereditary

hemochromatosis and for our studies regarding atherosclerosis. Our candidate-gene approach, focusing on plausible genes, did not reveal genetic determinants of serum hepcidin, but it learned that previously identified associations of common variants in these genes with the iron parameters are independent of serum hepcidin and the ratio hepcidin/ferritin. Our meta-analysis of GWAS including all currently existing cohorts with both genotype data and hepcidin measurements did result in identification of one common variant potentially associated with serum hepcidin. In this respect, application of a multivariate GWAS for hepcidin and (a selection of) the iron parameters might be valuable, especially since it does not require additional investments given that the data are already available. Our studies into the role of hepcidin in atherosclerosis showed that hepcidin and the ratio hepcidin/ferritin are associated with NIMA, and provided indications that these associations are causal. The evidence that we provided is however weak, so we warrant follow-up of these studies in larger and independent populations.

Chapter 8 contains a general discussion of the studies described in this thesis and provides directions for future research.

Samenvatting

Hepcidine speelt een sleutelrol in de systemische ijzerhomeostase. Het reguleert de hoeveelheid circulerend ijzer via beïnvloeding van twee processen: (i) de opname van ijzer uit de darm, en (ii) de afgifte van ijzer uit macrofagen die ijzer van verouderde erytrocyten recyclen. Hepcidine bepaalt dus de distributie van ijzer over het lichaam, waarbij een verhoogde concentratie van serum hepcidine leidt tot een afname van serum ijzer en een toename van de hoeveelheid ijzer opgeslagen in de reticulo-endotheliale macrofagen, en vice versa. Hepcidine speelt een rol bij verschillende aangeboren ijzerstofwisselingsziektes, waarbij hepcidine-deficiëntie leidt tot een ijzeroverschot en hoge serumspiegels van hepcidine resulteren in een lage concentratie van circulerend ijzer en daardoor een ijzergebreksanemie. Daarnaast hangt de hepcidine concentratie samen met ijzerstofwisselingsziektes die kunnen ontstaan als gevolg van andere ziektes, zoals chronische nierziekten, kanker, en het metabool syndroom. Er is eerder beschreven dat hepcidine een rol speelt in de ontwikkeling van atherosclerose, uiteindelijk leidend tot hart- en vaatziekten (HVZ). Manipulatie van de concentratie van serum hepcidine kan dus mogelijk waardevol zijn voor toepassing in de kliniek. Er is echter eerst meer inzicht nodig in de regulatie van serum hepcidine voordat het eventueel in de toekomst als therapeutisch target gebruikt kan worden.

Alhoewel hepcidine aan het begin van de 21^{e} eeuw en dus meer dan 10 jaar geleden is ontdekt, is de huidige kennis over de regulatie van de productie van hepcidine nog steeds incompleet. De meeste studies die tot nu toe zijn uitgevoerd, waren gebaseerd op gebruik van *in vitro* en diermodellen. Bij de start van dit onderzoek waren er slechts enkele studies uitgevoerd die concentraties van hepcidine in patiëntengroepen of kleine aantallen gezonde individuen hebben bestudeerd. In dit proefschrift hebben we ons gericht op concentraties van serum hepcidine in de algemene populatie met als doel biochemische en genetische determinanten van serum hepcidine te identificeren, gebruikmakend van epidemiologische studies (Deel I). Ten tweede hebben we de rol van hepcidine als potentiële determinant van atherosclerose onderzocht; meer specifiek is gepoogd de effecten van serum hepcidine op non-invasieve metingen van atherosclerose (NIMA) op te helderen (Deel II).

Hoofdstuk 1 bevat een algemene introductie van deze thesis. Het geeft een overzicht van de huidige kennis over regulatie van hepcidine en over de veronderstelde relatie tussen ijzer,

hepcidine en atherosclerose. De doelen en de inhoud van dit proefschrift worden beschreven. Tevens wordt een beschrijving van de Nijmegen Biomedische Studie (NBS) gegeven, aangezien alle studies in dit proefschrift zijn gebaseerd op data van deze steekproef van inwoners van de gemeente Nijmegen.

Deel I: Determinanten van serum hepcidine

Hoofdstuk 2 beschrijft een studie waarin we referentiewaardes voor serum hepcidine hebben geconstrueerd en waarin we de samenhang hebben onderzocht tussen biomarkers in het bloed en serum hepcidine in een steekproef van bijna 3000 individuen uit de algemene populatie. Referentiewaardes werden bepaald in een subset van deze groep, waaruit we personen hebben geëxcludeerd met kenmerken die volgens eerdere studies de concentratie van serum hepcidine beïnvloeden. We toonden aan dat concentraties van serum hepcidine lager zijn in premenopausale dan in postmenopausale vrouwen, maar constant blijven met toenemende leeftijd in mannen. Daarnaast vonden we een trend van toenemende concentraties van hepcidine gedurende de dag. We hebben regressieanalyses toegepast om de associaties van serum hepcidine met de ijzerparameters [serum ferritine, serum ijzer, totale ijzerbindingscapaciteit (TIBC), transferrinesaturatie (TS)], body-mass index (BMI), alanine aminotransferase (ALAT) als een marker van leverschade, geschatte glomerulaire filtratierate (eGFR) als een marker van nierfunctie, en de ontstekingsmarker C-reactief proteïne (CRP) te onderzoeken. Hieruit bleek dat serum ferritine het sterkst samenhangt met serum hepcidine in zowel mannen als vrouwen. Serum ferritine verklaarde ongeveer 60% van de totale variantie in de concentratie van serum hepcidine. Daarnaast observeerden we significante associaties met hepcidine, alhoewel minder sterk, voor CRP en TIBC in mannen en voor TIBC, ALAT en eGFR in vrouwen.

Hoofdstuk 3 beschrijft een kandidaatgen associatiestudie waarbij we ons hebben gericht op de genen *HFE* en *TMPRSS6*. De single nucleotide polymorfismen (SNPs) rs1800562 in *HFE* (p.Cys282Tyr) en rs855791 in *TMPRSS6* zijn eerder geassocieerd met ijzer en de gedachte was dat deze associaties verklaard konden worden door een intermediair effect van deze SNPs op de concentratie van serum hepcidine. De resultaten van een Italiaanse studie leverden echter geen bewijs voor een intermediaire rol voor hepcidine in de SNP–ijzerparameter associaties. Wij repliceerden deze bevinding in de NBS. We toonden aan dat rs1800562 en rs855791 niet zijn geassocieerd met serum hepcidine, maar we vonden bewijs voor associaties van deze SNPs met de ratio's hepcidine/ferritine en hepcidine/TS. De ratio's hepcidine/ferritine en hepcidine/TS reflecteren de hoeveelheid hepcidine ten opzichte van respectievelijk de ijzervoorraad in het lichaam en de hoeveelheid circulerend ijzer. Correctie voor hepcidine of de ratio hepcidine/ferritine leidde niet tot een afname van de sterkte van het effect van de associaties van de SNPs met ijzer, hetgeen suggereert dat de SNPs een pleiotroop effect uitoefenen op de ijzerparameters en de hepcidine ratio's. Deze onverwach-

te bevinding in zowel de Italiaanse als onze Nederlandse studie wijst erop dat de associaties van rs1800562 en rs855791 met ijzer via mechanismen lopen die onafhankelijk zijn van serum hepcidine.

Het doel van **hoofdstuk 4** was om genetische determinanten van serum hepcidine te identificeren om meer inzicht te verkrijgen in de regulatie van serum hepcidine. We hebben een genoomwijde associatiestudie (GWAS) uitgevoerd voor serum hepcidine in de NBS en we hebben de resultaten hiervan gecombineerd met de GWAS resultaten van twee andere cohorten in een meta-analyse. Deze drie cohorten zijn de enige cohorten wereldwijd die zowel genoomwijde SNP data als metingen van serum hepcidine hebben. In totaal hebben we 6096 samples geïncludeerd. We hebben zes genetische varianten die een sterke associatie vertoonden met serum hepcidine gemeten in 3826 additionele, onafhankelijke monsters afkomstig uit twee van de drie cohorten. Op basis hiervan werd één interessante locus gevonden (linkage disequilibrium regio van *EML6* tot *SPTBN1* (alias *ELF*), sterkst geassocieerde SNP rs354202) die potentieel geassocieerd is met de serum hepcidine concentratie. Het ELF eiwit is essentieel voor TGF-β signalering door son of mothers against decapentaplegic (SMAD) eitwitten in muizen, en de bone morphogenetic protein-SMAD pathway speelt een centrale rol in hepcidineregulatie. De effectschattingen en p-waardes van de associaties van rs354202 met de ratio's hepcidine/ferritine en hepcidine/TS waren niet genoomwijd statistisch significant. De polymorfismen rs1800562 (p.Cys282Tyr) in *HFE* en rs855791 (p.Ala736Val) in *TMPRSS6* vertoonden sterke associaties met de ratio hepcidine/ferritine, maar niet met hepcidine. We pleiten voor follow-up van rs354202 in additionele associatiestudies en bevelen de start van functionele studies aan als de associatie van rs354202 met serum hepcidine overtuigend is aangetoond.

In **hoofdstuk 5** presenteren we de resultaten van een studie waarin we een selectie van multivariate (MV) analysemethoden voor GWAS hebben vergeleken. In een MV-GWAS worden meerdere traits tegelijkertijd geanalyseerd om hun gezamenlijke associatie met genetische varianten te onderzoeken. Het doel van onze studie was om onderzoekers te ondersteunen in hun keuze van de meest geschikte MV-GWAS methode met betrekking tot hun vraagstelling en data. We focusten op een selectie van zes methoden die beschikbaar zijn als gratis softwarepakketten. We gebruikten gesimuleerde data om de methoden te vergelijken, waarbij we empirisch de significantieniveaus hebben bepaald zodat de type I fout op α=5% werd gefixeerd voor alle methoden. Op deze manier hebben we ervoor gezorgd dat de power van de methoden eenduidig vergeleken kon worden. We hebben data gesimuleerd voor 1000 personen, drie quantitative traits en één quantitative trait locus (QTL). Onze resultaten tonen aan dat alle methoden de power kunnen verhogen vergeleken met een univariate analyse die op de correcte manier is gecorrigeerd voor het testen van meerdere traits, zelfs als slechts één van de drie traits is geassocieerd met de QTL. Vier van de methoden presteerden vergelijkbaar voor alle geteste simulatiescenario's: MV-PLINK, MV-SNPTEST, MV-BIMBAM en MultiPhen. Deze methoden presteerden het beste voor bijna alle simula-

tiescenario's, waarbij ze een sterke toename in power lieten zien voor simulatiescenario's met een genetische correlatie die een tegengestelde richting had vergeleken met de residuele correlatie. De twee andere methoden, TATES en PCHAT, presteerden meer constant over alle simulatiescenario's, maar ze resulteerden altijd in lagere power dan de andere vier methoden. Kortom, we bevelen het gebruik van MV-GWAS methoden aan, omdat het de power voor identificatie van genetische determinanten van de trait(s) van interesse vergroot, zelfs als genetische correlaties tussen de traits laag zijn.

Deel II: Effecten op atherosclerose

In **hoofdstuk 6** hebben we de associaties van hepcidine en de ijzerparameters met niet-invasieve metingen van atherosclerose (NIMA) onderzocht in mannen en vrouwen apart. NIMA waren: aanwezigheid van plaque, dikte van de intima media, en de enkel-arm-index bij rust en na inspanning. We includeerden 766 NBS deelnemers voor wie metingen van hepcidine, ijzerparameters, NIMA en potentiële confounders beschikbaar waren. Multivariabele logistische en lineaire regressieanalyses werden toegepast om de associaties te bestuderen, gebruikmakend van kwartielen van hepcidine en de ijzerparameters. We hebben gecorrigeerd voor de volgende potentiële confounders: tijd van bloedprikken, CRP, hoog sensitief CRP, BMI, eGFR, ALAT, folaat, aanwezigheid van anemie, tijd tussen baseline en follow-up, en traditionele risicofactoren gebaseerd op de Framingham risicoscore, namelijk leeftijd, systolische bloeddruk, roken, diabetes, totaal cholesterol, high-density lipoprotein cholesterol, en gerapporteerd gebruik van antihypertensiva, lipidenverlagende medicatie of anticoagulantia. De analyses voor de vrouwen hebben we daarnaast nog gecorrigeerd voor het gebruik van hormoonvervangende therapie. Hepcidine en de ratio hepcidine/ferritine waren statistisch significant geassocieerd met de aanwezigheid van plaque in vrouwen. De ratio hepcidine/ferritine was significant en negatief geassocieerd met de enkel-arm-index bij rust in mannen en vrouwen. Onze resultaten suggereren dat de distributie van ijzer over het lichaam zoals bepaald door hepcidine en de ratio hepcidine/ferritine een rol speelt in atherosclerose in vrouwen en in mindere mate in mannen.

In **hoofdstuk 7** hebben we associaties van hepcidine en de ijzerparameters met NIMA vanuit een genetisch perspectief bestudeerd. Het doel was om de causaliteit van de associaties geïdentificeerd in **hoofdstuk 6** te bestuderen door een Mendeliaanse randomisatie (MR) benadering toe te passen. Hiervoor hebben we 12 genetische varianten gebruikt die tot de topresultaten van een recente meta-analyse van GWAS voor ijzerstatus behoorden. We konden geen MR analyse uitvoeren voor hepcidine, omdat er tot op heden geen genetische determinanten voor serum hepcidine bekend zijn. Daarnaast hebben we de associaties van atherosclerose-geassocieerde SNPs met ijzer en hepcidine bestudeerd. Tot slot hebben we genetische correlaties geschat van hepcidine en de ijzerparameters met atherosclerose. Behalve voor rs651007, geassocieerd met een afname in de ferritineconcentratie en een ver-

laagd risico op atherosclerose, observeerden we geen SNP associaties die overeenkomen met de veronderstelde richting van het effect tussen ijzer en NIMA. Associatieanalyse van de zes NIMA-geassocieerde SNPs met hepcidine en ijzerparameters liet zien dat twee van deze zes SNPs nominaal statistisch significant zijn geassocieerd met de ratio hepcidine/ferritine. De richtingen van het effect van deze SNPs op hepcidine/ferritine waren positief, en de associaties van deze SNPs met NIMA komen overeen met een verhoogd risico op atherosclerose. Alle geschatte genetische correlaties waren bijna nul, behalve die van hepcidine en ferritine met de enkel-arm-index bij rust en na inspanning. Hiervoor vonden we negatieve correlaties, alhoewel niet significant. Deze negatieve correlaties wijzen erop dat verhoogde hepcidine- en ferritineconcentraties zijn geassocieerd met een verlaagde enkel-arm-index, hetgeen overeenkomt met een verhoogd risico op atherosclerose. Onze resultaten suggereren een potentieel causale rol voor hepcidine en ferritine in atherosclerose, maar niet voor de andere ijzerparameters.

Samengevat, onze populatiestudies hebben geleid tot nieuwe inzichten in biochemische en genetische determinanten van serum hepcidine en de effecten van hepcidine op atherosclerose in de algemene populatie. We ontdekten dat van alle ijzerparameters ferritine het sterkst samenhangt met hepcidine. Het belang van de balans tussen hepcidine en ferritine wordt aangetoond door studies waarin de ratio hepcidine/ferritine wordt gerapporteerd als biomarker voor ziekte, zoals bijvoorbeeld voor hereditaire hemochromatose en voor onze studies met betrekking tot atherosclerose. Onze kandidaatgen associatiestudie, waarin we ons richtten op de biologisch plausibele genen *HFE* en *TMPRSS6*, leidde niet tot de ontdekking van genetische determinanten voor serum hepcidine. We leerden er wel van dat de eerder geïdentificeerde associaties van polymorfismen in deze genen met de ijzerparameters onafhankelijk zijn van serum hepcidine en de ratio hepcidine/ferritine. Onze meta-analyse van GWAS, waarin we alle momenteel bestaande cohorten met zowel genotype data als metingen van serum hepcidine includeerden, resulteerde in identificatie van één polymorfisme dat potentieel is geassocieerd met serum hepcidine. Met betrekking tot identificatie van genetische determinanten voor serum hepcidine kan toepassing van een MV-GWAS voor hepcidine en (een selectie van) de ijzerparameters waardevol zijn, vooral omdat geen additionele investeringen nodig zijn daar de data daarvoor reeds beschikbaar zijn. Onze studies naar de rol van hepcidine in atherosclerose toonden aan dat hepcidine en de ratio hepcidine/ferritine zijn geassocieerd met NIMA. We vonden aanwijzingen dat deze associaties causaal zijn. Het bewijs hiervoor is echter niet sterk, daarom bevelen we vervolgonderzoek van onze studies aan in grotere en onafhankelijke populaties.

Hoofdstuk 8 bevat een discussie van de studies beschreven in dit proefschrift en mogelijkheden voor vervolgonderzoek.

About the author

Tessel Elizabeth Galesloot was born in Deventer on January 28th, 1986. From 1998 until 2004, she attended secondary school at the Geert Groote College in Deventer, which was later merged in the Etty Hillesum Lyceum together with all other secondary schools in Deventer. In 2004, she started with the study Medicine at the Radboud University Nijmegen (RUN). She finished her first year (cum laude propedeuse) and then switched to the study Biomedical Sciences, also at the RUN. She completed two majors, pathobiology and epidemiology, and performed three internships during her studies. Two internships were performed within the Radboud university medical center (Radboudumc), one at the Department of Experimental Rheumatology and one at the Department for Health Evidence. The third internship was performed at Future Diagnostics BV in Wijchen, The Netherlands. In 2010, Tessel obtained here master degree (cum laude). From 2010 till 2014, she worked on her PhD project at the Department for Health Evidence of the Radboudumc, of which the results are described in this thesis. During this time, she was also part-time appointed at the department of Human Genetics and the Cancer Epidemiology group as a genetic epidemiologist, and she was practical coordinator of the Nijmegen Biomedical Study. In the third year of her PhD, in June 2013, Tessel was a visiting scientist at the NHS Blood and Transplant research group, led by prof. W. Ouwehand, embedded in the Department of Haematology, and the Cardiovascular Epidemiology Unit of the Department of Public Health and Primary Care, led by prof. J. Danesh, at the University of Cambridge. Tessel is now appointed as a postdoctoral researcher at the Department for Health Evidence. She works on the genetic epidemiology of urological cancers and continues her work as practical coordinator of the Nijmegen Biomedical Study.

PhD portfolio

Name PhD student: Tessel Galesloot
Department: Health Evidence
Graduate School: Radboud Institute for Health Sciences

PhD period: September 2010 – August 2014
Promotor(s): Prof. dr. D.W. Swinkels & Prof. dr. L.A.L.M. Kiemeney
Copromotor(s): Dr. ir. S.H. Vermeulen

	Year(s)	ECTS
TRAINING ACTIVITIES		
A Courses & Workshops		
– RIHS Introduction Course, RIHS, Nijmegen	2010	1.4
– Basic course on 'R', Erasmus MC, Rotterdam	2010	0.7
– Advances in Genome-Wide Association Studies, NIHES, Rotterdam	2011	1.4
– Presentation Skills, Radboud University Nijmegen (RUN), Nijmegen	2011	1.5
– Advanced Conversation, RUN, Nijmegen	2011	1.5
– Principles of Epidemiologic Data-analysis, Erasmus MC, Rotterdam	2012	0.7
– Loopbaanmanagement, RUN, Nijmegen	2013	0.6
– Talentworkshop, De Nieuwe Voorde, Laag Zuthem	2013	0.4
– Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK), Radboudumc, Nijmegen	2013	1.4
– Next Generation Sequence Data Analysis Course, Max Delbrück Center (MDC) for Molecular Medicine, Berlin, Germany	2014	1.4
B Seminars & lectures		
– Seminars Department of Laboratory Medicine, Nijmegen (5x oral presentation of ± 45 minutes)	2011-2014	NA
C Symposia & congresses		
– European Iron Club Meeting, Nijmegen	2010	0.6
– Radboud iron symposium, Radboudumc, Nijmegen (oral presentation)	2011	0.15
– Werkgroep Epidemiologisch Onderzoek Nederland (WEON), IJmuiden (poster presentation)	2011	0.6
– RIHS PhD Retreat, Wageningen (poster presentation)	2011	0.3
– Conference of the European Society of Human Genetics (ESHG), Nürnberg, Germany (poster presentation)	2012	0.9
– WEON, Rotterdam (poster presentation)	2012	0.6
– RIHS PhD Retreat, Wageningen (organization, chairing sessions)	2012 & 2013	0.6
– International BioIron Society Meeting, London, UK (oral presentation)	2013	1.4
– European Mathematical Genetics Meeting (EMGM), Leiden (poster presentation)	2013	0.6
– International Genetic Epidemiology Society Meeting, Chicago, Illinois, USA (poster presentation)	2013	0.6
– WEON, Leiden (poster presentation)	2014	0.6
– European Iron Club Meeting (EIC), Verona, Italy (oral presentation)	2014	0.9
D Other		
– Weekly epidemiology journal club at the Department for Health Evidence, Radboudumc, Nijmegen	2010-2014	3.6
– Seminars and colloquia at the Department for Health Evidence, Radboudumc, Nijmegen	2010-2014	3.6
– Member of the RIHS PhD council, RIHS, Nijmegen	2012-2013	3.0
TEACHING ACTIVITIES		
D Lecturing		
– Teacher in MSc course Genetic Epidemiology, Biomedical Sciences, RUN, Nijmegen	2010, 2012	NA
– Teacher in BSc course Medical Genomics, Biomedical Sciences, RUN, Nijmegen	2011-2013	NA
E Supervision of internships / other		
NA	NA	NA
TOTAL		**29.05**

List of publications

Cremers RG & **Galesloot TE**, Aben KK, Van Oort IM, Vasen HF, Vermeulen SH, Kiemeney LA. Known susceptibility SNPs for sporadic prostate cancer show a similar association with 'hereditary' prostate cancer. The Prostate 2014; in press.

Rafnar T, Sulem P, Thorleifsson G, Vermeulen SH, Helgason H, Saemundsdottir J, Gudjonsson SA, Sigurdsson A, Stacey SN, Gudmundsson J, Johannsdottir H, Alexiusdottir K, Petursdottir V, Nikulasson S, Geirsson G, Jonsson T, Aben KK, Grotenhuis AJ, Verhaegh GW, Dudek AM, Witjes JA, van der Heijden AG, Vrieling A, **Galesloot TE**, De Juan A, Panadero A, Rivera F, Hurst C, Bishop DT, Sak SC, Choudhury A, Teo MT, Arici C, Carta A, Toninelli E, de Verdier P, Rudnai P, Gurzau E, Koppova K, van der Keur KA, Lurkin I, Goossens M, Kellen E, Guarrera S, Russo A, Critelli R, Sacerdote C, Vineis P, Krucker C, Zeegers MP, Gerullis H, Ovsiannikov D, Volkert F, Hengstler JG, Selinski S, Magnusson OT, Masson G, Kong A, Gudbjartsson D, Lindblom A, Zwarthoff E, Porru S, Golka K, Buntinx F, Matullo G, Kumar R, Mayordomo JI, Steineck DG, Kiltie AE, Jonsson E, Radvanyi F, Knowles MA, Thorsteinsdottir U, Kiemeney LA, Stefansson K. Genome-wide association study yields variants at 20p12.2 that associate with urinary bladder cancer. Hum Mol Genet 2014;23:5545-57.

Galesloot TE, van Steen K, Kiemeney LA, Janss LL, Vermeulen SH. A comparison of multivariate genome-wide association methods. PLoS One 2014;9:e95923.

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. Identification of novel genetic Loci associated with thyroid peroxidase antibodies and clinical thyroid disease. PLoS Genet 2014;10:e1004123.

Galesloot TE, Holewijn S, Kiemeney LA, de Graaf J, Vermeulen SH, Swinkels DW. Serum hepcidin is associated with presence of plaque in postmenopausal women of a general population. Arterioscler Thromb Vasc Biol 2014;34:446-56.

Roke Y, van Harten PN, Franke B, **Galesloot TE**, Boot AM, Buitelaar JK. The effect of the Taq1A variant in the dopamine D_2 receptor gene and common CYP2D6 alleles on prolactin levels in risperidone-treated boys. Pharmacogenet Genomics 2013;23:487-93.

Galesloot TE, Geurts-Moespot AJ, den Heijer M, Sweep FC, Fleming RE, Kiemeney LA, Vermeulen SH, Swinkels DW. 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 2013;50:593-8.

van der Zanden LF, **Galesloot TE**, Feitz WF, Brouwers MM, Shi M, Knoers NV, Franke B, Roeleveld N, van Rooij IA. Exploration of gene-environment interactions, maternal effects and parent of origin effects in the etiology of hypospadias. J Urol 2012;188:2354-60.

Luciano M, Huffman JE, Arias-Vásquez A, Vinkhuyzen AA, Middeldorp CM, Giegling I, Payton A, Davies G, Zgaga L, Janzing J, Ke X, **Galesloot T**, Hartmann AM, Ollier W, Tenesa A, Hayward C, Verhagen M, Montgomery GW, Hottenga JJ, Konte B, Starr JM, Vitart V, Vos PE, Madden PA, Willemsen G, Konnerth H, Horan MA, Porteous DJ, Campbell H, Vermeulen SH, Heath AC, Wright A, Polasek O, Kovacevic SB, Hastie ND, Franke B, Boomsma DI, Martin NG, Rujescu D, Wilson JF, Buitelaar J, Pendleton N, Rudan I, Deary IJ. Genome-wide association uncovers shared genetic effects among personality traits and mood states. Am J Med Genet B Neuropsychiatr Genet 2012;159B:684-95.

Kloss-Brandstätter A, Erhart G, Lamina C, Meister B, Haun M, Coassin S, Seifert M, Klein-Franke A, Paulweber B, Kedenko L, Kollerits B, Swinkels DW, Vermeulen SH, **Galesloot TE**, Kronenberg F, Weiss G. Candidate gene sequencing of SLC11A2 and TMPRSS6 in a family with severe anaemia: common SNPs, rare haplotypes, no causative mutation. PLoS One 2012;7:e35015.

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. Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood 2011;117:e218-25.

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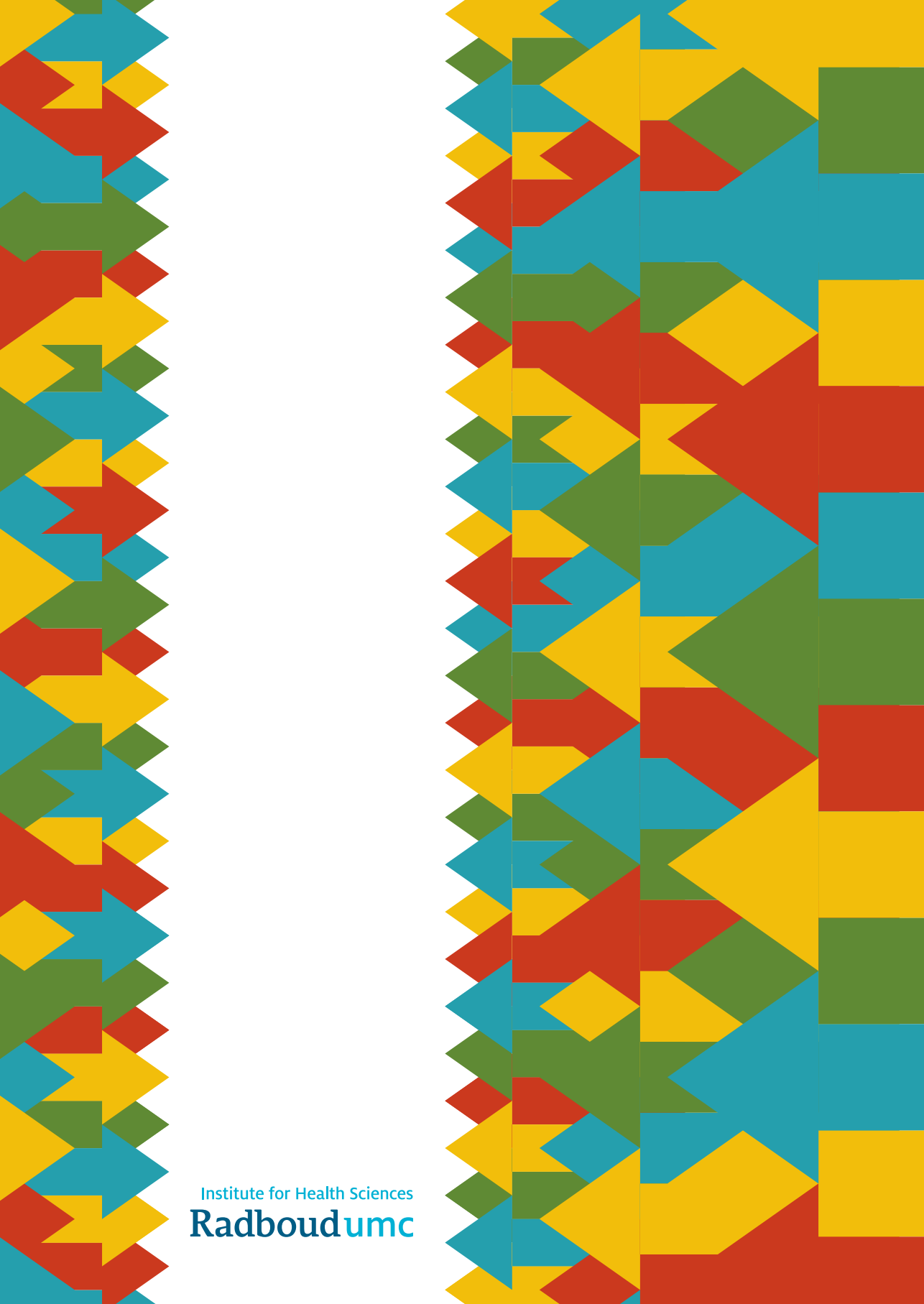
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