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PREMENOPAUSAL PLASMA FERRITIN LEVELS, *HFE* POLYMORPHISMS, AND RISK OF BREAST CANCER IN THE NURSES' HEALTH STUDY II

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Abstract

Background—Evidence from the Nurses' Health Study II (NHSII) suggests that red meat consumption is associated with increased breast cancer risk in premenopausal women. Iron may be responsible by contributing to oxidative stress or effects on immune function.

Methods—We conducted a case-control study nested within the NHSII examining prediagnostic plasma ferritin (n=795 cases, 795 controls), 15 hemochromatosis gene (*HFE*) SNPs (n=765 cases, 1,368 controls), and breast cancer risk. Cases were diagnosed after providing blood samples between 1996 and 1999. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for ferritin levels by conditional logistic regression and for *HFE* SNPs by unconditional logistic regression.

Results—We did not observe a significant association between ferritin levels and breast cancer (top vs. bottom quartile multivariate OR: 1.05, 95% CI: 0.77–1.45, *P*-value for trend: 0.77). Results did not change when restricted to women who were premenopausal at blood draw, and were similar when cases were examined by hormone receptor status and menopausal status at diagnosis. No *HFE* SNPs were significantly associated with breast cancer in a log-additive manner. Among controls, ferritin levels were nominally associated with SNPs rs9366637 (*P*-value for trend: 0.04), rs6918586 (*P*-value for trend: 0.06) and rs13161 (*P*-value for trend: 0.07), but results did not remain significant after adjusting for multiple testing.

Conclusions—Ferritin levels and *HFE* SNPs were not associated with breast cancer risk in this population.

Impact—Components of red meat other than iron are likely responsible for its positive association with breast cancer in premenopausal women.

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Keywords

plasma ferritin; *HFE* polymorphisms; breast cancer; biomarker; iron

INTRODUCTION

In the United States, high iron body stores are common due to iron supplementation, enrichment of foods with iron, and high levels of meat intake (1–6). While iron is an essential micronutrient for DNA synthesis in addition to respiratory and oxidative cell metabolism, its pro-oxidative properties can render it carcinogenic (6, 7). Free iron can catalyze the formation of mutagenic hydroxyl radicals that, in turn, can cause increased oxidative stress, DNA damage, and oncogene activation (2, 4–6, 8–10). Iron also suppresses host defenses, thereby permitting cancer cell proliferation, and acts as a nutrient for unrestricted tumor cell multiplication (8, 11–13). Iron has been carcinogenic in animal models, and in several studies iron stores were positively associated with risks of certain human cancers, including colorectal and liver (2, 3, 14–19). Heme iron is of particular concern given that the body continues to absorb it even if stores are adequate (20).

The role of iron in breast carcinogenesis specifically has received little attention. A recent case-control study indicated that animal-derived (largely heme) iron intake was associated with a 50% elevated risk of breast cancer (21). In a prior analysis among premenopausal women in the Nurses' Health Study II (NHSII) cohort, we observed that greater intake of red meat, a primary source of heme iron, was associated with increased the risk of breast cancers with positive estrogen and progesterone receptors (ER+/PR+) (22). Few studies have examined the relationship between body iron stores and subsequent risk of breast cancer (15, 23–25). Ferritin binds iron in a nontoxic and nonreactive manner to store iron in a bioavailable form (26, 27), and thus serves as an ideal clinical indicator of body iron stores, and one that incorporates individual differences in iron intake and genetic variation.

In addition to evaluating plasma ferritin levels, examining genetic variation in iron regulatory pathways may further clarify the relationship between iron and breast cancer. The protein encoded by the hemochromatosis gene (*HFE*; 6p21.3) is thought to regulate iron absorption by binding to the cell-surface transferrin receptor, thereby reducing its affinity for iron-bound transferrin and affecting cellular iron status (28). Individuals homozygous for variant rs1800562 (substitution of tyrosine for cysteine at amino acid 282; C282Y) comprise a large majority of those with iron overload disorders (10, 28). Homozygosity for variant rs1799945 (substitution of aspartic acid for histidine at amino acid 63; H63D) and compound heterozygosity of rs1799945 (H63D) and rs1800562 (C282Y) also predispose to hereditary hemochromatosis with lower penetrance (29). Individuals with either variant tend to have high mean body iron stores (29–33). Mutations in *HFE* have been studied for their possible correlation with various cancers, and some prior studies have found rs1800562 (C282Y) and rs1799945 (H63D) mutations to be associated with breast cancer incidence in particular (34–37). However, no such associations have been seen in other studies (29, 38–41).

To improve our understanding of the role of iron in breast carcinogenesis, we examined the association between predominantly premenopausal plasma ferritin levels and incidence of both pre- and postmenopausal breast cancer in a case-control study nested within the prospective NHSII cohort. We further assessed whether particular *HFE* polymorphisms were associated with risk of breast cancer.

MATERIALS AND METHODS

Study population

The NHSII was established in 1989 when 116,430 registered nurses, aged 25 to 42 years completed a baseline questionnaire. Since then, participants have completed biennial self-administered questionnaires that assess various exposures and ascertain disease diagnoses.

Between 1996 and 1999, blood and urine samples were collected from 29,611 cohort members between the ages of 32 and 54 who were cancer-free. These women were similar to the overall cohort with respect to lifestyle factors, excepting a slight difference in the prevalence of family history of breast cancer (19% versus 15% in the overall cohort) (42). Participants were mailed a short questionnaire as well as a blood and urine collection kit containing the supplies necessary to have blood drawn by a local laboratory or colleague.

Among those who supplied samples, 18,521 were premenopausal and provided two blood samples timed within the menstrual cycle – one follicular sample collected on the third to fifth day and one luteal sample collected seven to nine days prior to the anticipated start of their next cycle. Participants aliquotted follicular plasma eight to 24 hours following sample collection and stored it in their personal freezers until their luteal collection. All other women (n=11,090) provided a single 30mL untimed sample. Both untimed and luteal blood samples (along with urine samples) were shipped overnight with an ice pack to our laboratory, processed and then separated into plasma, and red blood cell and white blood cell components. Since collection, all samples have been stored in liquid nitrogen freezers (< −130°C). This study was approved at the Committee on the use of Human Subjects in Research at Harvard School of Public Health and Brigham and Women's Hospital. Participants implied informed consent by completing questionnaires and providing blood samples.

Cases

Participants reported diagnoses of breast cancer on biennial questionnaires. For non-responders, we searched the National Death Index. Women with breast cancer were considered to be cases if they had no previously reported cancer diagnosis and were diagnosed with breast cancer after blood draw and before June 2009. A total of 827 breast cancer cases were eligible for ferritin assays (plasma available) and/or genotyping (white blood cells available), of which 796 were confirmed by medical record and 31 were confirmed verbally by a nurse. Given that 99% of reported cases are confirmed by medical record review, we elected to include the latter cases. Data regarding invasiveness and hormone receptor status were abstracted from medical records. Mean time from blood draw to diagnosis was 6.1 years (range: 1 month to 13.3 years).

Two controls were matched to each case diagnosed through 2007 and one control was matched to the remaining cases. Matching was based on age (± 2 years), ethnicity (African-American, Asian, Hispanic, Caucasian, Other), menopausal status at blood draw and diagnosis, month/year of specimen collection (± 2 months), luteal day (timed samples only, date of next period - date of luteal blood draw; ± 1 day), and for each blood draw, time of day (± 2 hours) and fasting status (< 2 hours, 2 to 4, 5 to 7, 8 to 11, 12+).

We excluded 32 cases from analyses of plasma ferritin due to unavailable plasma or failed assays for the case sample or its matched control sample. Plasma ferritin analyses included one matched control per case, for a total of 795 cases and 795 controls. Analyses of genetic data excluded samples lacking white blood cells, non-Caucasian participants, and samples that failed genotyping (n=62 cases). Genetic analyses included a subset of cases with one

matched control and the majority of cases that had two matched controls, for a total of 765 cases and 1,368 controls.

Laboratory assays for plasma ferritin

Plasma ferritin levels were assayed at the Clinical & Epidemiologic Research Laboratory at Children's Hospital, Boston with an electrochemiluminescence immunoassay, a quantitative sandwich enzyme immunoassay technique on the Roche E Modular system (Roche Diagnostics, Indianapolis, IN). Case-control sets were assayed together and samples were ordered randomly and labeled so as to mask case-control status. Samples were assayed in two batches; coefficients of variation from masked replicate quality control samples included in each batch were ≤10.5% in both batches.

Reproducibility study

We assessed the reproducibility of plasma ferritin measurements over time among participants of the Nurses' Health Study (NHS), a cohort of 121,700 female registered nurses aged 30 to 55 years at baseline in 1976 (43). Between 1989 and 1990, we collected blood samples from 32,826 participants. A subset of 186 of those participants provided additional samples during the following three years. These women were postmenopausal, had not used postmenopausal hormone therapy for at least three months, and had no previous diagnosis of cancer (excepting nonmelanoma skin cancer) at the time of each blood collection. Two blood specimens from a random sample of 40 of these participants were assayed for ferritin levels at the same laboratory.

SNP selection and genotyping methods

To select *HFE* SNPs for evaluation, we utilized the Tagger algorithm (44) in the HaploView program (45) and dense genotyping data from the HapMap Release 28 CEU panel. We identified 15 SNPs to capture variation with a coefficient of determination (R^2) >0.8 with coverage between 20 kb upstream and 10 kb downstream of the *HFE* gene. We prioritized functional SNPs rs1799945 (H63D) and rs1800562 (C282Y), but otherwise restricted selection to tagging SNPs with a minor allele frequency greater than five percent in the reference panel.

SNP genotyping was performed at the Dana Farber/Harvard Cancer Center High Throughput Polymorphism Detection Core, using Taqman OpenArray SNP Genotyping Platform (Applied Biosystems). Samples from matched case-control pairs were handled identically and genotyped in the same batch in a blinded fashion. Blinded duplicate samples were genotyped across batches for quality control. All SNPs had genotype completion rates greater than 95% and concordance was 100% for the blinded quality control samples.

Covariate data

We obtained information about breast cancer risk factors from questionnaires completed at the time of blood draw and from biennial NHSII questionnaires. Age at menarche, weight at age 18, and height were asked at baseline in 1989. Age at first birth, parity, and diagnosis of benign breast disease are assessed biennially. Family history of breast cancer (in mothers and/or sisters) was asked in 1989 and 1997. Women were asked about the regularity and length of their menstrual cycles on the 1993 questionnaire. Weight was assessed at the time of blood draw.

Meat intakes were assessed by semiquantitative food questionnaire (FFQ) in 1991 and 1995. For each item listed on the FFQ, a commonly used unit or portion size is specified, and participants are asked how often, on average, over the past year, they have consumed that

amount of each food, choosing from nine possible frequencies, ranging from never or less than once per month to six or more times per day. Processed red meat intake was calculated by summing servings of bacon, hot dogs and other processed meats. Unprocessed red meat intake was calculated by summing beef or lamb as a main dish, pork as a main dish, beef or pork or lamb as a sandwich or mixed dish and hamburger. Total red meat intake was calculated by summing processed and unprocessed red meat.

Statistical analysis

We assessed the reproducibility of log-transformed plasma ferritin over time by dividing the between-person variance by the sum of the within-person and between-person variances to calculate the intraclass correlation coefficient (ICC).

We assessed statistical outliers in ferritin levels using the generalized extreme Studentized deviate many-outlier detection approach (46); none were detected. We then calculated quartile cut points according to the distribution in the controls.

We used conditional logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). Multivariate models adjusted for body mass index (BMI) at age 18 and weight change between age 18 and time of blood draw, ages at menarche and first birth, parity, family history of breast cancer, and history of benign breast disease. We conducted tests for trend by modeling quartile median concentrations as single variables and calculating the Wald statistic.

Given that red meat intake is a primary source of iron and is somewhat correlated with plasma ferritin levels in our data (Spearman correlation: 0.08), we did not include it in our primary analysis. We did, however, adjust for it in a secondary analysis. Because menstruation is a primary mechanism whereby women rid excess iron (47), we also adjusted for both cycle regularity and length in 1993 in an additional secondary analysis. To exclude any possible influence of undiagnosed disease on plasma ferritin levels, we also conducted analyses in which we excluded cases diagnosed within two years of blood draw. Additional sensitivity analyses restricted to women who were premenopausal at blood draw, and to women who did not take iron supplements or multivitamins around the time of blood draw.

For analyses of cases characterized by hormone receptor status and menopausal status at diagnosis, and analyses restricted to invasive breast cancer cases, we implemented unconditional logistic regression adjusting for matching factors, since results from multivariate unconditional and conditional logistic regression models were comparable.

All genetic analyses were restricted to Caucasian participants in order to diminish the likelihood of population stratification (n=765 cases). We evaluated each *HFE* SNP for deviation from Hardy-Weinberg equilibrium among controls using the Pearson's goodness-of-fit test with a cutoff of 0.01.

We calculated ORs and 95% CIs for each *HFE* SNP and breast cancer incidence from unconditional logistic regression models adjusted for age at blood draw. For each model, we coded the SNP genotype additively as copies of the minor allele (0, 1, 2). We report the nominal 2-sided *P*-values without adjusting for multiple comparisons, but we also calculated the number of effective independent tests using a method that accounts for linkage disequilibrium within the set of SNPs (48). The 15 SNPs corresponded to 12 independent tests. As such, a *P*-value significance threshold of 0.004 controls the experiment-wide type I error rate at the 0.05 level.

Because the two functional SNPs, rs1799945 (H63D) and rs1800562 (C282Y), act in a recessive manner for hemochromatosis, we also ran models for each assuming a recessive mode of inheritance. We furthermore created a genetic score in which homozygotes for either risk allele and compound heterozygotes received a score of one, and all other allele combinations received a score of zero. The r^2 between rs1799945 (H63D) and rs1800562 (C282Y) was −0.10. We modeled the genetic score as a dummy variable. For all models, statistical significance was determined using a likelihood ratio test (LRT) with one degree of freedom.

We used generalized linear models adjusted for age at blood draw to assess the associations between *HFE* SNPs and plasma ferritin levels among Caucasian controls for whom we had both plasma and genetic data (n=735 controls). Ferritin levels were natural log-transformed to improve the normality of the data. We conducted tests for trend by modeling the number of minor alleles as continuous variables and calculating the Wald statistic.

In order to evaluate any interaction between plasma ferritin levels and *HFE* genotypes for breast cancer risk, we dichotomized plasma ferritin at its median value and ran unconditional logistic regression models adjusted for age at blood draw of the per-allele effect of each SNP on breast cancer risk within each of the two levels of plasma ferritin. *P*-values for interaction were calculated using an LRT with one degree of freedom based on per-allele ORs and a continuous plasma ferritin variable.

All tests were 2-sided with *P*-values less ≤0.05 considered to be statistically significant (unless otherwise specified above). Analyses were conducted using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA) and R (http://www.r-project.org/) statistical package.

This study was approved by the institutional review boards at the Harvard School of Public Health and Brigham and Women's Hospital.

RESULTS

The 40 participants of the NHS who provided two blood samples did so an average of 22 months apart (range, 15 to 29 months). The within-person reproducibility over time for ferritin was high, with an ICC of 0.81.

Among the 795 cases and 795 controls in the plasma analyses, the mean age at blood draw was 45 years (range, 33 to 53 years); 76% were premenopausal at blood collection. Controls had a higher mean BMI at both age 18 and at blood draw and had gained more weight between the two measurements than cases. Cases had fewer children than controls and were more likely to have a family history of breast cancer and a personal history of benign breast disease (data not shown). Among controls, women with higher plasma ferritin levels were older, less likely to be premenopausal, had higher BMI at both age 18 and blood draw, ate more red meat, and were more likely to use iron supplements (Table 1).

In a simple conditional logistic regression model, we did not observe a significant association between plasma ferritin and breast cancer risk (top vs. bottom quartile OR: 1.04, 95% CI: 0.77–1.40, *P*-value for trend: 0.99) (Table 2). Adjustment for additional breast cancer risk factors did not materially change the results (OR: 1.05, 95% CI: 0.77–1.45, *P*-value for trend: 0.77), nor did further adjustment for total red meat intake (OR: 1.04, 95% CI: 0.75–1.43, *P*-value for trend: 0.87). Adjustment for menstrual cycle characteristics also did not change the results (OR: 1.07, 95% CI: 0.77–1.48, *P*-value for trend: 0.74). Results were similar in all sensitivity analyses and among invasive cancers, by hormone receptor status, and when characterized by menopausal status at diagnosis. An *a-posteriori* analysis

comparing women with ferritin levels ≥150 ng/mL to women with levels <150 ng/mL also provided null results (OR: 0.88, 95% CI: 0.55–1.41, *P*-value: 0.59).

Genotype frequencies were in Hardy-Weinberg equilibrium among controls. None of the *HFE* SNPs were associated with breast cancer incidence in a log-additive manner (Table 3). Further assessment of the functional SNPs rs1799945 (H63D) and rs1800562 (C282Y) coded in a recessive manner yielded null results as well (data not shown). The genetic score that penalized the possession of at least two risk alleles also was not associated with breast cancer risk (OR: 0.88, 95% CI: 0.58–1.33, *P*-value: 0.54).

We found a suggestion of associations between increasing numbers of minor alleles for SNPs rs9366637, rs6918586 and rs13161 and higher levels of plasma ferritin among controls (Table 3). Although only eight controls had two minor alleles for rs1800562 (C282Y), their average ferritin level was higher than controls with zero or one minor allele (108.6 vs. 41.1 ng/mL *P*-value: 0.009). A test for trend across the number of minor alleles, however, was not significant (*P*-value: 0.53). Ferritin levels among those with at least two risk alleles across rs1800562 (C282Y) and rs1799945 (H63D) were marginally higher than those with fewer than two risk alleles (57.3 vs. 40.8 ng/mL, *P*-value: 0.04).

Associations between each *HFE* SNP and breast cancer risk did not differ across levels of plasma ferritin (data not shown).

DISCUSSION

In this analysis, among women who were predominantly premenopausal at blood draw, we did not observe an association between plasma ferritin levels and risk of breast cancer. Results were also null in invasive cases and both ER+/PR+ and ER−/PR− tumors, as well as from all stratified and restricted analyses. We also did not find significant associations between 15 *HFE* SNPs and breast cancer risk.

Few studies have evaluated the association between body stores of iron and breast cancer risk and results have been inconsistent. In 1994, Knekt and colleagues did not observe an association between serum iron, total iron binding capacity or transferrin saturation and breast cancer risk in their cohort study (n=192 cases) (15). Huang and colleagues found similarly null results for serum iron and breast cancer in their case-control study from 1999 (n=35 cases) (49). More recently, two case-control studies observed modest positive associations between ferritin levels and breast cancer risk (24, 25). Moore and colleagues examined a Chinese population (n=130 cases, RR for log[ferritin]: 1.20, 95% CI: 1.00–1.44), but lacked sufficient power to detect differences by menopausal status (24). In a study of primarily postmenopausal Japanese atomic bomb survivors (n=107 cases, RR for log[ferritin]: 1.3, 95% CI: 1.0–1.7), Stevens and colleagues were unable to adjust for several possible confounders (25). Our study is the first to focus on premenopausal iron levels and our results are consistent with those of the null studies.

Despite the primarily null results from studies assessing body iron stores and breast cancer risk, several other studies have shown breast cancer tissue to have significantly higher levels of ferritin, transferrin and transferrin receptor proteins than normal or benign tissue (50–54). They have not shown, however, whether excess iron precedes cancer development or occurs as a result of carcinogenesis.

The association between red meat, a major source of heme iron, and breast cancer risk has been extensively studied and summarized in various pooled and meta-analyses (22, 55–57). In our prior work in the NHSII, higher red meat intake was associated with ER+/PR+ breast cancer risk (n=1,021 cases, RR for >1.5 servings per day versus ≤3 servings per week: 1.97,

95% CI: 1.35–2.88, *P*-value for trend: 0.001) (22). Our assessment of plasma ferritin levels in the same cohort showed no association with ER+/PR+ breast cancer, indicating that components of red meat other than iron may explain the positive association with hormone receptor-positive breast cancer.

Other studies of dietary iron intake have yielded conflicting results. Two early case-control studies observed inverse associations with breast cancer risk (58, 59). More recently, however, two case-control studies of dietary iron were null (24, 60), and a case-control analysis from the Shanghai Breast Cancer Study observed *animal-derived* iron intake to be positively associated with breast cancer risk (n=3,452 cases, OR comparing the highest versus lowest quartile: 1.49, 95% CI: 1.25–1.78, *P*-value for trend: <0.01) (21). Prospective cohort studies have been mostly null (49, 61), with the exception of a study in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, which observed a modest positive association between dietary iron and risk of breast cancer (n=1,205 cases, RR highest versus lowest quintile: 1.25, 95% CI: 1.02–1.52, *P*-value for trend: 0.03) (62).

Results of prior studies of rs1799945 (H63D) and rs1800562 (C282Y) mutations have been inconsistent (29, 34–41). For studies of rs1799945 (H63D), case numbers have ranged from 18 to 605. Among those that coded rs1799945 (H63D) in a dominant manner (29, 34, 37, 40), the effect sizes have ranged from 0.5 (95% CI: 0.2–1.2) to 4.4 (95% CI: 1.4–14.1). No studies that showed an inverse association yielded statistically significant results (29, 40), but two of the studies showing a positive association demonstrated statistical significance (29, 34). For studies of rs1800562 (C282Y), case numbers have ranged from 18 to 664 (35–41). The largest of the prior studies (nine rs1800562 (C282Y) homozygote cases and 90 homozygotes in the referent group) was the only one to observe a significantly increased risk of breast cancer relative to those with no rs1800562 (C282Y) variant (n=664 cases, hazard ratio: 2.39, 95% CI: 1.24–4.61) (36). Our study included fewer rs1800562 (C282Y) homozygotes (n=13), and we did not find any association with breast cancer risk. We also did not observe associations between the remaining *HFE* SNPs evaluated and breast cancer risk. We did, however, discover that some SNPs may be associated with plasma ferritin levels. That these SNPs were not associated with breast cancer risk supports the null results from our analyses of plasma ferritin and breast cancer risk.

Our study had some limitations, but it is unlikely that they are responsible for our null results. Although we were only able to collect one measurement of plasma ferritin as a representation of long-term exposure, our reproducibility data suggest that one sample is an adequate representation of plasma ferritin over at least several years. The reproducibility that we demonstrated is better than other biomarkers with well-established relationships to disease outcomes in epidemiological studies, such as cholesterol (ICC: 0.65) (63) and blood pressure (ICC: 0.60–0.64) (64). Plasma ferritin does not capture exposure to heme iron in particular, which could be important in breast carcinogenesis given its capacity to absorb even in the presence of ample body iron stores. It is also possible that because our population was predominantly premenopausal at blood draw, ferritin levels were not high enough to result in increased breast cancer risk; our prior observed association between red meat intake and breast cancer risk, however, was in premenopausal women (22). In addition, our analysis of women with ferritin levels ≥150 ng/mL relative to women with levels <150 ng/mL returned null results. Regarding the analyses of *HFE* SNPs and breast cancer risk, our study was underpowered to detect associations of small magnitude. For example, we had 85% power to detect an OR of 1.30 for SNPS with a minor allele frequency of 0.30 at an alpha of 0.004. For SNPs with a minor allele frequency of 0.10, we had 80% power to detect an OR of 1.45. We cannot rule out the possibility that the *HFE* SNPs we tested have weak associations with breast cancer risk. Our power to evaluate interactions between *HFE* SNPs and plasma ferritin was also low, so we could not rule out weak interactions on the risk of

breast cancer. This study also had several strengths, including a large number of premenopausal cases, extensive data on potential confounders, plasma ferritin measurements prior to breast cancer diagnosis, and incorporation of genetic variation with plasma ferritin levels.

Modifiable risk factors for breast cancer have remained elusive. That dietary iron is the primary source of human stores (65, 66), that its intake is modifiable, and that breast cancer is the most common malignancy in women, gives a potential carcinogenic role of iron accumulation critical public health implications. Our results do not support the hypothesis that body iron stores are associated with an increased risk of breast cancer risk. Although iron is one potential mechanism by which red meat intake may increase breast cancer risk, it is possible that there are other mechanisms involved. Future studies should explore other components that may be responsible for this association.

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Table 1

Characteristics of study controls by plasma ferritin quartile in the NHSII (Mean (SD) or %)

		Ferritin Quartile, ng/mL			
	Range:	**≤ 23.9**	**> 23.9 – ≤ 42.1**	**> 42.1 – ≤ 72.0**	**> 72.0**
Characteristic	**Median:**	**15.0**	**32.2**	**55.0**	**107**
Number		198	199	199	199
Age at Blood Draw, years		44.2 (4.1)	44.2 (4.4)	45.0 (4.5)	46.2 (4.4)
Age at Menarche, years		12.6 (1.4)	12.5 (1.5)	12.5 (1.4)	12.4 (1.5)
BMI at Age 18, kg/m^2		20.7 (2.7)	21.0 (3.2)	21.0 (2.8)	21.5 (3.2)
BMI at Blood Draw, kg/m^2		24.8 (5.4)	25.3 (5.3)	25.4 (5.2)	28.2 (6.8)
Weight Change Since Age 18, kg		10.9 (11.7)	11.7 (11.1)	12.0 (12.0)	18.0 (15.4)
Nulliparous		16.2%	20.6%	18.6%	19.6%
Parity, children[a]		2.5 (1.0)	2.4 (0.9)	2.3 (1.0)	2.2 (1.0)
Age at First Birth, years[a]		25.9 (3.9)	26.3 (4.5)	26.8 (4.9)	26.2 (5.1)
Ever Breast Fed[a]		80.7%	81.7%	80.9%	75.6%
Ever Use of Oral Contraceptives		83.3%	84.4%	91.0%	90.0%
Duration of Oral Contraceptive Use, months[b]		51.2 (41.7)	53.5 (45.3)	50.6 (47.8)	54.3 (44.4)
Family History of Breast Cancer		13.1%	11.6%	4.5%	11.1%
History of Biopsy-Confirmed Benign Breast Disease		16.2%	17.6%	16.1%	11.1%
Premenopausal at Blood Draw		91.4%	83.9%	73.4%	53.8%
Use of Iron Supplements at Blood Draw		6.7%	4.6%	7.1%	10.0%
Total Red Meat Intake, servings/week[c]		4.9 (3.1)	5.1 (3.2)	5.0 (3.1)	5.7 (3.6)
Processed Red Meat Intake, servings/week[c]		1.3 (1.3)	1.3 (1.5)	1.2 (1.3)	1.5 (1.7)
Unprocessed Red Meat Intake, servings/week[c]		3.6 (2.3)	3.8 (2.5)	3.8 (2.4)	4.2 (2.4)
Saturated Fat Intake, grams/day[c]		21.1 (7.1)	21.8 (7.7)	20.7 (7.1)	21.9 (8.4)
Energy Intake, kcal/day[c]		1,790 (456)	1,867 (504)	1,776 (478)	1,806 (523)

Abbreviations: BMI – Body Mass Index; SD – Standard Deviation

[a] Among 646 parous women

[b] Among 693 ever users of oral contraceptives

[c] Average intake for 1991 and 1995

Table 2

Odds ratios and 95% confidence intervals for breast cancer incidence according to quartile of plasma ferritin (ng/mL) in the NHSII

	Plasma Ferritin Quartile, ng/mL				
	≤ 23.9	**> 23.9 – ≤ 42.1**	**>42.1 – ≤ 72.0**	**> 72.0**	***P*-value[a]**
Overall					
Cases/Controls	196/198	217/199	177/199	205/199	
Simple[b]	1.00 (ref)	1.11 (0.84, 1.46)	0.89 (0.67, 1.19)	1.04 (0.77, 1.40)	0.99
Multivariate[c]	1.00 (ref)	1.04 (0.78, 1.39)	0.85 (0.62, 1.15)	1.05 (0.77, 1.45)	0.77
Invasive					
Cases/Controls	127/198	155/199	126/199	142/199	
Multivariate[d]	1.00 (ref)	1.21 (0.88, 1.66)	0.96 (0.69, 1.33)	1.06 (0.75, 1.49)	0.90
ER+/PR+					
Cases/Controls	82/198	105/199	88/199	95/199	
Multivariate[d]	1.00 (ref)	1.31 (0.91, 1.89)	1.08 (0.74, 1.59)	1.14 (0.77, 1.69)	0.88
ER−/PR−					
Cases/Controls	26/198	26/199	15/199	19/199	
Multivariate[d]	1.00 (ref)	0.93 (0.51, 1.70)	0.54 (0.27, 1.08)	0.70 (0.35, 1.39)	0.24
Premenopausal at Diagnosis					
Cases/Controls	113/117	127/121	88/100	78/64	
Multivariate[d]	1.00 (ref)	1.02 (0.70, 1.48)	0.87 (0.58, 1.30)	1.21 (0.77, 1.88)	0.47
Postmenopausal at Diagnosis					
Cases/Controls	62/66	62/51	71/73	104/111	
Multivariate[d]	1.00 (ref)	1.37 (0.79, 2.37)	1.11 (0.65, 1.88)	1.09 (0.65, 1.83)	0.87

[a] *P*-values from tests for trend

[b] Conditional logistic regression model accounting only for matching factors

[c] Conditional logistic regression model accounting for matching factors and further adjusted for age at menarche (<12, 12, 13, ≥14 years), BMI at age 18 (<18.5, 18.5–<21, 21–<23, ≥23 kg/m^2), weight change since age 18 (<5, 5–<20, ≥20 kg), parity and age at first birth (nulliparous, 1–2 children and <25 years, 1–2 children and 25–29 years, 1–2 children and ≥30 years, ≥3 children and <25 years, ≥3 children and ≥25 years), family history of breast cancer (yes, no), personal history of benign breast disease (yes, no)

[d] Unconditional logistic regression model adjusted for covariates in the other multivariate models and adjusted for matching factors: age at blood draw (continuous), race (Caucasian, other), menopausal status at blood draw (premenopausal, postmenopausal, unknown menopausal status), menopausal status at diagnosis (premenopausal, postmenopausal, unknown menopausal status), month of blood draw (continuous), luteal day at blood draw (<8, ≥8, untimed collected), time of day at blood draw (12am – 8am, 9am – 12pm, 1pm – 11pm), fasting status at blood draw (yes, no)

Table 3

Minor allele frequencies, odds ratios (ORs) and 95% confidence intervals (CIs) for *HFE* SNPs and breast cancer incidence and associations with plasma ferritin levels in the NHSII among women of European ancestry

rs Number	Location	Genotype	Frequency in Cases	Frequency in Controls	Per-Allele OR (95% CI)[a]	P-value[b]	Ferritin Level, ng/mL[c]	P-value[d]
		TT	35.1%	35.5%			41.73	
rs2794719	Intronic	GT	47.5%	47.4%	1.02 (0.89, 1.15)	0.81	40.00	0.66
		GG	17.4%	17.0%			44.89	
		CC	86.7%	88.7%			40.09	
rs9366637	Intronic	CT	12.9%	10.6%	1.15 (0.90, 1.48)	0.28	50.16	0.04
		TT	0.4%	0.7%			39.50	
		CC	72.3%	73.7%			40.52	
rs1799945 (H63D)	Non-Synonymous Coding	CG	25.6%	23.6%	1.04 (0.87, 1.23)	0.70	43.67	0.42
		GG	2.1%	2.6%			39.05	
		TT	43.9%	44.9%			40.43	
rs2071303	Splice Site, Intronic	CT	44.1%	44.1%	1.05 (0.92, 1.20)	0.50	40.90	0.24
		CC	12.0%	11.0%			47.37	
		GG	89.1%	87.1%			41.38	
rs1800562 (C282Y)	Non-Synonymous Coding	GA	10.2%	12.3%	0.85 (0.66, 1.11)	0.23	38.76	0.53
		AA	0.7%	0.6%			108.71	
		GG	59.6%	58.6%			42.51	
rs707889	Intronic	AG	36.8%	36.7%	0.94 (0.81, 1.10)	0.46	39.44	0.17
		AA	3.7%	4.7%			37.06	
		GG	80.2%	79.6%			41.43	
rs1045537	Non-Synonymous Coding	CG	19.1%	19.5%	0.95 (0.77, 1.18)	0.66	39.34	0.87
		CC	0.7%	0.9%			84.63	
		GG	78.9%	78.3%			40.78	
rs17596719	Downstream	AG	20.1%	20.6%	0.96 (0.79, 1.18)	0.71	42.42	0.27
		AA	0.9%	1.0%			67.78	

rs Number	Location	Genotype	Frequency in Cases	Frequency in Controls	Per-Allele OR (95% CI)[a]	P-value[b]	Ferritin Level, ng/mL[c]	P-value[d]
		TT	34.6%	35.4%			39.05	
rs6918586	Downstream	CT	50.5%	49.7%	1.02 (0.89, 1.16)	0.83	40.90	0.06
		CC	14.8%	14.9%			48.00	
		AA	39.1%	38.0%			43.34	
rs198852	Synonymous	AG	46.1%	48.5%	1.01 (0.88, 1.15)	0.93	39.93	0.36
		GG	14.9%	13.5%			41.01	
		GG	87.8%	88.5%			40.82	
rs2051542	Non-Synonymous Coding	AG	11.8%	10.9%	1.05 (0.81, 1.35)	0.73	45.29	0.35
		AA	0.4%	0.6%			39.40	
		GG	52.1%	53.5%			39.99	
rs198839	Upstream	GT	41.4%	39.2%	1.02 (0.88, 1.17)	0.81	42.52	0.16
		TT	6.5%	7.2%			47.04	
		AA	70.9%	72.5%			40.80	
rs198833	Downstream	AG	26.6%	24.5%	1.04 (0.88, 1.23)	0.66	42.19	0.44
		GG	2.5%	3.0%			48.05	
		TT	27.5%	28.8%			38.19	
rs13161	3′ Untranslated	CT	52.8%	51.3%	1.02 (0.90, 1.16)	0.72	41.77	0.07
		CC	19.7%	19.9%			44.90	
		GG	68.6%	68.7%			42.86	
rs707896	Intronic	AG	29.6%	28.8%	0.99 (0.83, 1.17)	0.86	37.48	0.10
		AA	1.8%	2.4%			42.37	

Note: Numbers may not add up as expected due to rounding

Note: The number of cases/controls for analyses of SNPs and breast cancer incidence varies across SNPs from 748–761/1,334–1,359

Note: The number of controls for analyses of SNPs and plasma ferritin levels varies across SNPs from 720–733

Note: A *P*-value significance threshold of 0.004 controls the experiment-wide type I error rate at the 0.05 level

[a] Obtained from unconditional logistic regression models adjusted for age at blood draw

[b] *P*-values determined by likelihood ratio tests with 1 degree of freedom

[c] Obtained from generalized linear models among controls adjusted for age at blood draw

[d] Type III Wald *P*-values generated from modeling the number of minor alleles as continuous variables