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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

Background—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. We report here the second study to investigate the role of hepcidin in the associations between common variants in *HFE* and *TMPRSS6* with iron parameters.

Methods—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.

Results—We found that *HFE* rs1800562 and *TMPRSS6* rs855791 are the 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 ($p<1\times10^{-5}$) and hepcidin/transferrin saturation ($p<1\times10^{-3}$), but not with serum hepcidin ($p>0.2$). Adjustment for

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hepcidin or the ratio hepcidin/ferritin did not decrease the strength of the SNP–iron parameter associations.

Conclusions—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 haemochromatosis gene (*HFE*) and in the transmembrane serine protease 6 gene (*TMPRSS6*) are associated with ferritin, iron, transferrin, and transferrin saturation (TS) (ie, 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] analysed 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 them 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 analyse the associations between common variants in and surrounding the *HFE* and *TMPRSS6* genes and iron parameters and hepcidin on a population level. The first objective 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

(1) considered hepcidin and its ratio to ferritin as intermediate variables in the association between the SNPs and iron parameters; (2) explored the presence of pleiotropy, that is, whether the SNPs both independently affect hepcidin and the iron parameters; and (3) evaluated the presence of an effect on iron parameters only (figure 1).

METHODS

Study population

This study was performed in participants from the NBS, a well phenotyped Dutch population based cohort. Details of the NBS have been described before.[20] For this study we used the subset of 1832 NBS participants that was selected to serve as controls in GWAS.[21] Questionnaire data on age, use of iron supplements, body mass index (BMI), presence of anaemia, and pregnancy, and measurements of hepcidin, iron, ferritin, TS and total iron binding capacity (TIBC), liver enzyme alanine aminotransferase (ALT), creatinine, and C-reactive protein (CRP) were available. The iron parameters were measured as described before.[22] Serum hepcidin was measured with an in-house developed and validated competitive enzyme linked immunosorbent assay (ELISA).[2223]

Genotyping and selection of SNPs

The NBS participants were genotyped with the Illumina HumanHapCNV370-Duo BeadChip[21]; density of genetic variants was increased by imputation using the CEU HapMap Phase II data as reference.

Genotype data for the SNPs within the genes *HFE* and *TMPRSS6* and the 10 kB surrounding regions were extracted for the purpose of this study, resulting in the inclusion of 35 SNPs for *HFE* and 66 SNPs for *TMPRSS6* (see online supplementary table S1).

Haplotype analyses were applied to uncover allelic interactions. We included only non-synonymous SNPs and SNPs known to influence expression of *HFE* or *TMPRSS6* based on expression databases (expression quantitative trait loci (eQTL)): the non-synonymous SNPs rs855791 (p.Ala736Val) and rs2235324 (p.Lys253Glu) and the eQTL rs2160906 for *TMPRSS6*, and the non-synonymous SNPs rs1800562 (p.Cys282Tyr) and rs1799945 (p.His63Asp) and the eQTL rs198853 for *HFE*.

Statistical analysis

The variables hepcidin, ferritin, and the ratios hepcidin/ferritin and hepcidin/TS were skewed towards higher values and therefore log-transformed to normalise their distributions.eOutliers, defined as values that differed more than three times the SD from the mean, were reduced to mean±3 SD (maximal number of outliers per trait: 26).

Two different subsets were created to investigate whether results for the whole cohort were influenced by extreme values on variables evidently influencing hepcidin concentration. Subset 1 was based on exclusion of persons with CRP >10 mg/L or ferritin <30 μg/L in agreement with the study of Traglia *et al.*[3] Subset 2 was selected based on the same exclusion criteria as previously[22]: pregnancy at time of blood sampling, ALT >50 U/L, CRP

>10 mg/L, estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, use of iron supplements, presence of anaemia, or BMI >30 kg/m^2.

Association analyses were performed using Plink V.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).[24] The associations between the SNPs and the traits were evaluated using linear regression analyses based on the genotypic model and adjusted for age, gender, and time of blood sampling, since these variables are independent determinants of serum hepcidin.[22] The resulting regression coefficients express the mean change in the independent variable for the heterozygous and minor homozygous genotype or haplotype relative to the reference genotype or haplotype. 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 or haplotype.

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 (ie, 35+66=101) would lead to a p value threshold of significance of 5×10^{-4}.

A full description of the methods used for this study can be found online (see online supplementary methods).

RESULTS

Characteristics of the study population

The mean age of the 1832 individuals was 62 years. Additional characteristics of the individuals included in the study are shown in table 1. Application of the exclusion criteria resulted in the inclusion of 1505 individuals in subset 1 and 1177 individuals in subset 2 (see online supplementary table S2). Characteristics of the subsets and the whole cohort were similar, except for the proportion of females (51% in the whole cohort vs 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).

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 1×10^{-18} and 1×10^{-3}) (see online supplementary table S3). For *TMPRSS6*, rs855791 was most strongly associated with both iron and TS of all variants tested ($p=3.4\times10^{-12}$ and 8.5×10^{-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) (see online supplementary table S3). Besides these two SNPs, other common variants in *HFE* and *TMPRSS6* showed associations (using the stringent Bonferroni corrected significance threshold of $p<5\times10^{-4}$) 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 (see online supplementary table S4). 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 (see online supplementary tables S5 and S6) 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 the most important variants within *HFE* and *TMPRSS6*, respectively, in their ability to affect the iron phenotypes.

Table 2 shows the associations between *TMPRSS6* rs855791 and *HFE* rs1800562 with the iron parameters. Both SNPs are c.G>A SNPs, with a minor allele frequency (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 (β (95% CI) AG vs GG −1.5 (−2.1 to −0.9); AA vs GG −2.5 (−3.2 to −1.8)) and TS (β (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 (β (95% CI) AG vs GG 2.3 (1.5 to 3.0); AA vs GG 10.7 (5.5 to 15.9)) and TS (β (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 (see online supplementary tables S7 and S8).

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 2 (see online supplementary tables S7 and S8 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 (see online supplementary table S9). *TMPRSS6* rs855791 and *HFE* rs1800562 were associated with an increase and decrease, respectively, in both log hepcidin/ferritin (*TMPRSS6* rs855791: β (95% CI) AG vs GG 0.04 (0.01 to 0.07), AA vs GG 0.08 (0.05 to 0.12); *HFE* rs1800562: β (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: β (95% CI) AG vs GG 0.05 (0.01 to 0.09), AA vs GG 0.12 (0.07 to 0.17); *HFE* rs1800562: β (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 (see online supplementary table S10).

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 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 2). Identical observations were done for subset 1 and 2 (see online supplementary tables S7 and S8). We did not correct the associations for the hepcidin/TS ratio because TS was calculated by dividing serum iron by TIBC.

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 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. In agreement with 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*.[3] 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.[3]

Our results and the results of Traglia *et al*[3] 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.[1625–28] For example, it was shown that *HFE* mutations can directly affect iron accumulation in hereditary haemochromatosis macrophages, independently of the presence of hepcidin.[2526] 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 hepcidin in the liver and lower serum hepcidin concentrations relative to the body iron stores, leading to the iron storage disorder hereditary haemochromatosis.[7–11] Mutations in *TMPRSS6* have been associated with inappropriately high urine and serum hepcidin concentration for the setting of systemic iron deficiency, which has been suggested to cause iron refractory iron deficiency anaemia. 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.[29] 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 (CRP >1 mg/dL).[29] We and Traglia *et al*[3] 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*[29] and Traglia *et al*[3] 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*[11] 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 ($p<0.01$); however, 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 the results of both Nai *et al*[29] and van Dijk *et al*.[11]

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*[3] 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 concentrations 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 signalling form of the molecule—that is, differic transferrin.[30] Besides, we used serum concentrations of hepcidin as well as serum concentrations of iron and ferritin, but the associations that we studied might be cell type or

tissue specific. Hepcidin is predominantly produced in hepatocytes[31–33] whereas the Hfe protein is expressed in a whole range of tissues and cell types[3435] and MT2 is expressed primarily in the liver.[36] 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 the 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.*[3] 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 to a direct SNP effect on certain serum iron parameters, rather than an indirect effect entirely dependent upon a change in serum hepcidin concentrations. We call for additional functional studies in a controlled setting that allow further elucidation of the role of hepcidin in the associations between the SNPs and iron parameters, which will contribute to our understanding of the underlying mechanisms and eventually facilitate the development of interventions for iron disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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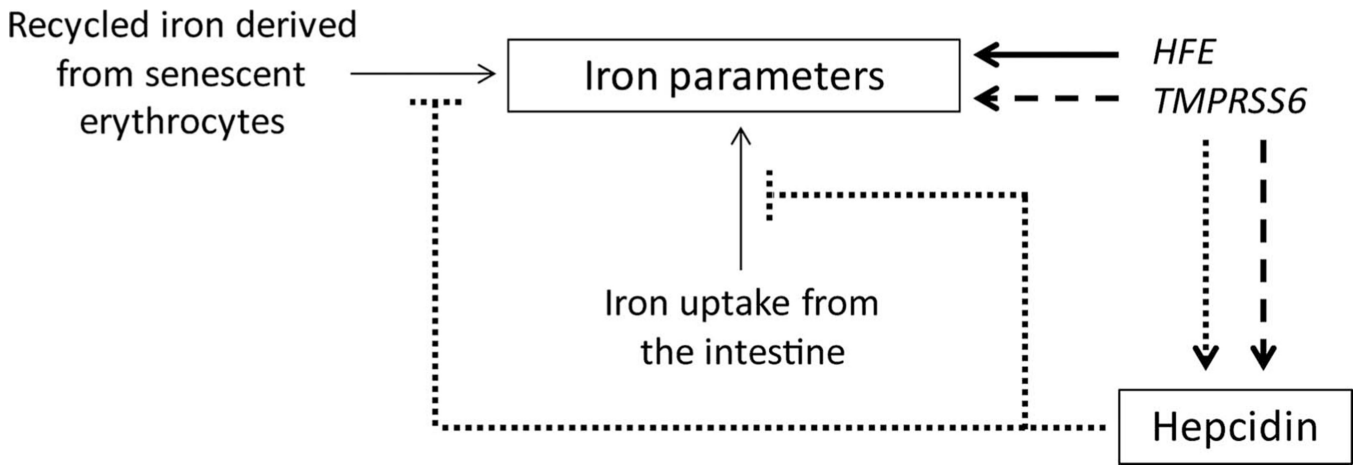

Figure 1.

Hypothetical roles of serum hepcidin in the associations of haemochromatosis gene *(HFE)* and the transmembrane serine protease 6 gene (*TMPRSS6*) variants with serum iron parameters. (1) Hepcidin and its ratio to ferritin might be intermediate in the association between *HFE* and *TMPRSS6* variants and iron parameters (dotted arrow). (2) The variants might both independently affect hepcidin and the iron parameters (dashed arrows), that is, presence of pleiotropy. (3) The variants may affect the iron parameters only and not hepcidin (bold arrow).

Table 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 12:00	378	21	NA
Between 12:00 and 17:00	1172	64	NA
After 17:00	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; 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.

TIBC, total iron binding capacity; TS, transferrin saturation.

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Table 2

Associations between rs855791 in the transmembrane serine protease 6 gene (*TMPRSS6*) and rs1800562 in the haemochromatosis gene (*HFE*) with iron parameters for the total study population (N=1832)

				AG		AA	
	Additionally adjusted for:	**Total N***	**p Value**	**N**	**β AG vs GG (95% CI)**	**N**	**β AA vs GG (95% CI)**
TMPRSS6 rs855791†							
Log(hepcidin)‡, nM	NA	1824	2.02E-01	928	0.01 (−0.03 to 0.05)	365	0.05 (−0.01 to 0.10)
Log(ferritin), μg/L‡	NA	1822	1.86E-01	926	−0.03 (−0.07 to 0.01)	365	−0.04 (−0.09 to 0.01)
	Log(hepcidin)	1822	1.36E-05	926	−0.04 (−0.06 to −0.01)	365	−0.07 (−0.10 to −0.04)
Log(hepcidin/ferritin)‡, nmoles/μg	NA	1822	8.60E-06	926	0.04 (0.01 to 0.07)	365	0.08 (0.05 to 0.12)
Log(hepcidin/TS)‡, nM/%	NA	1804	3.42E-05	919	0.05 (0.01 to 0.09)	358	0.12 (0.07 to 0.17)
Iron, μM	NA	1804	3.36E-12	919	−1.50 (−2.06 to −0.93)	358	−2.53 (−3.24 to −1.82)
	Log(hepcidin)	1804	6.71E-13	919	−1.51 (−2.08 to −0.95)	358	−2.60 (−3.31 to −1.90)
	Log(hepcidin/ferritin)	1804	7.90E-11	919	−1.42 (−1.98 to −0.86)	358	−2.37 (−3.08 to −1.66)
TS, %	NA	1804	8.47E-14	919	−2.92 (−3.96 to −1.89)	358	−4.96 (−6.26 to −3.66)
	Log(hepcidin)	1804	1.48E-15	919	−2.98 (−3.99 to −1.96)	358	−5.21 (−6.48 to −3.94)
	Log(hepcidin/ferritin)	1804	1.54E-12	919	−2.81 (−3.84 to −1.77)	358	−4.71 (−6.01 to −3.41)
TIBC, μM	NA	1804	1.27E-01	919	0.74 (−0.20 to 1.68)	358	1.16 (−0.02 to 2.33)
	Log(hepcidin)	1804	2.57E-02	919	0.82 (−0.07 to 1.71)	358	1.51 (0.40 to 2.63)
	Log(hepcidin/ferritin)	1804	7.87E-02	919	0.81 (−0.13 to 1.75)	358	1.30 (0.11 to 2.48)
HFE rs1800562†							
Log(hepcidin)‡, nM	NA	1824	3.89E-01	217	0.00 (−0.06 to 0.05)	4	−0.27 (−0.66 to 0.12)
Log(ferritin), μg/L‡	NA	1822	2.01E-04	216	0.04 (−0.01 to 0.10)	4	0.69 (0.33 to 1.04)
	Log(hepcidin)	1822	2.08E-15	216	0.05 (0.01 to 0.08)	4	0.88 (0.66 to 1.11)
Log(hepcidin/ferritin)‡, nmoles/μg	NA	1822	1.35E-07	216	−0.04 (−0.08 to −0.01)	4	−0.65 (−0.90 to −0.41)
Log(hepcidin/TS)‡, nM/%	NA	1804	5.73E-04	212	−0.07 (−0.13 to −0.02)	4	−0.58 (−0.96 to −0.20)
Iron, μM	NA	1804	1.39E-11	212	2.26 (1.50 to 3.02)	4	10.71 (5.53 to 15.88)
	Log(hepcidin)	1804	5.70E-12	212	2.26 (1.51 to 3.02)	4	11.09 (5.94 to 16.24)
	Log(hepcidin/ferritin)	1804	3.33E-10	212	2.18 (1.42 to 2.93)	4	9.33 (4.15 to 14.52)

				AG		AA	
	Additionally adjusted for:	**Total N***	**p Value**	**N**	**β AG vs GG (95% CI)**	**N**	**β AA vs GG (95% CI)**
TS, %	NA	1804	7.00E-18	212	5.36 (3.98 to 6.75)	4	22.60 (13.17 to 32.04)
	Log(hepcidin)	1804	3.20E-19	212	5.37 (4.02 to 6.73)	4	23.93 (14.68 to 33.18)
	Log(hepcidin/ferritin)	1804	1.85E-16	212	5.24 (3.87 to 6.62)	4	20.58 (11.10 to 30.06)
TIBC, μM	NA	1804	1.17E-06	212	−3.05 (−4.30 to −1.80)	4	−9.58 (−18.13 to −1.02)
	Log(hepcidin)	1804	6.81E-08	212	−3.06 (−4.25 to −1.88)	4	−11.51 (−19.61 to −3.41)
	Log(hepcidin/ferritin)	1804	3.44E-07	212	−3.13 (−4.38 to −1.88)	4	−10.94 (−19.54 to −2.33)

Associations are adjusted for age, gender, time of blood sampling, and additionally for serum hepcidin or the ratio hepcidin/ferritin.

N indicates number; β AG vs GG, regression coefficient for AG genotype versus GG genotype; β AA versus GG, regression coefficient for AA genotype versus GG genotype.

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.

SNP, single nucleotide polymorphisms; TIBC, total iron binding capacity; TS, transferrin saturation.