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Heritability of Serum Iron Measures in the Hemochromatosis and Iron Overload Screening (HEIRS) Family Study

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Abstract

Heritability is the proportion of observed variation in a trait among individuals in a population that is attributable to hereditary factors. The HEIRS Family Study estimated heritability of serum iron measures. Probands were *HFE* C282Y homozygotes or non-C282Y homozygotes with elevated transferrin saturation (TS > 50%, men; TS > 45%, women) and serum ferritin concentration (SF > 300 μg/L, men; SF > 200 μg/L, women). Heritability (h^2) was estimated by variance component analysis of TS, natural logarithm (ln) of SF, and unsaturated iron-binding capacity (UIBC). Participants (N=942) were 77% Caucasians, 10% Asians, 8% Hispanics, and 5% other race/ethnicities. Average age (SD) was 49 (16) y; 57% were female. For *HFE* C282Y homozygote probands and their family members, excluding variation due to *HFE* C282Y and H63D genotype and measured demographic and environmental factors, the residual h^2 (SE) was 0.21 (0.07) for TS, 0.37 (0.08) for ln SF, and 0.34 (0.08) for UIBC (all P < 0.0004 for comparisons with zero). For the non-C282Y homozygote proband group, residual h^2 was significant with a value of 0.64 (0.26) for ln SF (p=0.0096). In conclusion, serum iron measures have significant heritability components, after excluding known genetic and non-genetic sources of variation.

Keywords

HFE; familial aggregation; transferrin saturation; serum ferritin concentration

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Introduction

Hereditary hemochromatosis (HH) occurs in 0.2%–0.5% of US Caucasians [1,2]. Some persons with HH absorb excessive dietary iron and develop liver fibrosis and cirrhosis, hepatocellular carcinoma, diabetes mellitus, cardiomyopathy, and hypogonadotrophic hypogonadism [3]. Most persons with HH are homozygous for the C282Y mutation in the *HFE* gene on chromosome 6p21.3 [4–6]. H63D, another common *HFE* mutation, is infrequently associated with iron overload [7–9]. Other persons with hemochromatosis do not have either mutations C282Y or H63D [4]. Among *HFE* C282Y homozygotes, the spectrum of iron-related phenotypes is broad. Gender, age, diet, and blood loss account for some phenotypic variability [10–12]. Mutations in known iron-related genes other than *HFE* explain phenotypic variability in a small proportion of cases [13]. Thus, much of the phenotypic variability among C282Y homozygotes is likely attributable to environmental or non-*HFE* heritable factors.

Heritability (h^2) is defined as the proportion of total variance of a particular measurement in a population, taken at a particular time or age, that is attributable to variation in heritable factors [14]. Thus, heritability estimates are also affected by factors that may influence laboratory measurements used to assess iron phenotypes. Heritability analysis of a trait is performed using data from genetic relatives, incorporates data for an observed phenotype, and partitions observed variation into unobserved genetic and environmental factors [14]. Residual heritability is estimated after excluding variation in the trait due to measures included as covariates in the analysis.

The HEIRS Study is a multi-center, multi-ethnic study in which transferrin saturation (TS), serum ferrintin level (SF), unbound iron-binding capacity (UIBC), and *HFE* mutations were determined in 101,168 adults [15,16]. We hypothesized that genetic factors influence serum iron measures after excluding variation in these measures in HEIRS Study participants due to gender, age by gender interaction, C282Y and H63D genotype, and other clinical and demographic characteristics. Thus, we examined the heritability of serum iron phenotypes in participants in the HEIRS Family Study.

Methods

Study population

HEIRS Study participants ≥ 25 years old were recruited and screened as described in detail elsewhere cLaren, 2003 #195}. Participants with C282Y homozygosity or both TS and SF values above gender-specific thresholds (TS >50% and SF > 300 μg/L in men; TS > 45% and SF > 200 μg/L in women) participated in a clinical examination in which they completed personal and family medical history, and food frequency questionnaires. They received a brief physical examination, a blood draw, and appropriate genetic counseling. Based on results of the examination, a provisional diagnosis of iron overload was made (defined as confirmed elevations of both SF and TS with no evidence of inflammation, elevation of serum concentration of hepatic transaminases, or secondary iron overload defined as a lifetime history of anemia or more than 10 units of blood transfusion).

Participants categorized as having provisional iron overload, and all C282Y homozygotes, were defined as probands for the present study if a minimum number of first-degree relatives (biologic parents, full siblings, offspring) aged ≥ 19 years were also available for study. The HEIRS eligible family structures are described elsewhere; the minimum number of first-degree relative was two full siblings of the proband [17]. Family members and additional age-eligible first-degree relatives of eligible probands were invited for a similar clinical examination. Individual race/ethnicity was determined by self reports as described

elsewhere [15]. Institutional review boards at each Study site reviewed and approved the Study.

Phenotypes and *HFE* genotypes

Serum iron concentration, TS, SF and UIBC were measured as previously reported [16]. SF values were transformed by natural logarithms for statistical analyses. C282Y and H63D genotypes were determined in probands and family members using a PCR-RFLP technique [4,18]. Lack of a detectable C282Y or H63D mutation is designated as *HFE* wild-type (wt/wt). Verification of reported familial relationships and integration of results from genome-wide linkage scan error checking were performed as described previously [17].

Statistical Analysis

Heritability analyses of TS, ln SF, and UIBC were performed using a variance component approach as implemented in Sequential Oligogenic Linkage Analysis Routines (SOLAR) software [19]. Stratified analyses were based on proband *HFE* genotype; families with a C282Y homozygote proband were analyzed separately from those with a non-C282Y homozygote proband. Models are described in the Supplementary Statistical Methods. Bivariate analyses were conducted to calculate estimates of genetic (r_G) and environmental (r_E) correlation *between* serum iron measures, after accounting for effects of *HFE* C282Y and H63D genotype and the additional covariates. For genetic correlations between serum iron measures, two-sided tests for the null hypothesis $\rho_G = 0$ versus the alternative hypothesis $\rho_G \neq 0$ were conducted as well as tests for the null hypothesis $\rho_G = 1$ versus the alternative hypothesis $\rho_G \neq 1$. To examine environmental correlations between serum iron measures, two-sided tests for the null hypothesis $\rho_E = 0$ were conducted.

Heritability (h^2) and residual h^2 were estimated using total additive genetic heritability under a polygenic model. Because non-C282Y homozygote proband families were ascertained through probands with elevated serum iron measures, all variance component analyses in the non-C282Y homozygote proband group included proband ascertainment correction as implemented in the SOLAR software [19]. P values ≤ 0.05 were defined to be statistically significant.

Results

Characteristics of Study Subjects

The Study population included 174 families with a mean family size of 5.4 members per family for analysis. Characteristics of the 942 participants are displayed in Table 1. There were 77% whites, 10% Asians, 8% Hispanics, and 5% other race/ethnicities. Average age (SD) was 49 (16) y; 57% were female. The distribution of *HFE* genotypes was 22% C282Y/C282Y (31% in C282Y homozygote proband families), 7% (8%) C282Y/H63D, 2% (1%) H63D/H63D, 34% (43%) C282Y/wt, 8% (5%) H63D/wt, and 25% (12%) wt/wt.

Phenotype analysis in *HFE* C282Y homozygote proband families

Mean (SD) TS and UIBC levels in these participants were 42.9% (22.4) and 172 μg/L (84), respectively (Table 1). The median and interquartile range for SF were 124.5 μg/L and 51.0–281.0 μg/L. Table 2 displays estimates of heritability (model A) and residual heritability (models B-D) for these measures. Heritability and residual heritability were significantly greater than zero (all $P<1\times10^{-3}$) for all of measures and h^2 (SE) was 0.18 (0.07) for TS, 0.28 (0.07) for ln SF, and 0.34 (0.08) for UIBC. Residual heritability was similar using model B, consistent with the small proportion of variation due to these covariates (0.02 for TS, 0.18 for ln SF, and 0.02 for UIBC). In model C, residual heritability was greater for all measures than with model B: 0.28 (0.06) for TS, 0.34 (0.07) for ln SF, and 0.40 (0.07) for UIBC. As

expected, C282Y (and H63D) genotype accounted for increased variability in serum iron measures. The proportion of variation due to age, gender, age by gender, and *HFE* genotype was 0.41 for TS, 0.36 for ln SF, and 0.45 for UIBC. Addition of interaction terms age × *HFE* genotype and gender × *HFE* genotype changed the model C results very slightly (data not shown). Under model D, after excluding variation in the iron measures due to many potential predictors, residual heritability was 0.21 (0.07) for TS, 0.37 (0.08) for ln SF, and 0.34 (0.08) for UIBC. Finally, the proportion of variation due to covariates consistently increased across models; the proportions under model D were 0.45 for TS, 0.41 for ln SF, and 0.50 for UIBC.

Genetic and environmental correlations between iron measures under models C and D are presented in Table 3. As expected, all correlations were strongly negative between (inversely) related measures TS and UIBC. Furthermore, TS and ln SF had correlations >0, whereas, UIBC and ln SF had correlations <0. Genetic correlations with ln SF ranged from −0.61 to −0.54 for UIBC, and from 0.73 to 0.76 for TS. All genetic correlations were different from 0 and from 1 ($P < 0.05$).

Phenotype analysis in *HFE* non-C282Y homozygote proband families

Mean (SD) TS and UIBC levels in participants were 44.5% (16.0) and 163 μg/L (63), respectively (Table 1). The median and interquartile range for SF were 208.5 μg/L and 83.0 – 370.5 μg/L, respectively, and h^2 (SE), after correcting for proband ascertainment, was 0.10 (0.19) for TS, 0.41 (0.15) for ln SF, and 0.47 (0.19) for UIBC (Supplementary Table 4). Heritability for TS did not differ significantly from zero, and this finding persisted for residual heritability across models (all $P \geq 0.1$ for TS, models A–D). Heritabilities and residual heritabilities for ln SF and UIBC were significantly different from zero (i.e., $P \leq 0.05$) under all models except model D for UIBC. For ln SF and UIBC, residual heritability was similar to heritability, after accounting for variation due to age, gender, and age by gender (model B). After accounting for *HFE* C282Y and H63D genotype, residual heritability was consistently lower; residual heritability under model C was 0.04 (0.12) for TS, 0.42 (0.23) for ln SF, and 0.32 (0.19) for UIBC. This decrease in residual heritability was associated with greater proportions of variation due to covariates after inclusion of *HFE* genotype: 0.24 for TS, 0.40 for ln SF, and 0.24 for UIBC. Interaction terms, age × *HFE* genotype and gender × *HFE* genotype, altered the results from model C very slightly (data not shown). Under model D, after excluding variation in the iron measures due to many potential predictors, residual heritability was 0.01 (0.12) for TS, 0.64 (0.26) for ln SF, and 0.19 (0.18) for UIBC. The proportion of variation due to covariates consistently increased across models, with the proportion under model D equaling 0.32 for TS, 0.43 for ln SF, and 0.29 for UIBC. We did not report bivariate analyses for estimating genetic and environmental correlations between serum iron measures due to small sample limitations.

Discussion

We modeled the total additive effects of heritable factors, i.e., the sum of average parental effects that yield mean values inherited in the offspring, in *HFE* C282Y and non-C282Y homozygote proband families. High heritability indicates that variation in an observed phenotype of the study population is caused by genotypic variation [14]. In the present study, we demonstrate that heritability estimates of TS, ln SF, and UIBC, were significantly greater than zero, after excluding variation in these phenotypes, due to gender, age, C282Y and H63D genotype, and other clinical and demographic characteristics. This implies that genetic variation plays a role in inter-individual differences in these measures. ln SF and UIBC had higher estimated heritability than TS. The genetic correlation between TS and UIBC was higher than that between either TS and ln SF or ln SF and UIBC. The latter results were expected because TS is calculated from the measured values of serum iron and

UIBC. Results of this study complement those of the HEIRS Family Study genome-wide linkage scan in which evidence was reported for linkage of ln SF to chromosome 5q, of UIBC to chromosome 4p, and of TS, SF, and UIBC to the chromosome 6p region containing *HFE.* After adjustment for *HFE* genotype and other covariates, there was also evidence of linkage of SF to chromosome 16p and of UIBC to chromosomes 5q and 17q ($P \leq 0.004$ for all) [17]. In another HEIRS Study [20], denaturing high-performance liquid chromatography (DHPLC) was used to detect mutations in 20 selected regions in six candidate genes known to influence iron metabolism in 789 participants. DHPLC analyses detected numerous mutations of *HFE*, *SLC40A1*, *HAMP*, *HJV*, *TFR2*, and *FTL.* Although the effect on iron metabolism of many of the missense mutations remains is unreported, their individual or cumulative allele frequencies do not account for most iron phenotype heterogeneity in HEIRS Study participant subgroups [20]. In the current study, probands without *HFE* C282Y homozygosity were ascertained because they had elevated values of SF and TS measured. Causes for these elevations may have been due to non-*HFE* iron overload or other reasons, but clinical assessment through liver biopsies or determination of phlebotomy-mobilizable iron was not available for all participants. The disparate results of analyses for the two family cohorts may reflect the fact that probands with C282Y homozygosity are genetically defined, whereas probands without C282Y homozygosity were defined using phenotype criteria. Thus, genetic differences may have contributed to the differences in heritability results for the two family cohorts. *HFE* hemochromatosis occurs predominantly in Caucasian populations [8,21,22]. In the present study, 96% of the participants in the families of probands with C282Y homozygosity reported Caucasian race/ethnicity. In families of probands without C282Y homozyogosity, only 29% reported Caucasian race/ethnicity.

There are extensive published data regarding strain-specific differences of iron phenotype in mice. For example, BMP6 has emerged as a key regulator of hepcidin expression through this pathway, and mice lacking BMP6 develop substantial iron loading [23–25], as do Smad4-knockout mice [26]. In humans, multiple proteins are involved in iron metabolism and mutations in genes that encode transferrin receptor-2, hepcidin, hemojuvelin, and ferroportin cause different types of hemochromatosis [27–29]. The high prevalence of elevated TS and SF values among Asians in the HEIRS Study led the investigators to hypothesize that this could be explained by *HFE* IVS5+1 G/A, splice site mutation previously reported in a Vietnamese man with iron overload [30,31]. A subsequent study indicated that this hypothesis was incorrect [31]. Asian patients from Pakistan, Bangladesh, Sri Lanka, and Thailand with iron overload have been found to have mutations in *HJV, HAMP, and SLC40A1;* none had pathogenic *HFE* mutations. Nonetheless, it is likely that such cases are rare. Taken together, there observations suggest that presently unknown genes exert a significant influence on iron phenotypes [32].

The present results are consistent with other reports that indicate that iron phenotypes are associated with attributes other than *HFE* genotype. For example, a study of *HFE* C282Y homozygotes and their families detected a residual heritability for serum ferritin of 0.35 and concluded that male sex is the major factor associated with hyperferritinemia in hemochromatosis [33]. In a genetically isolated population in the southwest of the Netherlands, there was evidence of heritability of serum iron, TS and SF, after adjustment for age, sex, and C282Y and H63D genotypes [34]. In a candidate gene study of 592 unrelated C282Y homozygotes, there was a significant association of serum ferritin with the common single-nucleotide polymorphism *rs235756* in the region of *BMP2*, a gene on chromosome 20p12 that encodes bone morphogenetic protein-2 [35]. The results of two genome-wide association studies performed on samples from Australians of European descent revealed that three variants (*rs3811647, rs1799852, rs2280673*) in the transferrin gene *TF*, plus the *HFE* C282Y mutation, explained approximately 40% of genetic variation

in serum transferrin [36]. In a study regarding adult male and female twins recruited from the Australian Twin Registry (562 monozygotic and 571 dizygotic twin pairs), significant sources of variation in iron measures included age, sex, age-sex interaction, body mass index, and both of the common *HFE* alleles, C282Y and H63D [37]. After correction for age and body mass index, 23% and 31% of the variance in serum iron level, 66% and 49% of the variance in transferrin levels, 33% and 47% of the variance in TS, and 47% and 47% of the variance in SF could be explained by additive genetic factors, for men and women, respectively. C282Y and H63D variation accounted for less than 5% of the corrected phenotypic variance, except for TS (12% in women and 5% in men) [37]. Taken together, these results provide substantial although indirect evidence that as-yet-unidentified genes have influence on serum iron measures, in addition to confirming the effects of *HFE* genotype [17,37]. Concordance of iron measures between same-sex siblings with C282Y homozygosity also suggests that the variable phenotype in C282Y homozygotes may be caused by non-*HFE* genetic factors [38].

In the present study, 8.6% of participants in families of probands with C282Y homozygosity and 1.5% of participants in families of probands without C282Y homozygosity reported that they had been treated by quantitative phlebotomy (Table 1). Thus, a covariate was added to model D for both participant groups. Some general limitations to the method of analysis include the following: rigorous assumptions about the model may be untestable; accuracy of a heritability estimate is dependent upon the sampling error, a function of sample size and pedigree structure; and heritability of a trait depends on the population. Nevertheless, the heritability parameter can be used successfully to compare traits within and across populations [14]. Because proband ascertainment differed across families, separate analyses were conducted in families with an *HFE* C282Y homozygote proband and in those with a non-C282Y homozygote proband. The pattern of heritability across different models was more consistent for C282Y homozygote proband families than for non-C282Y homozygote proband families, and may reflect the influence of ascertainment criteria. Although ascertainment corrections were implemented in families ascertained through probands with elevated TS and SF levels, selection of families in this manner may affect the external generalizability of heritability estimates. It is possible that genes that modulate iron accumulation in hemochromatosis patients may differ from those that control serum iron measures in the healthy population. Thus, it would be interesting to estimate heritability of serum iron values in a cohort of C282Y homozygotes. This issue was not explored because of the lack of families with C282Y alleles segregating at the *HFE* locus. In contrast, our approach to estimating heritability relied on the occurrence of multiple phenotypes within families. Regardless, the estimated heritability of TS, SF, and UIBC that we observed in the present study confirms and extends the results of studies of other diverse populations [33–38].

We conclude that TS, SF, and UIBC phenotypes in HEIRS Family Study participants have significant heritability, even after excluding effects of C282Y and H63D genotypes and other known factors that influence these measures. This suggests that other genetic variants contribute to iron phenotype variability. Gene discovery studies could identify other genes or alleles that affect phenotype variations of iron absorption and metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of HEIRS Family Study participants[*,†]

	Family Study Participants by *HFE* Genotype		
Characteristic	**Participants in C282Y/C282Y Proband Families (n = 666)**	**Participants in Non-C282Y/C282Y Proband Families (n = 276)**	**All Participants (n = 942[‡])**
Proband[§]	103 (15)	68 (25)	171 (18)
SF, TS screen positive	56 (8.4)	68 (25)	124 (13)
SF, TS screen negative	47 (7.1)	0 (0)	47 (5.0)
Female	388 (58)	150 (54)	538 (57)
Race or Ethnicity			
Caucasian	642 (96)	81 (29)	723 (77)
Asian	0 (0.0)	98 (36)	98 (10)
Hispanic	17 (2.6)	58 (21)	75 (8.0)
Other	7 (1.1)	38 (14)	45 (4.8)
HFE genotype			
C282Y/C282Y	206 (31)	4 (1.5)	210 (22)
C282Y/H63D	53 (8.0)	16 (5.8)	69 (7.3)
H63D/H63D	5 (0.8)	16 (5.8)	21 (2.2)
C282Y/wt	288 (43)	35 (13)	323 (34)
H63D/wt	34 (5.1)	45 (16)	79 (8.4)
wt/wt	79 (12)	160 (58)	239 (25)
Menopause (% among women)	178 (46)	81 (54)	259 (48)
History of hepatitis	17 (2.6)	9 (3.3)	26 (2.8)
Phlebotomy treatment	57 (8.6)	4 (1.5)	61 (6.5)
CRP >0.5 mg/dL	173 (26)	49 (18)	222 (24)
Age (y)	48.2 ± 16.0	49.8 ± 15.9	48.7 ± 16.0
Alcohol consumption (gm/d)	8.3 ± 18.5	6.3 ± 16.8	7.7 ± 18.1
Body mass index	27.5 ± 5.5	27.3 ± 6.2	27.4 ± 5.7
SF (μg/L) median (IQR)	124.5 (51.0, 281.0)	208.5 (93.0, 370.5)	137.5 (59.0, 329.0)
TS (%)	42.9 ± 22.4	44.5 ± 16.0	43.4 ± 20.7
UIBC (μg/dL)	172 ± 84	163 ± 63	169 ± 79

[*] Participants include both proband and non-proband family members from heritability analysis

[†] Values are count and column percentage for categorical variables, mean ± standard deviation for continuous variables, except where median and interquartile range (IQR) are used, as noted.

[≠] Total sample size is n = 942. Depending on completeness of data, the sample size varied from 602 to 666 in C282Y/C282Y proband families, and from 236 to 276 in non-C282Y/C282Y proband families.

[§] Three probands are excluded from the 174 families for analysis due to incomplete or inconsistent data for inclusion. Probands listed as screen positive are those for whom the TS and SF values were both above gender-specific cutoffs at the Initial Screening; screen negative are those for whom TS and SF values did not meet this criterion.

Table 2

Heritability analysis results for *HFE* C282Y homozygote proband families

Proband	Phenotype	Model*	N (Individuals)	Residual Heritability h^2	Standard Error of h^2	*P* (Residual h^2)	Proportion of Variance in Phenotype Due to Covariates
C282Y/C282Y	TS (%)	A	666	0.181	0.065	3.3×10^{-4}	-
		B	664	0.169	0.064	6.6×10^{-4}	0.020
		C	664	0.277	0.064	$<1 \times 10^{-6}$	0.410
		D	596	0.205	0.072	3.5×10^{-4}	0.450
	Ln SF (μg/L)	A	666	0.280	0.070	$<1 \times 10^{-6}$	-
		B	664	0.287	0.066	$<1 \times 10^{-6}$	0.182
		C	664	0.335	0.065	$<1 \times 10^{-6}$	0.360
		D	596	0.368	0.079	$<1 \times 10^{-6}$	0.407
	UIBC (μg/dL)	A	666	0.343	0.078	$<1 \times 10^{-6}$	-
		B	664	0.329	0.078	$<1 \times 10^{-6}$	0.023
		C	664	0.402	0.068	$<1 \times 10^{-6}$	0.454
		D	596	0.342	0.077	$<1 \times 10^{-6}$	0.496

*Model covariates: Model A (no covariates); Model B (age, gender, age × gender); Model C (age, gender, age × gender, *HFE* genotype); Model D (age, gender, age × gender, *HFE* genotype, Field Center, body mass index, menopausal status, phlebotomy treatment, hepatitis, decile of alcohol intake, C-reactive protein); *HFE* C282Y-H63D genotypes were coded as five indicator variables in all models. Field Centers were coded as four indicator variables in all models.

Table 3

Genetic (r_G), and environmental (r_E) correlations between serum iron measures in families with *HFE* C282Y/C282Y probands

Proband	Measure 1*	Measure 2*	Model	N (Individuals)	r_G Correlation (Standard error)	$P^{\dagger}$ ($\rho_G = 0$)	$P^{\dagger}$ ($\rho_G = 1$)	r_E Correlation (Standard error) †	$P^{\dagger}$ ($\rho_E = 0$)
C282Y/C282Y	TS	Ln SF	C	664	0.726 (0.114)	5.4×10^{-6}	0.0038	0.344 (0.059)	$<1\times10^{-6}$
			D	596	0.759 (0.148).	0.00028	0.049	0.370 (0.065)	3.1×10^{-6}
	TS	UIBC	C	664	−0.945 (0.033)	$<1\times10^{-6}$	0.035	−0.864 (0.019)	$<1\times10^{-6}$
			D	596	−0.955 (0.054)	6.7×10^{-6}	0.21	−0.869 (0.020)	$<1\times10^{-6}$
	Ln SF	UIBC	C	664	−0.614 (0.102)	0.000011	1.1×10^{-6}	−0.462 (0.058)	$<1\times10^{-6}$
			D	596	−0.544 (0.133)	0.0015	5.1×10^{-6}	−0.504 (0.065)	$<1\times10^{-6}$

*Model covariates: Model C (age, gender, age × gender, *HFE* genotype); Model D (age, gender, age × gender, *HFE* genotype, Field Center, body mass index, menopausal status, phlebotomy treatment, hepatitis, decile of alcohol intake, C-reactive protein); C282Y-H63D genotypes were coded as five indicator variables in all models. Field Centers were coded as four indicator variables in all models.

†P values for two-sided tests of the genetic correlation between serum iron measures, $\rho_G = 0$ vs. $\rho_G \neq 0$, and $\rho_G = 1$ vs. $\rho_G \neq 1$.

‡P values for two-sided tests of the environmental correlation between serum iron measures, $\rho_E = 0$ vs. $\rho_E \neq 0$.