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HFE genotypes and dietary heme iron: No evidence of strong gene—nutrient interaction on serum ferritin concentrations in middle-aged women

Daphne L. van der A [a,b], Petra H.M. Peeters [a], Diederick E. Grobbee [a], Mark Roest [c], Hieronymus A.M. Voorbij [c], Yvonne T. van der Schouw [a,*]

[a] *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, The Netherlands*
[b] *Center for Nutrition and Health, National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[c] *Department of Clinical Chemistry, University Medical Center Utrecht, The Netherlands*

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Abstract *Background and aim:* Hereditary hemochromatosis (HH) is a disorder characterized by inappropriately high intestinal iron absorption. In populations of Northern European descent, HH is most commonly caused by mutations (C282Y/H63D) in the HFE gene.
Methods and results: We investigated the effects of dietary heme iron intake and HFE mutations on serum ferritin concentrations in a population-based random sample of 1611 women aged >50 years using analysis of covariance (ANCOVA). Higher heme iron intake was associated with significantly higher serum ferritin concentrations ($P_{trend} < 0.001$). Also, women with the compound or C282Y homozygous genotype had significantly higher serum ferritin concentrations (geometric mean 115.2 μg/L (95% CI 81.4—162.9 μg/L) than women carrying normal alleles (geometric mean 76.6 μg/L (95% CI 72.5—80.9 μg/L). We observed the highest serum ferritin concentrations among postmenopausal women who are compound heterozygous or C282Y homozygous, and who consume relatively high amounts of heme iron (geometric mean 183.9 μg/L (95% CI 97.2—347.8 μg/L).

* Corresponding author. Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, HP Str.06.131, PO Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: +31 30 250 9360; fax: +31 30 250 5485.
E-mail address: y.t.vanderschouw@umcutrecht.nl (Y.T. van der Schouw).
URL: http://www.juliuscenter.nl

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Conclusions: Even when there are currently no clinical signs, women with the compound or C282Y homozygous genotype may still be at risk for developing iron overload sometime after menopause.
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Introduction

In populations of Northern European ancestry, the iron storage disease, hereditary hemochromatosis, is predominantly due to defects in the HFE gene [1]. The two most common mutations are the C282Y mutation, which has an estimated allele-frequency of 6%, and the H63D mutation, which has an allele-frequency of about 14% [2,3]. Most patients with clinically diagnosed hemochromatosis are homozygous for the C282Y mutation. Therefore, for a long time the general belief was that the penetrance (i.e., the extent to which the mutation predicts disease) of the homozygous state was very high. However, large scale screening studies have demonstrated that the clinical penetrance of the HFE mutations lies more in the order of a few percent [4—7]. Possibly, mutations or polymorphisms in other genes modify the hemochromatosis phenotype [8—10], although the variability in disease expression may also be partially explained by environmental factors like dietary or supplemental iron intake and regular blood loss.

At present, serum ferritin most accurately reflects the accumulation of iron in the body [11]. Previous reports have shown a significantly higher serum ferritin concentration in individuals with the compound (i.e., heterozygosity for both the C282Y and H63D mutation) or C282Y homozygous genotype as compared to wild-type individuals [12,13]. More subtle effects on iron parameters are observed in subjects who are only single heterozygous. In women, clinical signs of hemochromatosis become particular clear post-menopausally, which might be due to the relative protection from the effects of HFE genotypes and dietary iron intake by the regular blood loss through menstruation.

The purpose of this paper is to examine the effects of dietary iron intake, HFE genotypes and blood loss through menstruation on body iron stores indicated by serum ferritin concentrations in middle-aged women.

Methods

Subjects

The study population consisted of participants of the Prospect-EPIC cohort [14], which is one of the two Dutch contributions to the European Prospective Investigation into Cancer and Nutrition (EPIC). Participants were recruited between 1993 and 1997 among women living in Utrecht and vicinity who attended the regional population-based breast cancer-screening program. A total of 17,357 women aged 49—70 years were included. At enrollment all women underwent a physical examination and filled out two detailed questionnaires. One was a general questionnaire relating to non-dietary factors, the other a validated food frequency questionnaire. In addition, women donated a 30-ml non-fasting blood sample, which was fractionated into serum, citrated plasma, buffy coat and erythrocyte aliquots of 0.5 ml each. The straws were stored under liquid nitrogen at −196 °C for future research.

A random sample of 1736 (10%) women was taken for biochemical analyses. For 64 (3.7%) women, serum, plasma or buffy coat samples were missing. We excluded women with missing questionnaires ($n = 16$), missing data for serum ferritin ($n = 3$) or HFE genotyping ($n = 41$), and women who reported low (<500 kcal) total daily energy intake ($n = 2$). For some women multiple reasons applied for exclusion and a total of 1611 women remained in the analyses.

All women signed an informed consent form prior to study inclusion. The study complies with the Declaration of Helsinki and was approved by the Institutional Review Board of the University Medical Center Utrecht.

Laboratory measurements

Serum ferritin was determined using an automated immuno-metric assay on the Immulite (Diagnostic Products Corporation, Los Angeles, Ca, USA). Serum iron was estimated using a colorimetric assay on a Hitachi 904 (Johnson & Johnson, Rochester, New York, USA). Serum transferrin values were obtained by immunochemical turbidimetry on a Hitachi 904. Total iron binding capacity (TIBC) (μmol/L) was calculated as serum transferrin (g/L) × 25.14, and transferrin saturation as the ratio of serum iron to TIBC. As a marker for inflammation, high-sensitivity C-reactive protein (hsCRP) was measured in citrated plasma using the Behring BNII nephelometric method (Dade Behring, Deerfield, Il, USA). HsCRP values below the detection limit of 0.2 mg/L ($n = 66$) were set to 0.1 mg/L.

Genomic DNA was extracted from buffy coats with the use of the QIAamp® Blood Kit (Qiagen Inc., Valencia, California, USA).

C282Y-genotyping was performed by PCR following a radioactive hybridization assay using allele-specific probes. PCR was performed in a volume of 25 μl containing 1 μl DNA (1:5 dilution), 2.5 μl of PCR Gold Buffer (Applied Biosystems, Foster City, CA), 0.5 mmol/L dNTPs (Amersham Biosciences, Buckinghamshire, UK), 2.5 mmol/L $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.4 μmol/L of each primer (5′-CCT CCT TTG GTG AAG GTG ACA-3′ (forward) and 5′- CAC AAT GAG GGG CTG ATC CA-3′ (reverse)) (Isogen Bioscience BV, Maarsen, the Netherlands) and 1.25 U of Amplitaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR cycling conditions were as follows: 4′ 95 °C, 10 cycli (40″ 94 °C, 40″ 55 °C, 2′ 72 °C), 23 cycli (20″ 94 °C, 40″ 55 °C, 2′ 72 °C), 10′ 72 °C. The following allele-specific probes were used to make a distinction between the *Cys*282 and *Tyr*282 allele: HFE*Cys*282: 5′-GAT ATA CGT **G**CC AGG TGG A-3′ and HFE*Tyr*282: 5′-GAT ATA CGT **A**CC AGG TGG A-3′[15]. For full details of the hybridization protocol we refer to Roest et al. [16].

H63D-genotyping was performed by TaqMan PCR assay using the following primers: 5′-GAA GCT TTT GGG CTA CGT GGA T-3′ (forward) and 5′-TTC TAC TGG AAA CCC ATG GAG TTC-3′ (reverse) (Applied Biosystems, Foster City, CA). FAM™ (5′-TCG TGT TCT ATG AT**C** ATG A-3′) MGBNFQ and VIC® (5′-TTC GTG TTC TAT GAT **G**AT GA-3′) MGBNFQ dual-labeled TaqMan® probes (Applied Biosystems, Foster City, CA) were used to distinguish between the *His*63 and *Asp*63 allele. The PCR was performed in a volume of 25 μl containing 1 μl DNA (1:5 dilution), 2.5 μl PCR buffer II (Applied Biosystems, Foster City, CA), 2.0 mmol/L dNTPs (Amersham Biosciences, Buckinghamshire, UK), 4 mmol/L $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.4 μmol/L of each primer, 1.25 μmol/L of each labeled probe and 0.5 U of Amplitaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR cycling conditions were as follows: 10′ 96 °C, 40 cycli (15″ 92 °C, 1′ 60 °C), 10′ 72 °C. Detection of allele specific fluorescent signals was performed on an automated fluorometer (FLUOstar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany) using the following excitation/emission wavelengths: 520/550 nm for VIC and 492/520 nm for FAM.

Dietary data

The food frequency questionnaire (FFQ) was developed to estimate the usual frequency of consumption of 79 main food items over the preceding 12 months. Subjects could indicate the consumption frequency of each food item on a scale ranging from never to daily with weekly, monthly and yearly as intermediate categories. The questionnaire also comprised questions regarding nutritional habits, preparation methods and additions. To estimate habitual portion sizes, color photographs of 28 dishes in 2–4 different-sized portions were included. Food consumption data were converted into macro- and micronutrients using an updated version of the computerized Dutch food composition table 1996 [17]. Single use of iron, vitamin C, and calcium supplements over the preceding 12 months was coded as a bivariate yes/no variable. Overall, the questionnaire enabled estimation of the average daily consumption of 178 food items. The FFQ was validated for food groups and nutrients [18,19]. All nutrients were adjusted for total energy intake using the regression residual method [20].

General questionnaire and anthropometric measurements

The general questionnaire contained questions regarding demographic factors, lifestyle habits, obstetric and gynecological history and past and current morbidity. Women were classified according to their smoking habits as current, past or never smokers. They were asked about the presence of menstrual bleeding periods in the 12 months prior to enrollment in the study. These bleeding periods could either be natural or hormone-induced. In addition, information was gathered regarding use of oral contraceptives (OC) and postmenopausal hormone therapy (HT). Both variables were coded ever/never. Daily use of aspirin was reported on separate medication lists, and was coded yes/no. Height and weight were measured, without shoes and wearing light indoor clothing, to compute body mass index (BMI) defined as weight divided by height squared (kg/m^2).

Statistical methods

The observed genotype distributions were compared with those expected under Hardy Weinberg equilibrium [21,22] using a chi-square goodness-of-fit test with one degree of freedom. C282Y and H63D genotypes were combined to give an overall HFE genotype.

Baseline characteristics of the study population were presented by means and standard deviations for normally distributed variables, and by medians and interquartile ranges (IQR) for variables that

showed skewed distributions. Categorical variables were expressed as frequencies. In the case of iron intake we categorized intake levels into two (separated at the median) and three (tertiles reflecting low, moderate and high intake) equal groups. HFE genotypes were grouped into three categories based on their expected effect on iron stores: normal (wildtypes), small (single heterozygotes and H63D homozygotes), and large (compound heterozygotes and C282Y homozygotes). We created two dummy variables for the three HFE categories. Serum ferritin concentrations were logarithmically transformed to produce approximately normal distributions.

Geometric mean concentrations of serum ferritin were calculated for each category of heme iron intake and HFE genotype, using analysis of covariance (ANCOVA). Since ferritin is also an acute phase protein, we constructed models with and without exclusion of individuals having hsCRP concentrations >6 mg/L as a measure of infection. Factors previously shown to be associated with serum ferritin concentrations, such as age, BMI, alcohol, aspirin use, smoking, use of OC, postmenopausal HT, vitamin C, calcium, coffee and fiber [23—31] were considered as potential confounders, and adjusted means were computed. A combination of heme iron intake and HFE genotype was made to explore the possibility of interaction. We used linear regression to evaluate linear trends for the effect of heme iron intake and HFE genotypes and to test interaction terms of heme iron intake (tertiles reflecting low, moderate and high intake) and HFE genotype (three categories). P values were two-sided and $P < 0.05$ was considered statistically significant.

Since menstrual bleeding may mask the effect of iron intake and HFE genotypes on serum ferritin concentrations, we studied the associations separately for menstruating and non-menstruating women.

All statistical procedures were performed using the statistical package SPSS (SPSS for Windows, Rel. 11.0.1.2001. Chicago: SPSS Inc.).

Results

Genotype distributions of both C282Y ($\chi^2 = 0.00$, $P = 0.95$) and H63D ($\chi^2 = 3.54$, $P = 0.06$) were in Hardy Weinberg equilibrium. The frequency of the C282Y-allele was 0.06, the frequency of the H63D-allele was 0.14.

The general characteristics of the population are summarized in Table 1. Mean serum ferritin concentration in menstruating women was 43.8 μg/L (IQR 20.6—79.4 μg/L) whereas in non-menstruating women this was 103.5 μg/L (IQR 64.6—165.0 μg/L).

Serum ferritin concentrations and transferrin saturation calculated for all HFE genotypes and stratified by regular menstrual bleeding are presented in Table 2. To our knowledge, none of the women was previously diagnosed with hemochromatosis.

Figure 1 shows the association between dietary heme iron and serum ferritin, and HFE genotypes and serum ferritin. The ranges of heme iron intake for each category are 0.00—1.42 mg/day for low intake, 1.42—2.09 mg/day for moderate intake and 2.09—5.38 mg/day for high intake. Women who consumed high levels of heme iron had higher concentrations of serum ferritin (93.9 μg/L; 95%CI 87.1—101.2 μg/L), than women who consumed moderate or low levels of heme iron (82.3 μg/L; 95% CI 76.3—88.7 μg/L and 63.6 μg/L; 95% CI 59.0—68.5 μg/L, respectively, $P_{trend} < 0.001$) (Figure 1A).

Serum ferritin concentrations were 76.6 μg/L (95% CI 72.5—80.9 μg/L) in subjects carrying normal alleles, 81.9 μg/L (95% CI 75.9—88.3 μg/L) in women single heterozygous or homozygous for the H63D mutation, and 115.2 μg/L (95% CI 81.4—162.9 μg/L) in subjects with the compound or homozygous C282Y genotype ($P_{trend} = 0.03$) (Figure 1B). There was no statistically significant difference in mean transferrin saturation between the low, moderate and high heme intake groups. On the other hand, there was a statistically significant association between HFE genotypes and transferrin saturation. Additionally, we investigated the relation between non-heme iron intake and serum ferritin concentration as well as transferrin saturation, but no statistically significant associations were present in this population.

When we excluded women with raised hsCRP concentrations (>6 mg/L) from the analyses, results did not change materially.

Figure 2 shows geometric mean serum ferritin concentrations for combinations of heme iron intake and HFE genotype. It suggests that at each intake level of heme iron, the compound and C282Y homozygous genotype is associated with higher concentrations of serum ferritin. The effects might be more pronounced in women who consume relatively high levels of heme iron, although the interaction was not statistically significant (interaction tests: HFE 'single heterozygote/H63D homozygote' × heme iron $P = 0.66$; HFE 'compound/C282Y homozygote' × heme iron $P = 0.67$). The hypothesis that effects on body iron stores can only be observed in women postmenopausally, was investigated by performing

Table 1 Baseline characteristics of the 1611 women participating in the Prospect-EPIC cohort

	Total population (n = 1611)	Menstruating (n = 403)	Non- menstruating (n = 1208)
General characteristics			
Age at intake, years[a]	56 (52–62)	51 (50–53)	59 (54–64)
BMI, kg/m^2 [b]	25.9 ± 4.0	25.3 ± 3.8	26.1 ± 4.0
Alcohol intake, g/d[a,c]	3.9 (0.4–13.5)	6.2 (1.0–17.1)	3.1 (0.3–11.4)
Smoking status, *N* (%)			
Never	679 (42)	142 (35)	537 (45)
Past	557 (35)	161 (40)	396 (33)
Current	375 (23)	100 (25)	275 (23)
Daily aspirin use, *N* (%)	49 (3)	7 (2)	42 (4)
OC use ever, *N* (%)	1017 (63)	328 (81)	689 (57)
HT ever, *N* (%)	397 (25)	131 (33)	266 (22)
Use of iron supplements, *N* (%)	58 (4)	13 (3)	45 (4)
Use of vitamin C supplements, *N* (%)	148 (9)	42 (10)	106 (9)
Use of calcium supplements, *N* (%)	79 (5)	13 (3)	66 (6)
Biochemical characteristics			
Ferritin, μg/L[a]	90.9 (49.6–145.0)	43.8 (20.6–79.4)	103.5 (64.6–165.0)
Transferrin saturation, %[b]	22.8 ± 7.4	21.8 ± 8.8	23.1 ± 6.9
High sensitive-CRP, mg/L[a]	1.2 (0.6–2.7)	1.1 (0.5–2.7)	1.2 (0.6–2.6)
Dietary characteristics			
Energy, kcal/d[b]	1799 ± 429	1840 ± 451	1786 ± 421
Total iron, mg/d[b,c]	10.5 ± 1.5	10.6 ± 1.5	10.4 ± 1.4
Heme iron, mg/d[b,c]	1.8 ± 0.8	1.8 ± 0.8	1.8 ± 0.8
Non-heme iron, mg/d[b,c]	8.7 ± 1.3	8.9 ± 1.3	8.7 ± 1.3
Fiber, g/d[b,c]	22.5 ± 4.4	22.0 ± 4.4	22.7 ± 4.4
Dietary calcium, mg/d[b,c]	1093 ± 320	1064 ± 318	1102 ± 320
Dietary vitamin C, mg/d[b,c]	116 ± 45	114 ± 46	117 ± 45
Caffeine, mg/d[b,c]	389 ± 188	394 ± 200	387 ± 184

[a] Median (IQR).
[b] Mean ± standard deviation.
[c] Energy-adjusted.

separate analyses for menstruating and non-menstruating women (Figure 3). Since the number of subjects is reduced among menstruating women, heme iron intake was divided into two equal groups (< or ≥ median of 1.77 mg/day). We observed no difference in mean serum ferritin concentration between subjects carrying normal alleles and subjects with the intermediate HFE genotype at each level of heme iron intake. Compound and C282Y homozygous subjects show the

Table 2 Serum ferritin levels (μg/L) and transferrin saturation (%) according to HFE genotype and menstrual status in 1611 women participating in the Prospect-EPIC cohort

Genotype	Menstruating (n = 403)			Non-menstruating (n = 1208)		
	n (%)	Serum ferritin, μg/L[a]	Transferrin saturation, %[b]	*n* (%)	Serum ferritin, μg/L[a]	Transferrin saturation, %[b]
Wt/wt	270 (67)	43.1 (21.6–78.3)	21.0 ± 8.3	765 (63)	101.0 (63.5–159.5)	22.2 ± 6.4
H63D/wt	92 (23)	48.1 (19.3–91.9)	22.7 ± 9.7	289 (24)	106.0 (66.2–165.5)	23.6 ± 6.6
C282Y/wt	30 (7)	40.0 (17.8–71.1)	23.2 ± 8.7	116 (10)	113.5 (70.3–180.5)	25.0 ± 7.6
H63D/H63D	6 (2)	52.9 (32.9–100.5)	26.1 ± 6.7	17 (1)	136.0 (80.5–223.5)	28.4 ± 8.6
C282Y/H63D	4 (1)	16.7 (11.3–236.9)	24.9 ± 8.9	17 (1)	157.0 (60.9–258.0)	31.3 ± 6.7
C282Y/C282Y	1 (0.2)	738	55.1	4 (0.3)	241.0 (96.1–342.0)	42.3 ± 5.1

[a] Median (IQR).
[b] Mean ± standard deviation.

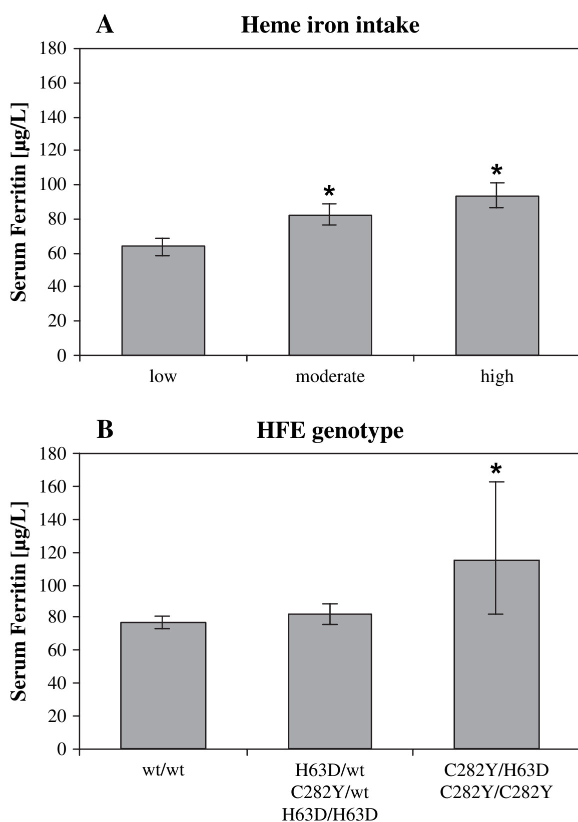

Figure 1 Serum ferritin concentrations (geometric mean and 95% confidence intervals (CI)) according to levels of heme iron intake (A) and HFE genotype (B). Ranges of heme iron intake are low (0.00—1.42 mg/d), moderate (1.42—2.09 mg/d), high (2.09—5.38 mg/d). $^{*}P < 0.05$ indicates significant difference from subjects with either low heme iron intake (A) or wt/wt genotype (B).

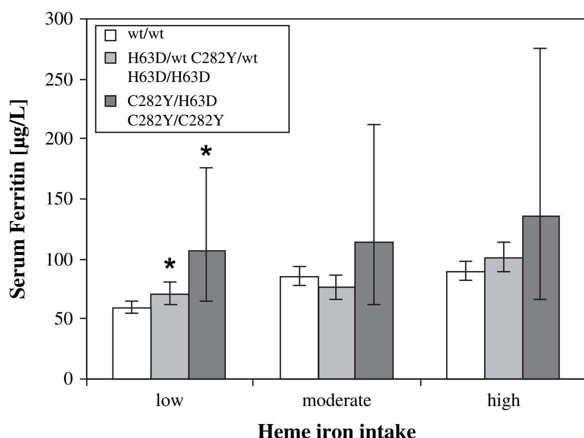

Figure 2 Serum ferritin concentrations (geometric mean and 95% confidence intervals (CI)) according to combined categories of heme iron intake and HFE genotypes. Ranges of heme iron intake are low (0.00—1.42 mg/d), moderate (1.42—2.09 mg/d), high (2.09—5.38 mg/d). *Different from the wt/wt genotype within the same cluster, $P < 0.05$.

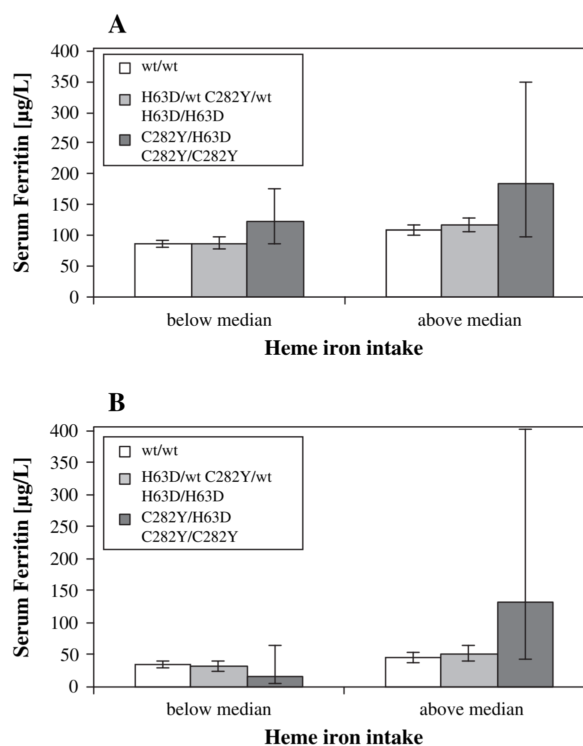

Figure 3 Serum ferritin concentrations (geometric mean and 95% confidence intervals (CI)) according to combined categories of heme iron intake and HFE genotypes. Analyses were stratified for non-menstruating (A) and menstruating (B) women. Median heme iron intake is 1.77 mg/d.

highest serum ferritin concentrations, both at low and at high heme iron intake.

All geometric mean serum ferritin concentrations were adjusted for factors previously described to affect serum ferritin concentrations. However, none of the variables attenuated the relation between heme iron intake or HFE genotypes and serum ferritin substantially, and therefore, unadjusted values are presented.

Discussion

To our knowledge this is the first study that characterizes HFE genotypes, serum ferritin concentrations and dietary heme iron intakes in a large population based sample of middle-aged women. We demonstrate that certain HFE genotypes are associated with elevated body iron levels as measured by serum ferritin. Apart from that, we found increased concentrations of serum ferritin with higher intakes of dietary heme iron. An interaction between these two factors, however, could not be demonstrated.

Menstruation lowers serum ferritin concentrations in general. Non-menstruating women with the compound or C282Y homozygous genotype, who consume ≥1.77 mg/day heme iron, present the highest mean serum ferritin concentration. Thirty-six percent of the women who reported presence of menstrual periods in the 12 months prior to inclusion indicated that this had occurred less than eight times. Because of these unstable menstrual patterns and the relatively small number of subjects, in this group serum ferritin concentrations are more prone to fluctuations. Unfortunately, we did not have any information about blood donation, which has been shown to significantly influence body iron stores [32]. In general, the proportion of blood donors is excessively low among women of this age and therefore a possible distorting effect on the results will be negligible.

Ferritin is an acute-phase protein, and its synthesis may be stimulated by inflammatory-cytokines produced as a result of infection and inflammation [33], thereby interfering with the reliability of serum ferritin as a reflection of iron stores. To control for inflammation we excluded women with high hsCRP concentrations (>6 mg/L) to see whether this had any influence on our results. However, this was not the case.

In recent years, various studies were set up to identify diet and host-related factors that influence iron absorption, resulting in a list of potential enhancers and inhibitors [34]. Because of this complexity, it is very difficult to find associations between dietary iron intake and iron status. Fleming et al. [26] demonstrated in a free-living elderly population that heme iron and supplemental iron intake were significant predictors of serum ferritin after adjustment for potential confounders. Each milligram of heme iron intake was associated with 46% higher serum ferritin (95%CI 22%, 74%) and use of iron supplements was associated with 63% higher serum ferritin (95% CI 28%, 109%). Recently, Liu et al. demonstrated, also in a population of healthy postmenopausal women, that heme iron intake is associated with higher serum ferritin concentrations [31].

We observed a positive association between iron intake and serum ferritin concentrations, as did many other studies [23,26,28,31,35–37], however, our findings conflict with several other reports that failed to find an association between iron intake and serum ferritin [38,39].

The HFE gene has been shown to encode a major histocompatibility complex (MHC) class I-like protein that, in its normal form, is able to form a non-covalent interaction with β_2-microglobulin (β_2M) [1]. The association of HFE with β_2M is needed for a proper cell-surface expression of HFE. How the HFE protein functions to regulate iron absorption still remains unclear. Studies have demonstrated that the HFE-β_2M complex is involved in the uptake of transferrin-bound iron by sensing the level of body iron stores [40]; i.e., the HFE protein may influence the affinity of plasma transferrin for its receptor [41]. In this population of middle-aged women, however, we were unable to detect an association between heme iron intake and transferrin saturation.

It is thought that iron homeostasis in the normal healthy population is tightly controlled by changes in absorptive efficiency. Hallberg et al. [42] demonstrated that beyond a serum ferritin concentration of 60 μg/L, iron absorption decreases to a level just sufficient to cover basal iron losses. In addition, a recently published study by Hunt et al. [43] demonstrated that C282Y heterozygotes do not absorb dietary iron more efficiently than do wildtype subjects. Our results showing no evidence of strong gene–nutrient interaction are in line with the results from Hunt et al., however, we do demonstrate that higher intakes of dietary heme iron are associated with higher concentrations of serum ferritin, even when mean concentrations exceed the point of 60 μg/L. It is important to note that the relationship between dietary heme iron intake and iron status holds for individuals with the wildtype genotype and the ''intermediate'' genotype. As slightly increased levels of body iron stores may be potentially harmful by playing a role in the etiology of cardiovascular disease [44–46], cancer [47,48], and diabetes [49,50], it is essential to get more insight into the factors that influence these body iron stores.

In conclusion our findings in a large cohort of middle-aged women support the view that dietary heme iron intake, as well as being compound or C282Y homozygous, affect serum ferritin concentrations. Women still experiencing menses seem to be protected from iron overload by this monthly blood loss.

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