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Iron-processing genotypes, nutrient intakes, and cadmium levels in the Normative Aging Study: Evidence of sensitive subpopulations in cadmium risk assessment

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ABSTRACT

Background: Because iron and cadmium share common transport mechanisms, iron-processing protein variants such as HFE C282Y, HFE H63D, and Transferrin P570S may influence cadmium metabolism. Our aim was to evaluate associations between common HFE and Transferrin polymorphisms and toenail cadmium levels among older men.

Methods: In a longitudinal cohort of men age 51–97, the Normative Aging Study (NAS), we evaluated toenail cadmium concentrations and missense single nucleotide polymorphisms (SNPs) in the HFE and Transferrin genes. We fit age-adjusted models to estimate associations between genotypes and toenail cadmium concentrations. We then considered potential interactions with smoking status, hemoglobin, and nutritional intakes known to modulate cadmium absorption. For the significant interactions, we also evaluated genotype specific effect estimates.

Results: HFE and Transferrin genotypes were not associated with toenail cadmium concentrations in the main effect analyses, but there were significant interactions between HFE H63D and hemoglobin ($p_{interaction}$ = 0.021), as well as HFE H63D and vitamin C intake ($p_{interaction}$ = 0.048). Genotype specific effect estimates suggested: 1) an inverse relationship between hemoglobin and cadmium levels among HFE H63D homozygotes, and 2) an inverse relationship between vitamin C intake and cadmium levels that strengthens with the number of HFE H63D variant alleles a subject carries.

Conclusions: These findings suggest that sensitive subpopulations defined by diet, hemoglobin level, and genotype may absorb more cadmium from their environment and thus should be considered in cadmium risk analyses. These findings are particularly relevant given the high prevalence of the H63D variant worldwide.

1. Background

Iron, an important nutrient, and cadmium, a toxic contaminant, are both metals with divalent cation forms that are found in food. (ATSDR, 2008; Mackenzie and Garrick, 2005; Schumann et al., 2007) Low body iron stores, and insufficient dietary intake of several other nutrients are known to increase the absorption of cadmium from foods (Fox et al., 1980; Andersen et al., 2004; Flanagan et al., 1978), and evidence described below suggests that iron-processing gene variants might also enhance intestinal cadmium uptake. Therefore, these factors may be risk factors for cadmium mediated diseases such as renal failure, osteoporosis (ATSDR, 2008), and possibly neurodevelopmental/

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neurocognitive dysfunctions (Pihl and Parkes, 1977; Capel et al., 1981; Thatcher et al., 1982; Ciesielski et al., 2012; Viaene et al., 2000; Ciesielski et al., 2013).

Polymorphisms in the HFE gene are present in the majority of cases of the iron overload syndrome Hereditary Hemochromatosis. (Bacon et al., 1999) Properly functioning HFE protein binds to the transferrin receptor and lowers its affinity for the iron transport protein transferrin. At least two HFE variant proteins (those from individuals with C282Y and H63D single nucleotide polymorphisms) have a reduced ability to perform this function. (Feder et al., 1998) Disruption of HFE function may increase transferrin mediated iron uptake in some tissues (Feder et al., 1998), and trigger a cascade of events that enhances iron absorption through intestinal transport proteins such as Divalent Metal Transporter-1 (DMT-1). (Pietrangelo, 2010; Byrnes et al., 2002; Brasse-Lagnel et al., 2011) Because cadmium can also be transported by these intestinal metal transporters (Gunshin et al., 1997; Vesey, 2010; Bressler et al., 2004), and can bind transferrin (Harris and Madsen, 1988), HFE variants may also alter cadmium absorption, toxicokinetics, or biomarker levels.

Previously, Akesson et al. reported that Hemochromatosis patients had increased blood cadmium levels, but only if they received regular phlebotomy treatments. (Akesson et al., 2000) The mechanism underlying these results is unclear but it may involve HFE and Transferrin function. Given the nearly ubiquitous nature of low level cadmium exposure (Jarup and Akesson, 2009; CDC, 2013) and the high prevalence of HFE (Merryweather-Clarke et al., 1997) and Transferrin (Lee et al., 1999) variants, research is needed to determine if these variants can alter cadmium absorption in the context of low iron or hemoglobin levels, even in the absence of clinical hemochromatosis. If carriers absorb more cadmium from food, they may be more susceptible to developing cadmium mediated health effects from chronic low level exposure. Thus carriers of these variants may represent sensitive subpopulations to consider in cadmium risk assessments.

In this study we evaluated whether common HFE and Transferrin variants: HFE C282Y, HFE H63D (Bradley et al., 1998), and Transferrin P570S (Lee et al., 1999) predicted cadmium levels among the participants in the Normative Aging Study (NAS). (Bell et al., 1972) We also considered interactions between these genotypes and several factors that have been previously shown to influence cadmium exposure, transport, or absorption, including: hemoglobin levels, intake of several nutrients, and smoking. (ATSDR, 2008; Fox et al., 1980; Andersen et al., 2004; Sarhan et al., 1986; Berglund et al., 1994)

2. Methods

2.1. Data source and study population

The Normative Aging Study (NAS) is a longitudinal cohort study started in 1963 at the Veterans Administration (VA) Outpatient Clinic in Boston MA. (Bell et al., 1972; Wang et al., 2007) Between 1963 and 1968, 2280 males between the ages of 21 to 80, were enrolled in the NAS. The study was intended to focus on the aging process in generally healthy adult males, so participants were screened prior to enrollment to assure that they were free of most chronic health conditions. These generally healthy participants were screened for conditions such as cancer, asthma, cardiovascular disease, gout, diabetes, hypertension, and peptic ulcers. (Jain et al., 2007) They were not screened for hemochromatosis, but we expect that not many (if any) would have been identified due to low penetrance of HFE mutations (Nadakkavukaran et al., 2012; Asberg et al., 2007), and the later life onset of symptomology in classic (HFE related) hemochromatosis. (Pietrangelo, 2010) Every 3 to 5 years NAS participants have medical and laboratory evaluations, and respond to health related questionnaires. Between June 1992 and May 2010, 756 participants (ages 51–97) provided toenail clippings which were analyzed for cadmium content. Over 96% of these measurements had toenail clipping dates listed and all of these dates were within one year of the NAS study visit. The new portion of the study (analyzed here), was approved by the Human Research Committees of the Harvard School of Public Health and the Department of Veterans Affairs Boston Healthcare System.

2.2. Genotyping

We genotyped participants for the following single nucleotide polymorphisms (SNPs): HFE C282Y (RS1800562), HFE H63D (RS1799945), and Transferrin P570S (RS1049296), using methods described previously. (Park et al., 2009) Briefly, we extracted DNA from white blood cells with PureGene Kits (Gentra Systems, Minneapolis MN, USA), and then amplified the SNPs along with 100 bp flanking sequences using multiplex polymerase chain reaction (PCR). Primers designed with SpectroDESIGNER software (Sequenom, San Diego, CA, USA), and primer sequences have been previously published (Park et al., 2009)

2.3. Cadmium biomarker

We assessed toenail cadmium levels as described previously. (Mordukhovich et al., 2012) Briefly, toenail clippings were collected from all toes and pre-cleaned by sonicating in 1% Triton X-100 followed by rinsing with distilled deionized water and drying at 60 °C for 24 h. Toenails were then weighed and dissolved in HNO_3 for 48 h at room temperature. The samples were diluted to up to 5 mL with deionized water and then analyzed with an inductively coupled plasma-mass spectrometer (DRC 11, Perkin Elmer, Norwalk CT). Indium was used as the internal standard. Quality control was ensured by analyzing a calibration verification standard [National Institute of Standard and Technology Standard References Material 1643e (trace elements in water, Gaithersburg, MD)], a 1 ng/mL mixed element standard solution, continuous calibration standards, and a procedural blank. Daily analytic variation was assessed with Certified Reference Material GBW 07601. Five replicate measurements were averaged to yield each toenail cadmium concentration. Toenail cadmium levels in this population likely reflect exposure from 10 to 18 months prior to the date of collection. This estimate is based on studies of occupational cadmium exposure (10–12 month lag), (Grashow et al., 2014) and the fact that toenail growth rates decrease with age. (Mordukhovich et al., 2012; Slotnick and Nriagu, 2006)

2.4. Statistical analysis

2.4.1. Genotype frequencies

We calculated allele and genotype frequencies among the 756 participants, and performed chi-square tests for specified proportions to compare the observed genotype frequencies with the expected genotype frequencies based on Hardy-Weinberg equilibrium. This serves as a quality control check, because deviation from Hardy-Weinberg equilibrium can be an indicator of genotyping errors or issues with data quality. (Hosking et al., 2004) An exact test was used for HFE C282Y because there were < 5 in the homozygous variant group.

2.4.2. Descriptive statistics

For our initial analyses we evaluated the first toenail cadmium measurement for each of the 756 participants (i.e. if a participant provided samples from multiple visit dates, we used only the first sample provided). We excluded 5 cadmium values due to insufficient weight of the toenail sample. Because the distribution of toenail cadmium concentrations was roughly lognormal, we log transformed cadmium values for *t*-test and regression analyses. Measurement imprecision resulted in some negative cadmium values. We did not left censor the data by eliminating values below the limit of detection, as this implicitly assumes that the sources of measurement error are distinct and incomparable on either side of this detection limit. (Whitcomb

and Schisterman, 2008) To assure there were no zero or negative values prior to log transformation we added a small constant (0.11 μg/g) to all the toenail cadmium concentrations. Among the initial log transformed cadmium values there were 6 extreme values (3 above and 3 below mean). These extreme values were identified visually, and they were noticeably separated from the bulk of the distribution. We excluded these points since they may have had undue influence on the results.

We calculated the means and standard deviations for baseline characteristics/covariates and toenail cadmium among the remaining 745 participants. We then calculated the means and standard deviations for toenail cadmium concentrations by genotype. Using Student's *t*-tests, we compared toenail cadmium levels among wildtypes to those of hetero and homozygote subjects separately and also to a combined group (hetero- and homozygote participants combined). We also calculated means and standard deviations for baseline characteristics/covariates by genotype and tested for significant differences with Student's *t*-tests. Log normally distributed variables were log transformed for this comparison. The distribution of packyears (cumulative cigarette smoking history - calculated as the *packs-per-day* times *years-smoked* (NCI, 2018)) could not be transformed to a normal distribution because there were many zero values. Therefore we used Wilcoxon-Mann-Whitney tests for this variable. Differences in smoking status (never, former or current smoker) by genotype were tested with chi-square or Fisher's exact tests as appropriate. There were only 4 HFE C282Y homozygotes so this group of 4 participants was not tested for significant differences from wildtypes. The nutritional variables for one participant were excluded from all analyses because they were consistently 1000 fold smaller than typical values, suggesting a data entry error.

Several participants had toenail cadmium measurements from samples collected at more than one study visit. Among the log transformed cadmium values in this larger dataset (n = 1383) there were 10 extreme values (5 above and 5 below mean) which we excluded as outliers. We calculated means and standard deviations for toenail cadmium by genotype in this dataset as well.

2.4.3. Models

To further characterize the relationship between genotypes and toenail cadmium levels we constructed unadjusted and age-adjusted regression models using GAM in R (version 2.10.1, www.r-project.org). (Wood, n.d.) The study group did not enroll any women, and > 97% of participants were Caucasian thus we did not control for sex or race. In order to evaluate the linearity of the age - toenail cadmium relationship we constructed a bivariate model with age modeled as a penalized spline selected using a Generalized Cross Validation (GCV) process. (Craven and Wahba, 1979; Wood, n.d.) GCV is an iterative process which selects a penalized spline to model a variable if there is evidence of a non-linear relationship. Otherwise GCV will select a linear term. In this model GCV selected a linear term for age, so for consistency we modeled age with a linear term in each of our age-adjusted models. We evaluated genotypes in two ways: (1) assuming an allele dose effect (wildtype = 0, heterozygous variant = 1, homozygous variant = 2) and (2) assuming a dominant inheritance pattern (wildtype = 0, hetero or homozygous variant = 1). Several participants had cadmium levels measured at multiple study visits. To incorporate this data into our analyses and account for clustering by participant, we also evaluated mixed models with a random intercept for each participant using the GAMM function in R. (Wood, n.d.)

We explored potential interactions between the genotypes and factors thought to affect cadmium exposure, absorption, or transport (Anon, n.d.; Fox et al., 1980; Andersen et al., 2004; Sarhan et al., 1986; Berglund et al., 1994), including: smoking status (never, former, or current smoker), packyears of cigarette smoking, hemoglobin level (g/dL), and intake of various nutrients as estimated by food frequency questionnaire (FFQ): zinc (mg/day), iron (mg/day), calcium (mg/day), manganese (mg/day), vitamin c (mg/day), and crude fiber (g/day). These variables were chosen because they had been described in the literature as influencing Cd exposure, absorption, or transport (Anon, n.d.; Fox et al., 1980; Andersen et al., 2004; Sarhan et al., 1986; Berglund et al., 1994), and they were available in the NAS data. Linear interaction terms were added to the age-adjusted models. If the interaction with allele dose or dominant-coded genotype was significant we evaluated interaction terms in which genotype was coded as a categorical variable so that we could obtain and compare effect estimates in strata defined by genotype (Table 5).

As the initial results identified interactions between these genotypes and factors that are known to affect iron absorption, we conducted a supplementary interaction analysis with copper intake (Table 6). To our knowledge copper intake has not as yet been linked with cadmium absorption, which is why it was not included in the main analyses. However, copper is thought to influence iron absorption and transport (Collins et al., 2010), and thus was worth exploring in a secondary analysis.

Because our regression models utilized a log-transformed outcome variable (logCd), the betas can be interpreted as follows: 100(beta) = % change in Cd level associated with a one unit increase in the exposure variable, when all other variables in the model are held constant. (UCLA-Statistical-Consulting-Group, 2018) These percentages can be found in Table 5, and we note that there are two models for each exposure. In our discussion we interpret the results from both models which results in a range estimate rather than a point estimate for our percentages. Thus, these ranges do not reflect uncertainty in the individual models, rather they reflect uncertainty from model integration (the individual model uncertainties are presented in Table 5).

Regression analyses were performed using R (version 2.10.1, www.r-project.org), and other analyses were performed using SAS 9.1.3 (SAS institute Cary, NC), unless stated otherwise.

3. Results

The observed genotype frequencies among the 756 participants (Table 1) did not differ significantly from what would be expected based on Hardy-Weinberg equilibrium (HFE C282Y: $\chi 2 = 0.01$, $p > 0.999$ [exact test used since there was a cell count < 5], HFE H63D: $\chi 2 = 4.04$, $p = 0.133$, Transferrin P570S: $\chi 2 = 3.50$, $p = 0.174$).

751 (99.3%) of the 756 participants, had at least one toenail sample of sufficient weight for cadmium analysis. After removing 6 outliers (n = 745, see methods), the initial toenail cadmium concentrations had

Table 1
Genotype frequencies.

	N	(%)
HFE C282Y[a]		
CC wildtype	608	(85.9)
CY heterozygous variant	96	(13.6)
YY homozygous variant	4	(0.6)
HFE H63D[b]		
HH wildtype	541	(76.5)
HD heterozygous variant	148	(20.9)
DD homozygous variant	18	(2.6)
Transferrin P570S[c]		
PP wildtype	388	(69.5)
PS heterozygous variant	147	(26.3)
SS homozygous variant	23	(4.1)

[a] 48 were missing genotype information. Test of Hardy-Weinberg equilibrium: $\chi^2 = 0.01$, $p > 0.999$ (exact test was used here since there was a cell count < 5).

[b] 49 were missing genotype information. Test of Hardy-Weinberg equilibrium: $\chi^2 = 4.04$, $p = 0.133$.

[c] 198 were missing genotype information. Test of Hardy-Weinberg equilibrium: $\chi^2 = 3.50$, $p = 0.174$.

Table 2
Baseline characteristics.

	N[a]	Mean	(SD or %)
Age (years)	745	71.1	(7.0)
Education (years)	585	14.6	(2.9)
Packyears cigarettes	745	21.5	(27.0)
Never smoker	135		(19.9%)
Former smoker	513		(75.4%)
Current smoker	32		(4.7%)
Crude fiber (g/day)	627	5.74	(2.64)
Iron intake (mg/day)	382	19.8	(11.6)
Zinc intake (mg/day)	382	18.2	(14.5)
Calcium intake (mg/day)	382	932	(447)
Manganese intake (mg/day)	629	4.68	(2.45)
Vitamin C intake (mg/day)	382	353	(327)
Hemoglobin (g/dL)	740	14.8	(1.2)
Toenail cadmium (μg/g)	745	0.0385	(0.0721)

[a] $N = 745$, From the original 756, 5 were excluded for insufficient toenail sample weight and 6 were excluded as toenail cadmium concentration outliers.

a mean of 0.0385 μg/g and a standard deviation of 0.0721 μg/g. Additional descriptive statistics for the 745 participants are listed in Table 2. When compared to wildtype participants, packyears of cigarettes were significantly higher among HFE C282Y heterozygotes ($p = 0.004$) and significantly lower among Transferrin P570S heterozygotes ($p = 0.027$). Iron intake was higher among HFE H63D heterozygotes ($p = 0.033$). No significant differences were found in smoking status by genotype (data not shown).

Cadmium levels by genotype are presented in Table 3 for the first toenail cadmium value dataset and for the larger mixed model dataset which allowed for the inclusion of multiple cadmium measurements for a given participant. We found no significant differences in toenail cadmium by genotype in the first toenail cadmium values dataset whether we compared heterozygotes and homozygotes separately to wildtypes or combined those with 1 or 2 variant alleles into a single group for comparison with wildtypes.

In the unadjusted and age-adjusted regression models for the first toenail cadmium values dataset, none of the allele dose or dominant coded genotypes were significantly associated with toenail cadmium level (Table 4). These genotypes were also not associated with toenail cadmium levels in the mixed model analyses. (Table 4).

Only one interaction was statistically significant in both analyses, and this was between allele dose coded HFE H63D and hemoglobin (Table 5; first toenail Cd analysis $p_{interaction} = 8.38 \times 10^{-3}$, mixed model analysis $p_{interaction} = 2.15 \times 10^{-2}$). The beta for hemoglobin was more negative among HFE H63D homozygotes than it was among wildtype or heterozygote participants, and this pattern was consistent across both analyses.

There were additional interactions between HFE H63D and vitamin C intake that were significant in the more powerful mixed model analysis (allele dose coded genotype $p_{interaction} = 4.78 \times 10^{-2}$, and dominant coded genotype $p_{interaction} = 4.54 \times 10^{-2}$). The beta for vitamin C became more negative as the number of HFE H63D alleles (0, 1, or 2) increased, and this pattern was present in both analyses (Table 5). There was also an interaction that was only significant in the first toenail cadmium values dataset between allele dose coded Transferrin P570S and hemoglobin (first toenail Cd analysis $p_{interaction} = 2.40 \times 10^{-2}$). The beta for hemoglobin was more negative among Transferrin P570S homozygotes and this pattern was present in both analyses (Table 5). There were no significant interactions identified for smoking status, packyears of cigarette smoking, or intakes of zinc, calcium, iron, manganese, or crude fiber.

When the models presented in Table 5 were re-run excluding the 32 current smokers the results were very similar (Table 6), however, the HFE H63D - vitamin C interaction just missed significance.

In the supplementary copper analyses we found a significant interaction between HFE H63D and copper intake in the more powerful mixed model analysis (allele dose coded genotype $p_{interaction} = 1.17 \times 10^{-2}$, and dominant coded genotype $p_{interaction} = 1.40 \times 10^{-2}$). The beta for copper intake became more negative as the number of HFE H63D alleles (0, 1, or 2) increased, and this pattern was present in both analyses (Table 7).

Table 3
Mean toenail cadmium levels (μg/g) by genotype.

	First toenail Cd values			Mixed model analyses		
	N	Mean	(SD)	N	Mean	(SD)
Total	745	0.0385	(0.0721)	1373	0.0335	(0.0677)
HFE C282Y[a]						
CC (wild type)	598	0.0343	(0.0582)	1135	0.0313	(0.0618)
CY (heterozygous variant)	95	0.0486	(0.1031)	170	0.0372	(0.0803)
YY (homozygous variant)	4	0.1889	(0.3675)	5	0.1515	(0.3290)
HFE H63D[a]						
HH (wild type)	531	0.0381	(0.0754)	1002	0.0334	(0.0715)
HD (heterozygous variant)	147	0.0352	(0.0590)	274	0.0304	(0.0538)
DD (homozygous variant)	18	0.0466	(0.0836)	31	0.0345	(0.0658)
Transferrin P570S[a]						
PP (wild type)	383	0.0366	(0.0668)	763	0.0299	(0.0572)
PS (heterozygous variant)	144	0.0314	(0.0556)	280	0.0341	(0.0790)
SS (homozygous variant)	21	0.0490	(0.0996)	44	0.0519	(0.0817)

48 were missing HFE C282Y genotype information in the first toenail Cd values data (63 in the mixed model dataset). 49 were missing HFE H63D genotype information in the first toenail Cd values data (66 in the mixed models dataset). 197 were missing Transferrin P570S genotype information in the first toenail Cd values data (286 in the mixed models dataset). 6 datapoints were excluded from the first toenail Cd values data as toenail Cd concentration outliers (10 in the mixed models dataset). 5 datapoints were excluded due to insufficient toenail sample weight.

[a] Toenail cadmium levels in heterozygotes and homozygotes did not differ significantly from those in wildtypes at $\alpha = 0.05$ (Student's *t*-test) in the first toenail Cd values. Differences were not tested in the mixed models dataset due to clustering of values by participant.

4. Discussion

4.1. Interpretations

In this study we observed that HFE and Transferrin polymorphisms (HFE C282Y, HFE H63D, and Transferrin P570S) did not predict toenail cadmium concentrations among older males. However, we found evidence that these genetic variants may modify the effect of two factors which influence cadmium absorption: hemoglobin levels and vitamin C intake.

The significant interaction between HFE H63D and hemoglobin, when combined with the genotype specific effect estimates, suggests an inverse relationship between hemoglobin and cadmium levels among those who are homozygous for the H63D variant. Specifically, the two effect estimates presented in Table 5 predict a 13.8–14.4% increase in toenail cadmium for each 1 g/dL decrease in hemoglobin among HFE H63D homozygotes. Thus, for this subgroup of participants, a two standard deviation decrease in hemoglobin (2.4 g/dL) would correspond to a 33–35% increase in toenail cadmium. Although we did not assess mechanisms in this study, the prior literature suggests that differences in cadmium absorption may explain these findings. Alternatively it is possible that differences in cadmium intake might explain these patterns, but there is clearer support for a toxicokinetic

Table 4
Effect estimates for HFE and TF variants.

	First toenail Cd values				Mixed model analyses			
	Unadjusted		Adjusted[a]		Unadjusted		Adjusted[a]	
	β[b]	*p*	β[b]	*p*	β[b]	*p*	β[b]	*p*
Allele dose coding								
HFE C282Y	0.0456	0.152	0.0455	0.154	0.0275	0.289	0.0261	0.319
HFE H63D	0.0072	0.765	0.0079	0.742	0.0049	0.800	0.0067	0.733
TF P570S	−0.0027	0.913	−0.0040	0.870	0.0210	0.274	0.0186	0.338
Dominant coding								
HFE C282Y	0.0366	0.280	0.0363	0.284	0.0190	0.487	0.0169	0.539
HFE H63D	0.0042	0.880	0.0051	0.857	0.0019	0.932	0.0039	0.864
TF P570S	−0.0078	0.787	−0.0095	0.744	0.0168	0.463	0.0137	0.554

Allele Dose coding: 0 = no variant alleles, 1 = 1 variant allele, 2 = 2 variant alleles.
Dominant coding: 0 = no variant alleles, 1 = 1 or 2 variant alleles.
[a] Adjusted for age.
[b] β for genotype with log(toenail Cd) as the outcome.

interpretation. For example: increased iron absorption, as indicated by low hemoglobin levels, may lead to increased cadmium absorption among those who are homozygous for the H63D variant. Previous studies have linked low iron stores with increased cadmium levels (reviewed in (Kim and Park, 2014)), but this relationship has generally not been observed in men (Satarug et al., 2004; Olsson et al., 2002). Our results are consistent with these findings, but they suggest that elevated iron uptake may increase cadmium uptake in a subset of men who have variant HFE proteins.

Because we have posited a putative toxicokinetic interpretation we should emphasize that iron regulation is complex, incompletely understood, and altered by the variants under study. In normal physiology, iron stores in different tissues, as well as erythropoetic activity in the bone marrow, can influence liver-based hepcidin production to modulate iron absorption in the gut. (Ganz, 2013) Stated simply, low hemoglobin levels would be expected to increase the production of red blood cells in the bone marrow, leading to lower Hepcidin production and increased iron absorption in the intestine. (Ganz, 2013) Low iron stores in different tissues can have a similar effect on hepcidin and iron absorption, (Ganz, 2013) but the genotypes under study can also have this same impact in the absence of low iron stores. (Pietrangelo, 2010) Thus, we emphasize that all our findings could be mediated by increased iron absorption, but we will attempt to avoid over speculating about the potential physiologic influences that we have not measured/analyzed (i.e. tissue iron levels).

We should note that these findings are also consistent with the work of Akesson et al. (Akesson et al., 2000), who conducted a small case control study with 21 Hereditary Hemochromatosis (HH) patients and 21 controls. In this study the authors found that blood cadmium concentrations were elevated among the HH cases, but only if they received

Table 5
Genotype stratified effect estimates for significant interactions.

	First toenail Cd values		Mixed model analyses	
	β_{hgb}[a]	% change in Cd associated with a 1 g/dL increase in Hgb (95% CI)	β_{hgb}[a]	% change in Cd associated with a 1 g/dL increase in Hgb (95% CI)
Hemoglobin - HFE H63D Allele Dose	$p_{interaction} = 8.38 \times 10^{-3}$		$p_{interaction} = 2.15 \times 10^{-2}$	
HH (wild type)	0.0062	0.6 (−1.6, 2.8)	0.0013	0.1 (−1.4, 1.6)
HD (heterozygous variant)	−0.0015	−0.1 (−4.6, 4.4)	0.0046	0.5 (−2.5, 3.4)
DD (homozygous variant)	−0.1445	−14.4 (−23.0, −5.9)	−0.1384	−13.8 (−20.9, −6.8)
Hemoglobin - Transferrin P570S Allele Dose	$p_{interaction} = 2.40 \times 10^{-2}$		$p_{interaction} = 1.28 \times 10^{-1}$	
PP (wild type)	−0.0021	−0.2 (−2.8, 2.3)	−0.0016	−0.2 (−1.9, 1.5)
PS (heterozygous variant)	0.0061	0.6 (−3.6, 4.8)	0.0016	0.2 (−2.7, 3.0)
SS (homozygous variant)	−0.1827	−18.3 (−27.4, −9.1)	−0.0923	−9.2 (−15.8, −2.6)

	First toenail Cd values		Mixed model analyses	
	β_{vitC}[b]	% change in Cd associated with a 100 mg/day increase in VitC (95% CI)	β_{vitC}[b]	% change in Cd associated with a 100 mg/day increase in VitC (95% CI)
Vitamin C - HFE H63D Allele Dose	$p_{interaction} = 3.09 \times 10^{-1}$		$p_{interaction} = 4.78 \times 10^{-2}$	
HH (wild type)	−0.0093	−0.9 (−2.1, 0.2)	−0.0016	−0.2 (−0.9, 0.6)
HD (heterozygous variant)	−0.0182	−1.8 (−4.1, 0.5)	−0.0203	−2.0 (−3.8, −0.3)
DD (homozygous variant)	−0.0580	−5.8 (−15.9, 4.3)	−0.0249	−2.5 (−9.8, 4.8)

P values for interaction were obtained from linear regression models that were adjusted for age and contained an interaction term between genotype and hemoglobin (or Vitamin C intake) with log(toenail Cd) as the outcome. Genotypes were coded as follows: Allele Dose coding: 0 = no variant alleles, 1 = 1 variant allele, 2 = 2 variant alleles. Genotype specific effect estimates (βs) were extrapolated from interaction terms in age adjusted models with categorically coded genotypes.
[a] βs for a 1 g/dL increase in Hemoglobin with log(toenail Cd) as the outcome.
[b] βs for a 100 mg/day increase in Vitamin C intake with log(toenail Cd) as the outcome.

Table 6
Analyses from Table 5 with current smokers excluded.

	First toenail Cd values		Mixed model analyses	
	β_{hgb}[a]	% change in Cd associated with a 1 g/dL increase in Hgb (95% CI)	β_{hgb}[a]	% change in Cd associated with a 1 g/dL increase in Hgb (95% CI)
Hemoglobin - HFE H63D Allele Dose	$p_{interaction} = 2.56 \times 10^{-3}$		$p_{interaction} = 3.46 \times 10^{-3}$	
HH (wild type)	0.0097	1.0 (−1.3, 3.3)	0.0047	0.5 (−1.4, 2.3)
HD (heterozygous variant)	−0.0091	−0.9 (−5.4, 3.6)	−0.0075	−0.7 (−4.4, 2.9)
DD (homozygous variant)	−0.1462	−14.6 (−23.0, −6.2)	−0.1373	−13.7 (−21.2, −6.2)
Hemoglobin - Transferrin P570S Allele Dose	$p_{interaction} = 2.62 \times 10^{-2}$		$p_{interaction} = 1.34 \times 10^{-1}$	
PP (wild type)	−0.0016	−0.2 (−2.9, 2.5)	−0.0016	−0.2 (−2.2, 1.9)
PS (heterozygous variant)	0.0128	1.3 (−3.1, 5.7)	0.0122	1.2 (−2.6, 5.0)
SS (homozygous variant)	−0.2208	−22.1 (−32.4, −11.7)	−0.1439	−14.4 (−23.2, −5.5)

	First toenail Cd values		Mixed model analyses	
	β_{vitC}[b]	% change in Cd associated with a 100 mg/day increase in VitC (95% CI)	β_{vitC}[b]	% change in Cd associated with a 100 mg/day increase in VitC (95% CI)
Vitamin C - HFE H63D Allele Dose	$p_{interaction} = 3.53 \times 10^{-1}$		$p_{interaction} = 6.47 \times 10^{-2}$	
HH (wild type)	−0.0095	−1.0 (−2.1, 0.2)	−0.0025	−0.3 (−1.0, 0.5)
HD (heterozygous variant)	−0.0175	−1.7 (−4.3, 0.8)	−0.0215	−2.1 (−4.1, −0.2)
DD (homozygous variant)	−0.0582	−5.8 (−15.8, 4.1)	−0.0241	−2.4 (−9.8, 5.0)

P values for interaction were obtained from linear regression models that were adjusted for age and contained an interaction term between genotype and hemoglobin (or Vitamin C intake) with log(toenail Cd) as the outcome. Genotypes were coded as follows: Allele Dose coding: 0 = no variant alleles, 1 = 1 variant allele, 2 = 2 variant alleles. Genotype specific effect estimates (βs) were extrapolated from interaction terms in age adjusted models with categorically coded genotypes.

[a] βs for a 1 g/dL increase in Hemoglobin with log(toenail Cd) as the outcome.

[b] βs for a 100 mg/day increase in Vitamin C intake with log(toenail Cd) as the outcome.

regular phlebotomies. The HH cases in the Akesson et al. study had clinical hemochromatosis and were iron overloaded, but genetic testing revealed that most were homozygous for HFE C282Y rather than HFE H63D. Our study was quite different in design as we were evaluating a longitudinal cohort of men who were generally healthy at enrollment. To our knowledge none of the NAS subjects had clinical hemochromatosis, which is not surprising as the penetrance of even the C282Y variant is very low. (Nadakkavukaran et al., 2012; Asberg et al., 2007) In our study, we did not find a significant interaction between HFE C282Y and hemoglobin. However, there were only 4 HFE C282Y homozygotes in our study, which gave us limited power to detect such an interaction if it was present and primarily evident among the homozygotes (as was the case with the HFE H63D findings). Having said this, these two studies both suggest that having low hemoglobin levels, or interventions that reduce hemoglobin levels (frequent phlebotomy), may be associated with increased cadmium absorption among HFE variant homozygotes.

One putative explanation of this interaction is that the processes which increase intestinal iron absorption in response to low hemoglobin are altered when the H63D variant is the only available HFE protein. The mechanisms linking hemoglobin levels and iron metabolism are incompletely understood (Pietrangelo, 2010), but if low hemoglobin levels lead to increased activity at intestinal channels which can transport both iron and cadmium, such as DMT-1, (Gunshin et al., 1997; Vesey, 2010) this might increase internal cadmium levels. However, low hemoglobin did not predict increased cadmium levels among wildtypes or HFE H63D heterozygotes. Therefore, if the processes which increase duodenal iron absorption can mediate increased cadmium absorption they do not appear to do so when wildtype HFE is present.

Akesson et al. (Akesson et al., 2000) proposed a mechanism that could be relevant in the interpretation of these findings. They speculated that phlebotomy may have stimulated increased intestinal iron absorption among the hemochromatosis patients in their study. They also proposed that if iron reduction mechanisms (which convert iron III to iron II) at the duodenal mucosa could not be further enhanced to match this additional increase iron transport channels, this might mediate increased cadmium absorption. This could happen because aqueous cadmium ions are divalent (Anon, n.d.) and ready for transport, but some intestinal iron is trivalent and must be reduced to a divalent state prior to absorption. (Mackenzie and Garrick, 2005) Thus, because cadmium and iron can pass through common intestinal transport channels such as DMT-1 (Gunshin et al., 1997; Vesey, 2010), the relative amount cadmium absorption may increase when less of the

Table 7
Genotype stratified effect estimates for the significant interaction in the supplemental copper interaction analyses.

	First toenail Cd values		Mixed model analyses	
	β_{Cu}[a]	% change in Cd associated with a 1 mg/day increase in Cu (95% CI)	β_{Cu}[a]	% change in Cd associated with a 1 mg/day increase in Cu (95% CI)
Copper - HFE H63D Allele Dose	$p_{interaction} = 3.21 \times 10^{-1}$		$p_{interaction} = 1.17 \times 10^{-2}$	
HH (wild type)	0.0148	1.5 (−1.9, 4.9)	0.0179	1.8 (−0.7, 4.2)
HD (heterozygous variant)	−0.0093	−0.9 (−6.9, 5.0)	−0.0369	−3.7 (−7.7, 0.3)
DD (homozygous variant)	−0.2446	−24.5 (−50.6, 1.7)	−0.0864	−8.6 (−24.6, 7.3)

[a] βs for a 1 mg/day increase in copper intake with log(toenail Cd) as the outcome.

luminal iron is in a transportable divalent form. If hemochromatosis patients and HFE H63D homozygotes have a larger magnitude increase in duodenal iron absorption in response low hemoglobin then this putative mechanism may help explain the results from both studies. However, neither of our studies evaluated mechanisms, and this explanation is speculative. If laboratory experiments can more fully characterize the molecular mediators of metal metabolism the explanation of this epidemiologic finding may become more apparent.

The HFE H63D and Vitamin C interaction models suggest there is an inverse relationship between vitamin C intake and cadmium level, and the genotype specific effect estimates suggest that the strength of this relationship increases with the number of variant alleles. These effect estimates predict that a 100 mg/day increase in vitamin C intake corresponds to a 0.2–0.9% decrease in toenail cadmium among wildtype participants, a 1.8–2.0% decrease among HFE H63D heterozygotes, and a 2.5–5.8% decrease among HFE H63D homozygotes. Thus these models suggest that a two standard deviation increase in Vitamin C intake (654 mg/day) would correspond to a 1–6% decrease in toenail cadmium among wildtype participants, a 12–13% decrease among HFE H63D heterozygotes, and a 16–38% decrease among HFE H63D homozygotes. Vitamin C may limit cadmium absorption by reducing iron to divalent form (Fox et al., 1980) thereby enhancing iron absorption and iron stores. Cadmium absorption is known to be decreased in the context of sufficient iron stores. (Flanagan et al., 1978) Those with HFE H63D variants may have enhanced iron absorption capacity (Pietrangelo, 2010; Brasse-Lagnel et al., 2011) which could explain why the vitamin C effect is amplified among them. Vitamin C has also been proposed to have metal chelating abilities (Tajmir-Riahi, 1991), however it is not clear how a metal chelation mechanism could explain different effects among those with HFE H63D variants.

In our data, vitamin C intake was positively correlated with several other nutritional factors that have been shown to reduce cadmium absorption (Spearman Correlation with crude fiber intake: 0.43, iron intake: 0.46, calcium intake: 0.42, zinc intake: 0.51, and manganese intake: 0.51). Given these correlations, it is difficult to draw inferences about the independent effects of these nutritional factors on cadmium levels, and it is possible that the interaction detected for vitamin C may be due in part to the effects of these correlated nutritional variables. However, our results are consistent with previous data suggesting that sufficient vitamin C intake may limit cadmium absorption (Fox et al., 1980; Kim et al., 2010), and our results suggest this effect may be amplified in the presence of HFE H63D variants. Given that excess vitamin C could exacerbate iron overload in HFE H63D homozygotes, the clinical implications of this finding would likely be limited even if this is later shown to be a causal relationship.

We might have expected to also detect interactions between HFE C282Y and hemoglobin/vitamin C, because people with this genotype may also have an increased iron absorption capacity. (Pietrangelo, 2010; Brasse-Lagnel et al., 2011) However, we note that there were a small number of HFE C282Y variants in this study, so we had limited power to detect these interactions if they were present.

The interaction between allele dose coded Transferrin P570S and hemoglobin was only significant in the first toenail cadmium values dataset, and the lack of significance of this interaction in the more powerful mixed model analysis suggests that it should be interpreted with caution. However, the corresponding genotype specific effect estimates in both analyses suggest that low hemoglobin may be associated with increased toenail cadmium among Transferrin P570S homozygotes. These effect estimates predict a 9.2–18.3% increase in toenail cadmium with each 1 g/dL decrease in hemoglobin among Transferrin P570S homozygotes. Although the initial *p*-values for interaction suggest cautious interpretation, the consistency of the effect estimates among Transferrin P570S homozygotes in both models is intriguing and sufficient to warrant validation attempts.

Recently Rentschler et al. evaluated associations between Transferrin variants and cadmium levels in women from Argentina and Bangladesh. (Rentschler et al., 2013) Consistent with our findings they did not report a significant association between RS1049296 (Transferrin P570S) and urine cadmium concentration, but it is not clear from the report if they considered interactions with iron status for this variant. They did however present iron status stratified results for a Transferrin receptor variant: RS2804141. This SNP was associated with higher urine cadmium levels among premenopausal but not postmenopausal Andean women, and it was also associated with higher urine cadmium levels among pregnant Bangladeshi women with low ferritin levels. Taken as a whole, these findings, as well as our findings, and those of Akesson et al. (Akesson et al., 2000) suggest that some variants in iron processing proteins can increase cadmium levels when iron absorption mechanisms are upregulated.

This conclusion suggested a new testable prediction: other factors that are known to influence iron metabolism may interact with these variants to influence Cd levels. Copper is one such factor, (Collins et al., 2010) and to our knowledge it has not yet been shown to influence cadmium absorption (which is why it was not included in the original analyses). Thus we conducted a supplementary analysis to look for interactions with copper intake and we found one significant interaction (Table 7). This interaction suggests that low copper intake may increase cadmium absorption among those with HFE H63D variants. This result appears corroborate the interpretations of our main analyses, but we will refrain from offering a detailed discussion of this exploratory finding.

Finally, we should discuss the role of smoking in this analysis. First we note that we observed some putative differences in cumulative cigarette smoking by genotype. Packyears of cigarettes were significantly higher among HFE C282Y heterozygotes (when compared to non-carriers) and they were significantly lower among Transferrin P570S heterozygotes (when compared to non-carriers). It would be interesting to see if these patterns are observed in other datasets, however it is not clear how these patterns would alter our interpretations. In the HFE C282Y analysis there were no findings to reconsider. Of course, it is possible that excess cadmium exposure through smoking could have masked some interesting patterns in these analyses. However, as we noted above, the HFE C282Y analyses were also limited in terms of power, and they should not be overinterpreted. The Transferrin P570S findings (Table 5) appear to be driven by the homozygotes, and it is not clear how this difference in smoking among heterozygotes would change our tentative interpretations of this result.

We also note that the percentage of former smokers in this study was high (75.4%), but we emphasize that this is not an unexpected result. First, we should mention that the NAS cohort was initiated in the decade of “peak tobacco” in the US. In the 1960s, per capita adult cigarette consumption was at an all-time high, and in 1965, over 60% of US males age 25–34 were smokers. (IOM, (Institute-of-Medicine), 2007) Second, we should add that there is a well-known connection between military service and elevated smoking rates. (Smith and Malone, 2009) Finally we should emphasize that the rate of smoking cessation (the percentage of ever smokers who had quit) consistently increased between 1965 and 1990. (Office-on-Smoking-and-Health, 2014) In accord with these national trends, most men in our study group had quit smoking by the time of their initial toenail submission, and only 4.7% were current smokers. Thus, it is not surprising that 75% of our participants were former smokers.

We should also note that the main findings (Table 5) were quite similar when the 32 current smokers were excluded from the analyses (Table 6). In short, excluding the smokers lowered the power of these analyses but the pattern of findings was not greatly altered.

4.2. Strengths and limitations

Our study benefited from a large sample size, and the use of mixed models which allowed us to incorporate more than one toenail cadmium concentration per participant while accounting for clustering.

These two factors enhanced the power of our study.

The homogeneity of the NAS study group with respect to race, sex, and age is both a strength and limitation of this study. Over 97% of the study population was Caucasian and all were males over the age of 50. This homogeneity should reduce the potential for confounding by factors associated with race, sex, and age, but it may also reduce the generalizability of the findings.

In our study, as in any observational study, a failure to assess relevant interactions may have influenced the results. To address this issue we considered a variety of interactions between the genotypes and factors known to influence cadmium absorption or transport. To our knowledge this is the largest epidemiologic study to evaluate HFE/Transferrin variants and cadmium levels, and the first to evaluate interactions between these variants and nutrients in cadmium processing.

The interactions reported here suggest the existence of sensitive subgroups in cadmium risk assessment, but we recognize that more work remains to be done. Future research may help to corroborate, characterize, and operationalize these subpopulations for risk assessment purposes, but we can start this process by making additional categorical comparisons in our data. Unfortunately, the categorical risk groups may be too small to produce stable estimates, but we can compare medians and avoid over-interpreting the results. Thus, we attempt this approach with our two primary findings (using R - version 2.10.1).

There were 18 HFE H63D homozygote participants in our study and 7 of them had below median hemoglobin levels. The median toenail cadmium level in this group was 0.0277 μg/g, which is 66% higher than that of the whole study group (0.0167 μg/g, $n = 745$). Additionally, there were 6 HFE H63D homozygote participants who had below median vitamin C intake. The median toenail cadmium level in this group was 0.0197 μg/g, which is 18% higher than that of the whole study group. Both of these results are consistent with the regression model findings, but the sample sizes are too small to warrant strong interpretations.

5. Conclusions

Our findings suggest that low hemoglobin levels may be associated with increased absorption of cadmium among HFE H63D homozygotes, and we found tentative evidence for a similar relationship between hemoglobin and cadmium among Transferrin P570S homozygotes. Finally we found evidence that low vitamin C intake may result in increased cadmium uptake, and this relationship appeared stronger among those with HFE H63D variants. More work is needed to investigate if these relationships are replicable and causal. If they are, they suggest the existence of sensitive subpopulations, which should be considered in future cadmium risk assessments. In the US population approximately 12.5–14.8% have an HFE H63D SNP, and 1.5–2.4% are homozygous for HFE H63D. (Steinberg et al., 2001) Furthermore the estimated allele frequency is 8.1% worldwide (Merryweather-Clarke et al., 1997), and thus there may be a large number of potentially sensitive people. Because cadmium toxicokinetics may differ in females (Vahter et al., 2007) and people under 50 years of age (Lauwerys et al., 1994) it would also be useful to further explore the potential influence of HFE and Transferrrin genotypes on cadmium absorption among women and younger participants.

Abbreviations

Cd	cadmium
GCV	Generalized Cross Validation
Hgb	hemoglobin
NAS	Normative Aging Study
HH	Hereditary Hemochromatosis

Ethics approval and consent to participate

The portion of the NAS study analyzed here, was approved by the Human Research Committees of the Harvard School of Public Health and the Department of Veterans Affairs Boston Healthcare System.

Consent for publication

Not applicable.

Availability of data and material

The datasets are not publicly available.

Competing interests

We are not aware of any actual or potential competing interests.

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Authors' contributions

TC performed the statistical analyses with the help of feedback from the other authors. JS, DB, RH, and RW served on the dissertation committee for TC and as this work was part of TCs dissertation, the analytic approach and results were discussed at dissertation committee meetings. TC wrote the manuscript draft. CA analyzed the ICP-MS data to determine Cd levels, helped to interpret the Cd data documentation needed for the epidemiologic analyses, and assured that the Cd determination methods were described appropriately. DS has substantial knowledge of the NAS data and helped make important adjustments in the manuscript. The authors provided comments/edits to the manuscript, and approved the final manuscript. RW was the senior author.

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