Association between hemochromatosis genotype and lead exposure among elderly men: the normative aging study.

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There is considerable variability in the development of toxicity in response to lead exposure in the general population. Genetic factors that modify the absorption, metabolism, or excretion of lead may influence lead toxicity. Genetic variants that predispose individuals to accumulation of lead could occur in enzymes known to influence or regulate lead metabolism. For example, lead is known to bind to the enzyme aminolevulinic acid dehydratase (ALAD), and the absorption of lead is inversely related to calcium stores and dietary vitamin D intake (Chisolm et al. 1998; Mahaffey et al. 1986). Genetic variants in the ALAD and vitamin D receptor genes have been associated with lead exposure biomarkers (Hu et al. 2001; Schwartz et al. 2000a, 2000b; Smith et al. 1995; Wetmur et al. 1997).

Another potential candidate gene for susceptibility to lead exposure is the gene that is altered in hemochromatosis (Onalaja and Claudio 2000; Wright 1999). Hemochromatosis is an autosomal recessive genetic disease that produces an increase in the absorption of ingested iron. Affected subjects may develop iron overload, leading to diabetes, heart disease, and liver disease, but generally do not present until mid- to late adulthood. A hemochromatosis gene (HFE) variant (C282Y) accounts for most cases (Feder et al. 1996). The HFE variant H63D is also associated with hemochromatosis but with a lower penetrance (Wahed et al. 1997).

Both polymorphisms are very common in the U.S. population. Approximately 7–17% of the U.S. general population are heterozygous for C282Y (Bradley et al. 1998; Cox and Kelly 1998; Jouanolle et al. 1997; Phatak et al. 1998), and the prevalence of the H63D heterozygous genotype in the general population has been estimated to be 10–32% (Beutler 1997; Jouanolle et al. 1997). The recent cloning of the HFE gene has made available rapid screening tests using polymerase chain reaction (PCR) techniques to identify subjects who carry either the C282Y or the H63D allele (Burke et al. 1998; Merryweather-Clarke et al. 1999). The high combined prevalence of the two alleles suggests that these two polymorphisms could play a major role in the general population in both the distribution of body iron and the distribution of any metals that share absorptive pathways with iron, such as lead.

Given the high prevalence of HFE variants in the general population and the known association between iron absorption and lead absorption, we hypothesized that these genetic variants may be important modifiers of lead toxicodynamics among heterozygotes. Previous reports suggested that subjects with clinical hemochromatosis had higher lead levels or equivalent blood lead levels (Akeson et al. 2000; Barton et al. 1994). However, a pilot study on 100 subjects we conducted for a grant application showed a trend toward lower blood lead levels among heterozygotes for C282Y and H63D. We therefore left our a priori hypothesis two-tailed. To test this hypothesis, we genotyped a population of elderly men enrolled in an established cohort study of lead biomarkers and chronic disease.

Materials and Methods

This study was conducted on a subsample of the Normative Aging Study (NAS), a multidisciplinary longitudinal study of aging established by the Veterans Administration in 1963 (Bell et al. 1972). Briefly, 2,280 men were enrolled in the NAS. Participants received their first medical examination between 1963 and 1968. Subsequently, subjects have reported for medical examinations and standard blood and urine tests every 3–5 years. During these visits, NAS participants fill out questionnaires on smoking history, education level, food intake, and other risk factors that may influence health. Beginning in 1991, those who gave their informed consent presented to the Ambulatory Clinical Research Center of Brigham and Women’s Hospital for a K X-ray fluorescence (KXRFL) measurement of lead content in the tibia and patella. Study subjects were thus measured for bone lead between 1991 and 1997. For this study, we conducted a cross-sectional analysis using data stemming from the most recent measurement of bone lead for each subject.

Although each NAS subject had signed an approved consent form signifying willingness to have blood archived for unspecified future testing, we were genotyping these archived blood samples for carriers of a known genetic variant.
disease. To maintain the confidentiality of the study subjects and to protect them from any undue possible consequences with respect to employment or health coverage, we created a new data file for the cohort that was kept anonymous and separate from the NAS master data file. Data on potential confounders of the association between \textit{HFE} genotype and bone lead levels were downloaded into this file, as were the bone lead levels and genotype. All identifiers were then deleted. These procedures are consistent with those recommended by the American Society of Human Genetics when genotyping archived blood samples [American Society of Human Genetics (ASHG) 1996; Clayton et al. 1995]. The study and its anonymization procedures were approved by the Human Research Committee of Brigham and Women’s Hospital.

\textbf{Bone lead levels measured by KXRF.} Bone lead measurements were taken at two sites, the mid-tibial shaft and the patella, with an ABIOMED KXRF instrument (ABIOMED, Inc., Danvers, MA, and the Harvard Metals Epidemiology Research Group). The tibia and patella have been targeted for bone lead research because these two bones consist mainly of pure cortical and trabecular bone. A 30-min measurement was taken at the mid-shaft of the left tibia and at the left patella after each region had been washed with a 50% solution of isopropyl alcohol. The tibial midshaft was taken as the point equidistant between the tibial plateau and the medial malleolus. The KXRF beam collimator was sited perpendicular to the flat bony surface of the tibia and at the patella.

As a quality control measure, once a week a 15-ppm phantom was positioned and measured 20 consecutive times overnight as a first-order calibration check. Analysis of means and standard deviations was performed to disclose any significant shift in accuracy or precision. Once each month, the entire set of calibration phantoms (0, 5, 10, 15, 20, 30, 40, 50, and 100 ppm; true values checked by inductively coupled plasma–mass spectrometry) was measured and a calibration curve was calculated as a final check on calibration.

\textbf{Blood lead levels measured by graphite atomic absorption spectrometry.} Fresh blood for lead measurement was taken in a special lead-free tube containing Ethylenediaminetetraacetic acid (EDTA) and sent to ESA Laboratories, Inc. (Chelmsford, MA). Blood samples were analyzed by Zeeman background-corrected flameless atomic absorption spectrophotometer (graphite furnace). The instrument was calibrated before use with National Institute of Standard and Technology materials. Ten percent of the samples were run in duplicate, 10% were controls, and 10% were blanks. In tests on reference samples from the Centers for Disease Control and Prevention, precision (percent relative standard deviation) ranged from 8% for concentrations < 30 µg/dL to 1% for higher concentrations. Blood lead levels are measured at each triennial NAS study visit. For this study, the blood lead measurement most proximal to the bone lead measurement in time was chosen.

\textbf{Hemochromatosis genotyping.} Puregene DNA isolation kits (Gentra Systems, Inc., Minneapolis, MN) were used to extract the DNA from the fresh blood sample. The H63D variant was genotyped by PCR and restriction fragment length polymorphism (RFLP) analysis as previously described (Cardoso et al. 1998). Briefly, the DNA sample was amplified with two primers, 5'-ATG GGT GCC TCA TCA GAG CAG-3' and 5'-AGT CCA GAA GTA CAC AGT-3', to generate a 210-bp fragment. The C282Y variant was also genotyped by a separate PCR and RFLP procedure (Cardoso et al. 1998). Wild-type alleles are designated H, and variant alleles are designated D. For C282Y, DNA sample was amplified with two primers, 5'-TGG GAA GGT TAA ACA GAT CC-3' and antisense primer 5'-TAC CTC CTC AGG CAC TCC TC-3' (Feder et al. 1996). A random sample of 10% of subjects was run in duplicate as a quality control measure. Genotypes were also determined on control blood known to be from subjects homozygous for the wild-type genotype and heterozygous and homozygous for each variant genotype. Wild-type alleles are designated C, and variant alleles are designated Y. Joint genotypes are expressed using both categories. For example, a subject wild-type for both C282Y and H63D is designated \textit{CC HH}, and a subject heterozygous for both variants is designated \textit{CY HD}.

\textbf{Data analysis.} We conducted a cross-sectional analysis of the association between \textit{HFE} variants and bone/blood lead concentration among elderly men. We first compared characteristics of subjects who had all the data of interest, including genotypes, bone/blood lead levels, and covariate data with subjects who were not included because of missing data. Allele and genotype frequencies and tests for Hardy-Weinberg equilibrium were performed. Univariate distributions of continuous variables were examined to determine departures from normality. For quality control purposes, we identified and omitted tibia and patella bone lead measurements with estimated uncertainties > 10 and 15 µg/g, respectively (these measurements usually reflect excessive patient movement during the measurement). Such procedures are standard in analysis of bone lead data (Hu 1998; Hu et al. 1991). Because we did not make an \textit{a priori} assumption that either cortical or trabecular bone lead is of greater significance with respect to \textit{HFE} variants, analyses were repeated for the summary measures of both tibia and patella lead.

The distributions of demographic and lifestyle characteristics and bone/blood lead levels by genotype (wild-type vs. C282Y or H63D carrier) were examined, and differences were tested by chi-square or Student’s \textit{t}-test as appropriate. Multivariate linear regression was used to model determinants of tibia lead, patella lead, and blood lead. To simplify the analysis, we decided \textit{a priori} to combine the data on the two alleles into a single indicator term (i.e., presence of one or two copies of either gene variant) if the bivariate analysis of genotype and bone/blood lead levels demonstrated consistent findings for both C282Y and H63D. Major core model determinants, based on the previous work of this laboratory, include age, education level, and cumulative smoking (Hu 1998; Hu et al. 1996). Each of these regressions was repeated, adding an indicator variable for hemochromatosis genotype. To assess whether the genotype may serve as an effect modifier of the relationship between our covariates and our lead biomarkers, we also compared the \(\beta\)-estimates of core-model determinants in regressions of our lead biomarkers stratified by genotype (C282Y or H63D carrier vs. wild-type). If a core model \(\beta\)-estimate was different between genotypes [i.e., the \textit{HFE} variant model \(\beta\)-estimate was outside the 95% confidence interval (CI) bounds of the wild-type model], an additional exploratory regression model was run that included a cross-product term for interaction between genotype and the core-model determinant of interest.

In models using blood lead as the dependent variable, patella or tibia lead levels have been included as independent variables because they are major predictors of blood lead levels in the NAS, probably because bone stores are the major source of lead exposure among this cohort of elderly men (Hu et al. 1998). However, if \textit{HFE} variants predict changes in bone lead levels, then these bone lead levels may be an intermediate variable between changes in blood lead associated with \textit{HFE} variants. Therefore, we did not include bone lead levels as a covariate in models to predict blood lead levels.

In the final models, we combined the two genotypes into a single indicator variable representing gene variant presence or dose. In one model, the presence of either variant was coded as \textit{yes}/\textit{no}. In the other model, we examined the dose effect of the variants by coding the genotype on an ordinal scale (none, one, or two variants present).

\textbf{Results} A total of 765 subjects in the NAS participated in the KXRF study and had archived blood. Of these, 730 were genotyped for H63D and C282Y. Archived blood from 35 subjects could not be reliably genotyped. The means and distributions of blood lead, bone lead, age,
education, and smoking among all subjects and the 35 who were not genotyped were similar (data not shown). The overall prevalence values for C282Y genotypes were wild-type, 87.1%; heterozygote, 12.2%; and homozygote, 0.7%. The prevalence values for H63D genotypes were wild-type, 74.9%; heterozygote, 22.6%; and homozygote, 2.5%. The distributions of both genotypes conformed to Hardy-Weinberg expected frequencies (C282Y: chi square = 0.93, p = 0.34; H63D: chi square = 1.69, p = 0.19). When collapsed into a single variable indicating the presence or absence of either variable, 261 (36.0%) of subjects in this sample carried at least one copy of either HFE variant.

Table 1 shows the distribution of the lead biomarkers and covariates stratified by genotype. The overall trend in the bone and blood lead levels was that carriers of HFE variants had lower bone and blood lead levels. Subjects with either one or two copies of either allele had lower bone or blood lead levels on average than did wild-type subjects (Table 1). As in previous studies, smoking, age, and education levels were important predictors of bone and blood lead concentration in the core regression models (Table 2).

In the multivariate regression models using either H63D or C282Y separately, lead biomarkers were consistently lower among subjects carrying at least one copy of either the C282Y or H63D allele (Table 3). Given these results, we collapsed the two variants into a single dichotomous variable indicating the presence or absence of either variant, and a three-level categorical variable indicating the dose of either variant (none, one, or two alleles) of either C282Y or H63D. Subjects with at least one HFE variant had significantly lower patella and blood lead levels than did subjects with wild-type genotypes (Table 3). Similar to the results of the crude analysis, subjects with HFE variants consistently had lower bone and blood levels than did subjects with wild-type alleles in all multivariate models (Table 3). To explore for effect modification, we then repeated the linear regression models stratifying on the presence or absence of an HFE variant. In the stratified model, the coefficients for age, smoking, and education differed between models of wild-type carriers and HFE variant carriers, whereas only the interaction term for age HFE (coded as carrier of either C282Y or H63D) in the model for tituba lead levels reached statistical significance (β = -0.3; 95% CI, -0.6 to -0.01; p < 0.05). The interaction is in the direction of a decreased age–bone lead slope among the HFE variant carriers compared with wild-type individuals. To better illustrate this interaction, we created smoothed plots of tibia lead versus age, adjusted for smoking and education. Figure 1 illustrates this interaction. To construct this figure, we repeated the stratified models using the residuals of age regressed by smoking and education as the independent variable. The residuals of tituba lead levels regressed by smoking and education were used as the dependent variable. The residuals were then plotted, and the smoothed plot was constructed using a lowess smoothing function with a bandwidth of 0.3. There was also evidence of modification by HFE genotype on the association between patella and blood lead levels. The β-coefficient for blood lead in predicting patella lead among wild-type genotype subjects was 0.08 (95% CI, 0.06 to 0.09), whereas among HFE variant carriers it was 0.05 (95% CI, 0.03 to 0.07). The interaction term for this difference was (–0.03; p = 0.041). We also constructed a smoothed plot of patella lead versus blood lead levels stratified by presence or absence of either HFE variant (Figure 2). The association between patella and blood lead levels is nearly linear among wild-type subjects but is nonlinear among HFE variants carriers, in which there appears to be a relatively flat association between patella lead and blood lead levels at low and high blood lead levels.

### Discussion

Our results indicate that among elderly men, the presence of a hemochromatosis variant allele (C282Y or H63D) predicts lower bone and blood lead concentrations. Because iron status is inversely associated with lead absorption, we believe that these results may be secondary to increased iron stores among HFE variant carriers causing decreased lead absorption in the gastrointestinal tract. During iron deficiency, regulatory mechanisms that cause an increase in iron absorption cause an increase in the percentage of ingested lead that is absorbed (Barton et al. 1978; Mahaffey-Six and Goyer 1972). Several clinical studies also have supported this association. An inverse association between dietary iron and blood lead level was found by Bradman et al. (2001) and Hammad et al. (1996) in separate studies. Similarly, our research group found an association between biomarkers of iron deficiency and elevated blood lead levels in children (Wright et al. 2004).
1999). Other clinical studies have demonstrated that high body iron stores are associated with decreased blood lead levels (Graziano et al. 1990). These previous studies formed the basis of our hypothesis that the HFE gene would be a candidate gene for modifying lead absorption and body burden.

Our results confirm that HFE genotype does indeed predict differences in lead biomarkers, although our results differ from those of previous studies in terms of the direction of the effect. Both previous reports (Akesson et al. 2000; Barton et al. 1994) suggested that subjects with hemochromatosis had higher blood lead levels. Our study differs from the previous reports in being a cohort-based study, in focusing mainly on subjects heterozygous for HFE variants, in being conducted exclusively in men, and in the older average age of the participants. All these factors may play a role in explaining the different results. In both previous reports, investigators compared subjects with hemochromatosis (i.e., homozygotes or compound heterozygotes) with control groups. In the earliest report, Barton et al. (1994) hypothesized that if iron deficiency and lead absorption were associated, lead absorption would be increased in subjects with hemochromatosis. In the study by Barton et al. (1994), blood lead levels were higher among subjects with clinical hemochromatosis than among controls. The mean blood lead level among 44 subjects with clinical hemochromatosis was 5.6 µg/dL and among 33 controls was 3.6 µg/dL. (p < 0.05). In contrast, Akesson et al. (2000) found no difference in blood lead levels between hemochromatosis subjects and controls. Instead, the investigators found an association between higher blood lead levels and longer duration of phlebotomy treatment. Phlebotomy is the treatment for clinical hemochromatosis. In attempting to explain this finding, the authors speculated that lower iron stores in phlebotomized subjects with hemochromatosis caused an up-regulation of the absorptive pathways by which iron and lead are absorbed, thus increasing blood lead levels.

Because there are no physiologic mechanisms for iron excretion, one possible explanation for the different results of the three studies is that age may serve as a proxy measure of increasing body iron stores, which in turn is inversely associated with lead absorption. Under this hypothesis, the interaction between tibia lead and age might reflect the down-regulation of both iron and lead absorption as body iron stores increase with age, a hypothesis also proposed by Akesson et al. (2000). Older subjects would, on average, have larger body iron stores and as they advance in age would absorb less environmental iron and less environmental lead. This decrease in absorbed lead with advancing age may cause the decrease in both bone and blood lead seen in the older subjects of the NAS cohort. HFE variant carriers may have higher iron stores earlier in life and may down-regulate iron and lead absorption sooner than subjects with wild-type genotypes. The study by Akesson et al. (2000) also suggests that body iron stores may regulate iron/lead absorption in subjects with clinical hemochromatosis. Subjects heterozygous for C282Y have evidence of higher body iron stores than do subjects with wild-type genotypes (Datz et al. 1998; Garry et al. 1997), and body iron stores still regulate iron absorption inversely even in subjects with clinical hemochromatosis (McLaren et al. 1991).

Therefore, subjects with HFE variants may have higher iron stores on average and lower lead absorption, because of the decreased risk of iron deficiency. Of interest, the mean age in our study was 70.8 years, whereas in the Barton et al. (1994) and Akesson et al. (2000) studies,

![Figure 1](image1.png)
*Figure 1. Smoothed plot of residuals for tibia lead levels versus age. (A) Relationship among wild-type subjects (CC HH). (B) Same relationship among subjects with at least one copy of C282Y or H63D. Values were adjusted for education level and smoking.*

![Figure 2](image2.png)
*Figure 2. Lowess smoothed plot of residuals for patella lead levels versus blood lead levels. Wild-type subjects (A) have the genotype CC HH. HFE variant carriers (B) have at least one copy of C282Y or H63D.*


the mean ages were 49.4 years and 55.5 years, respectively. Higher lead levels among subjects with hemochromatosis were noted only in the Barton et al. (1994) study, which had the youngest participants. Our study population may have been particularly predisposed to elevated iron stores because it was conducted solely in men, a group that may have a particularly low risk of iron deficiency. A similar study in a population at high risk for iron deficiency, such as women of child-bearing age, may be expected to have different results. We would note that in Barton et al. (1994), female subjects with hemochromatosis had higher blood lead levels than did male hemochromatosis subjects, whereas control female patients had lower blood lead levels than did male controls.

There are limitations to this study. We have no data on female subjects and can only speculate on sex-specific effects of HFE variants on lead dose biomarkers. There is also the possibility that population structure may have produced our results. Such an effect is less likely given the homogenous ethnicity of the NAS cohort. More than 95% of subjects are Caucasian. There are no known differences in blood lead levels than did male controls.

In summary, in a cohort study of elderly men, HFE variants predicted lower patella and blood lead levels. HFE variants also modified the effect of age on tibia lead levels, with younger subjects carrying HFE variants having higher tibia lead levels and older subjects with HFE variants having lower tibia lead levels. These results may be caused by the effect of the HFE gene product on increasing body iron stores with the eventual down-regulation of lead absorption.

REFERENCES

