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Blood Lead Levels, *ALAD* Gene Polymorphisms, and Mortality

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Abstract

Background—Previous analyses from the National Health and Nutrition Examination Survey (NHANES III) have found that elevated blood lead levels may be associated with cardiovascular mortality, cancer mortality, and all-cause mortality. The 5-aminolevulinic acid dehydratase (*ALAD*) G177C genetic polymorphism (rs 1800435) affects lead toxicokinetics and may alter the adverse effects of lead exposure. We examined whether the *ALAD* G177C single nucleotide polymorphism (SNP) affects the relationship between lead and mortality.

Methods—We analyzed a subset of 3349 genotyped NHANES III participants at least 40 years of age. Using Cox proportional hazards regression, we estimated the relative risk of all-cause, cardiovascular disease, and cancer mortality by *ALAD* genotype, and by blood lead levels (<5 µg/dL vs. ≥5 µg/dL). We also tested whether the *ALAD* genotype modified the relationship between blood lead level and mortality.

Results—The adjusted overall relative risk for participants with the variant *ALAD*^{CG/CC} genotype was decreased for all-cause mortality (hazards ratio = 0.68; [95% confidence interval = 0.50–0.93]) compared with persons having the common GG genotype. There was some suggestion that higher lead levels were associated with cancer mortality (1.48 [0.92–2.38]). We observed no convincing interaction effect between *ALAD* genotype and blood lead level on mortality risk.

Conclusion—The *ALAD*^{CG/CC} genotype may be associated with decreased mortality from all causes and from cancer. This association does not seem to be affected by lead exposure.

Lead is a naturally occurring heavy metal used in manufacturing, and in consumer products such as batteries, paints, metal products (eg, sheet metal), cable coverings, and ceramic glaze. Environmental exposure to lead in developed countries occurs primarily through contact with lead-contaminated soil, dust, drinking water, and deteriorating lead-based paint.¹ Toxic effects of lead exposure are well-established in human populations and animal models. At high levels, lead has adverse effects on hematopoietic, gastrointestinal, urinary, cardiovascular, and nervous systems.² Lower doses of lead exposure have been associated with aberrant cognitive development in children, anemia, hypertension, and neurologic

disorders.³⁻⁵ The International Agency for Research on Cancer (IARC) classifies inorganic lead as a probable human carcinogen (group 2A).⁶

The well-established acute toxicity of lead at high doses has resulted in stringent guidelines limiting occupational and environmental exposures. Allowable blood lead levels for adults with occupational exposures in most countries is 40–50 µg/dL.⁷ While these guidelines are designed to protect the general population, genetically susceptible individuals may not be fully protected by the current standards. One critical mechanism of lead toxicity is its inhibition of key enzymes within the heme biosynthetic pathway. The best characterized interaction is between lead and the second enzyme in the heme biosynthetic pathway, 5-aminolevulinic acid dehydratase (ALAD).

Rodents genetically modified to have an extra copy of the gene coding for ALAD accumulated higher levels of lead in the kidney (2.4-fold higher), liver (4.1-fold higher), and brain (2.5-fold higher) when compared with wild-type mice exposed to the same doses of lead.⁸ In humans, the gene that encodes ALAD exists in 2 polymorphic forms (*ALAD*₁ and *ALAD*₂). These may influence a person's susceptibility to lead poisoning.⁹ The difference between the 2 forms of *ALAD* is an amino acid substitution of asparagine for lysine residue 59, resulting from a single nucleotide change in position 177 (G → C transversion (rs1800435)) of the coding region.¹⁰ This substitution is thought to cause *ALAD*₂ to have an increased affinity for lead,¹¹ although it is not known how this increased affinity alters the circulating pool of lead in humans. Recent studies suggest higher mean blood lead levels among individuals with the *ALAD*₂ variant at levels of high lead exposures.¹²⁻¹⁴ This increase in blood lead levels was not observed among persons with *ALAD*₂ exposed to low levels of lead (as reviewed by Scinicariello et al¹⁵).

Mortality follow-up of National Health and Nutrition

Examination Survey II (NHANES)^{16,17} and NHANES III¹⁸ has suggested an increased risk of mortality at blood lead levels as low as 5–9 µg/dL. Since the *ALAD* G177C polymorphism affects lead toxicokinetics, we hypothesized that this polymorphism could modify the risk of mortality associated with exposure to lead. We tested this hypothesis using data from the Third National Health and Nutrition Survey (NHANES III).

METHODS

Third National Health and Nutrition Survey

NHANES III is a complex, multistage sample survey conducted by the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention (CDC) from 1984 through 1994.^{19,20} It collected detailed information from the civilian, noninstitutionalized population of the United States over the age of 2 months. Young children, older adults, non-Hispanic blacks, and Mexican-Americans were oversampled.¹⁹ Race/ethnicity was defined based on the combination of self-reported race (black, white, other) and ethnicity (not Hispanic, Mexican-American, and other Hispanic). The survey consisted of a household interview and a standardized physical examination performed in a mobile examination center. Details of the survey have previously been described.²¹

DNA Isolation and Genotyping Methods

As part of Phase 2 of NHANES III (1991–1994), 10,052 participants 12 years or older were examined in mobile units. The CDC/NCHS ethics review board approved a revised research plan to link genetic laboratory results to NHANES data through the NCHS Research Data Center to ensure confidentiality of the study participants.²² The generation of the NHANES III DNA bank has been previously described.²³ Briefly, white blood cells from 8200

Phase-2 participants were used to generate Epstein-Barr virus-transformed cell lines.^{24,25} Genomic DNA was successfully isolated and genotyped from 7159 participants (71%). Exclusions include participants from whom cells could not be successfully transformed and expanded (n = 1004) or whose samples were disqualified for other laboratory and quality control reasons (n = 21), and samples not genotyped (n = 16). DNA analysis for the overall NHANES III genotyping project was performed at 2 facilities because neither laboratory had the methodology to analyze all of the selected genetic variants.

Detailed genotyping information has been previously described.²³ Most polymorphisms were assayed by either the TaqMan assay (Applied Biosystems, Foster City, CA) or the MGB Eclipse assay (Nanogen, Bothell, WA). *ALAD* SNP rs1800435 was analyzed using the TaqMan assay; Chang et al²³ provide information on primer and probe sequences. Polymorphisms were genotyped by pyrosequencing and capillary fragment analysis. Duplicate samples were analyzed, along with no-template controls. The genotyping error rate on the blind quality control plates was <1%, with no evidence of contamination in the no-template controls. The average success rate for genotyping of the NHANES III samples was 95%. All genotyping data from the NHANES III genomic study are maintained in a NCHS database.²²

Blood Lead Level Determination

During the physical examination, venous blood was drawn for all survey participants 1-year-old and stored in EDTA-anticoagulant tubes prescreened for lead contamination. Blood lead concentrations were measured by graphite furnace atomic-absorption spectrophotometry and recorded as micrograms per deciliter ($\mu\text{g/dL}$). Detailed laboratory methods have been described previously.²⁶ Blood lead levels below detectable levels were assigned the value of 0.7 $\mu\text{g/dL}$.

Study Sample

This study was restricted to the 7159 participants who were successfully genotyped. We excluded persons younger than 40 years at interview (n = 3803) due to the small number of mortality events, and also those with no baseline blood lead measurements (n = 7). The remaining 3349 subjects made up our primary sample. Additional participants were excluded from our adjusted modeling due to missing data on *ALAD* genotype (n = 103), education (n = 22), and date of study entry (n = 1), resulting in a study population of n = 3223.

Definition of Outcome and Covariates

We assessed risk of mortality from all causes, major cardiovascular disease (ICD-10 codes I00-I78), and malignant neoplasm (ICD-10 codes C00-C97). Sampled persons were considered to have had an event if they were matched to a death record corresponding to the type of mortality. Information on determination of mortality status and cause has been previously described.²⁷⁻²⁹ Briefly, participants were passively followed for mortality through December 2000. A probabilistic methodology was employed to link NHANES III participants with the National Death Index records. The overall correct classification of vital status is reported to be 98.5%, based on a calibration study.²⁷ Age at death was defined as the time to event. Persons who were not matched to a death record were considered alive through the follow-up period and administratively censored at 31 December 2000.

Age was defined as the participant's age at the baseline examination. Other covariates were categorized as follows: race/ethnicity as non-Hispanic white, non-Hispanic black, Mexican-American, or other; education level as <12 years, completed high school, and > high school; total family income as <\$20,000/year and $\geq$ 20,000/year; and smoking status as never,

former, and current smokers. Alcohol consumption was analyzed as a continuous variable defined by summing the number of drinks of beer, wine, or liquor per week (more than 21 drinks were included in the 21 drinks/ week group). Blood lead levels were initially categorized (based on the current lead guidelines for exposure³⁰ and previous work on lead exposure in NHANES III¹⁸) as <5, 5–9, and >10 µg/dL. Because of small sample size in the >10 µg/dL category, we combined the top 2 categories, dichotomizing lead exposure (<5 and 5 µg/dL) for all reported analyses. *ALAD* variants were modeled as the referent category versus a combined category of heterozygous and homozygous variants (*ALAD*^{GG} vs. *ALAD*^{CG/CC}) based on the small number of persons with an *ALAD*^{CC} genotype.

Statistical Analysis

Hardy-Weinberg equilibrium was tested using a Rao-Scott Satterwaite Adjusted *F* test method developed for complex sample designs.³¹ We used Cox proportional hazard regression analysis, with attained age as the time scale, to examine the relative hazard of mortality from all causes, cardiovascular disease, and cancer. Mortality groups were analyzed using the 2 categories of blood lead, which are described earlier in the text. We stratified the baseline hazard by birth cohort in 6-year intervals to account for potential differences in the cumulative exposure to lead before the late 1970s, when lead exposures began to decline dramatically.³²

We used a multivariate proportional hazard model to examine the association between lead and mortality, adjusting for potential cofounders. The dose-response relationship between lead mortality was evaluated using a 5-knot cubic regression spline in the multivariate proportional hazards model.³³ Statistical analysis was conducted using the SAS System for Windows (version 9.1; SAS Institute Inc., Cary, NC) and SAS-Callable SUDAAN (version 9.01; CDC/ NCHS, Hyattsville, NC). All models included sample weights that accounted for the unequal selection probabilities and nonresponse within NHANES III.¹⁸ Interactions between *ALAD* and serum lead were tested using *T* tests for complex samples.³⁴

RESULTS

Selected characteristics of participants genotyped in the NHANES III cohort by blood lead categories are summarized in Table 1. The mean follow-up time was 7.8 years for those with low blood lead level (<5 µg/dL) and 7.5 years for those with high blood lead level (≥ 5 µg/dL). The median ages at baseline for the low and high blood lead level categories were 57 and 61 years, respectively. Blood lead levels were higher among men, non-Hispanic blacks, current smokers, persons with a total family income <\$20,000/year, and those completing less than 12 years of formal education. No difference was observed between the low and high blood lead categories for BMI, alcohol intake, or serum calcium and cholesterol levels. The *ALAD* gene was in Hardy-Weinberg equilibrium (*P* = 0.89).

Table 2 summarizes the association of blood lead levels and *ALAD* C177G polymorphism with mortality risk. Initially, *ALAD* genotype and blood lead level were modeled independent of one another for the 3 mortality outcomes. Risk estimates from these models were similar to estimates from models containing both. Therefore, all models were adjusted for age, education, sex, race/ethnicity, blood lead level, *ALAD* genotype, and smoking status. Participants with the *ALAD*^{CG/CC} genotype had decreased risk of all-cause mortality (hazard ratio = 0.68 [95% CI = 0.50–0.93]), with a similar decrease in cancer mortality (0.62 [0.29–1.29]).

Risk estimates were similar within race/ethnicity groups except for Non-Hispanic Blacks, where no association was observed (data not shown). However, due to small numbers these estimates were unstable in some cases, making them difficult to interpret. Higher blood lead

levels ($\leq 5 \mu\text{g/dL}$) were associated with a suggestive increased risk of cancer mortality (1.48 [0.92–2.38]). Blood lead levels $\leq 5 \mu\text{g/dL}$ were not associated with risk of all-cause or cardiovascular mortality.

To examine the joint effect of blood lead levels and *ALAD* genotype on mortality, we modeled the effect of lead within categories of *ALAD* (Table 2). Due to the limited number of deaths among those with the *ALAD*^{GC/CC} genotype and higher blood lead levels, these estimates are not stable. No statistically significant interaction was observed between *ALAD* genotype, blood lead levels, and risk of mortality.

We further explored the relationship of blood lead levels and *ALAD* genotype with mortality by modeling the proportional hazards. Blood lead levels were on a continuous scale with a 5-knot cubic regression spline adjusted for the same covariates as in the previously described models. Cancer mortality was again modestly increased as blood lead levels increased. Similarly, the *ALAD*^{GC/CC} variant was associated with an overall decreased relative hazard for all-cause and cancer mortality when modeled using the spline regression (results not shown).

DISCUSSION

We observed a suggestive increase in cancer mortality with blood lead levels at $5 \mu\text{g/dL}$ or above, while cancer mortality and all-cause mortality were lower with the *ALAD* G177C variant. Our a priori hypothesis for examining the *ALAD* G177C polymorphism was based on its known biologic interactions with lead.^{9,13,35–37} However, we did not find convincing evidence that *ALAD* modifies the effect of lead.

A small body of literature suggests a possible interaction between the *ALAD* G177C polymorphism and lead exposure in relation to health outcomes, including meningioma^{38,39} and adverse neurologic outcomes.⁴⁰ We saw no convincing evidence that the *ALAD* G177C polymorphism modified the effect of lead. While the apparent lack of effect modification by *ALAD* genotype could be due to a true lack of association, it is also possible that this study was not adequately powered to detect such an interaction. Also, given the small numbers, we were not able to examine risk of particular cancer subtypes.

Although we did not observe effect modification by *ALAD* genotype, we did observe a main effect of the genotype, with the *ALAD*^{GC/CC} allele associated with a decreased risk of all-cause mortality. The *ALAD*^{CC} allele has been reported to occur in 20% of whites and less frequently in populations of African and Asian descent.^{10,41} The overall weighted allele frequency of *ALAD*^{CC} in the NHANES III genotyped cohort was 8.1% among non-Hispanic whites, 1.3% among non-Hispanic blacks, and 4.5% among Mexican-Americans.²³ There is no difference in ALAD activity in the heme biosynthesis pathway among the different phenotypes [Lys-Lys(^{GG}), Lys-Asp(^{GC}), Asp-Asp(^{CC})],⁴² although the Asp-Asp phenotype has been associated with an increased affinity for lead, leading to enzymatic inhibition. ALAD has also been identified as a proteasome regulatory subunit (CF-2)⁴³ involved in regulating protein degradation and proteasomal activity.⁴⁴ Whether the activity of the various ALAD isozymes is altered with regards to normal proteasomal activity remains unclear.

The overall decreased risk of all-cause mortality among participants with the *ALAD*^{GC/CC} allele could also be a result of undetected changes in metabolic function within pathways where ALAD is active. For example, ALAD inhibition results in an accumulation of 5-aminolevulinic acid (ALA), the first committed precursor of heme. Overproduction and accumulation of ALA have been associated with hepatic porphyria, neurologic disorders (as

reviewed by Pischik and Kauppinen⁴⁵), and DNA damage^{46,47} through the production of reactive oxygen species. An increase in ALAD activity due to the G3C transversion could lead to a decrease in circulating ALA. ALAD is also considered a metalloprotein. A structural change from a charged lysine to a neutral asparagine could result in altered binding of not only lead but also other metals such as zinc or magnesium.⁴⁸ Finally, the association may not be related to the *ALAD* G177C polymorphism, but instead be an indication of linkage disequilibrium between this polymorphism and an unmeasured nearby variant.

The increased cancer mortality risk with blood lead level is consistent with previous studies of lead exposure and mortality in larger populations. A previous evaluation in the NHANES II population reported an increased risk for all-cause mortality (rate ratio = 1.24 [95% CI = 0.97–1.57]) and cancer mortality (1.70 [1.04–2.78]) among persons with blood lead levels 10–19 µg/dL; risk was similarly elevated among participants with blood lead levels 20–29 µg/dL.¹⁷ Schober et al¹⁸ reported a similar increased risk in all-cause mortality (HR = 1.59 [1.28–1.98]) and cancer mortality (1.69 [1.14–2.52]) in a larger sampling of participants with blood lead levels >10 µg/dL in NHANES III.¹⁸

An important limitation of this study is the potential for exposure misclassification. Blood lead levels were based on a one-time measurement at the time of examination. Lead has a half-life in blood of only about 1 month.⁴⁹ Long-term lead exposure is better measured in bone, where the half-life is between 5 and 19 years.⁵⁰ Nonetheless, lead is stored, released, and reabsorbed in the bone throughout a person's lifetime. As mammals age, bone lead stores are released into the blood through resorption, and this release has been associated with higher blood lead levels in older populations,⁵¹ including NHANES III.¹⁸ In addition, the general population was exposed to much higher levels of environmental lead prior to the reduction in lead in gasoline implemented in the late 1970s and completed in 1989. Although we attempted to control for these differences (using 6-year birth cohorts in our regression analysis), exposure misclassification bias may persist. This analysis was based on 7159 out of 10,052 participants in Phase 2 of NHANES III. While this was expected to be a random sample, there remains a possibility that genotyped participants differed in some systematic way from nongenotyped. It is also possible that we simply failed to measure the critical window of lead exposure related to chronic diseases.

Another concern is that race/ethnicity was defined in NHANES III using a combination of self-reported race and ethnicity. Ancestry-informative markers are not available, limiting our ability to adjust for admixture. We attempted to adjust for population substructure by stratifying on race/ethnicity. Results were generally consistent within the 4 groups.

Although this study had limited power for evaluating risk of the homozygous *ALAD*^{CC} variant, it remains one of the largest studies of the *ALAD* G177C polymorphism and acute lead dose to date. The primary strength of our study is that the NHANES III is representative of the US population. Among those genotyped, there is detailed survey information and well-preserved biologic samples that have allowed for comparisons of genotype and environmental exposures such as lead. Examination of the main effect of *ALAD* genotype on mortality needs to be replicated in other studies, and further research is needed to assess what mechanisms may contribute to this increased risk, if indeed it is real.

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TABLE 1
Selected Characteristics of the NHANES III Study Cohort by Blood Lead Level

Characteristic	<u>Blood Lead (ug/dL)</u>	
	<5	5
Sample size (no.)	2532	817
No. person-years	19,750	6128
Follow-up time (years); mean	7.8	7.5
Blood lead level ($\mu\text{g/dL}$); median (range: 0.2–52.9 $\mu\text{g/dL}$)	2.6	7.5
Sex; no. (%)		
Men	910 (41)	555 (68)
Women	1622 (59)	262 (32)
Age at baseline (years); median	57	61
Race/ethnicity; no. (%)		
Non-Hispanic white	1330 (81)	320 (71)
Non-Hispanic black	491 (8)	276 (17)
Mexican-American	594 (4)	195 (4)
Other	117 (7)	26 (8)
Education ^a ; no. (%)		
<12 years	1004 (22)	436 (39)
Completed high school	771 (33)	233 (36)
>High school	743 (45)	140 (25)
Missing	14	8
Total family income <\$20,000; no. (%)	1065 (26)	442 (40)
Smoking status at baseline; no. (%)		
Never	1370 (51)	261 (26)
Former	742 (31)	296 (39)
Current	420 (18)	260 (35)
BM1 (kg/m^2); mean	28	27
Alcohol intake (drinks/week); mean	5.9	5.9
Serum cholesterol (mg/dL); mean	215.8	212.9
Serum calcium (mmol/L); mean	1.24	1.24
ALAD ^a ; no. (%)		
ALAD ^{GG}	2173 (85)	718 (87)
ALAD ^{GC/CC}	283 (15)	72 (13)
Missing	76	27

^aPercentage based on nonmissing values.

ALAD indicates 5-aminolevulinic acid dehydratase; NHANES, National Health and Nutrition Examination Survey.

TABLE 2
Adjusted Relative Risk for All-cause, Cardiovascular Disease, and Cancer Mortality by Blood Lead Level and ALAD rs1800435 Polymorphism

	Mortality					
	All Causes		Cardiovascular		Cancer	
	No. Deaths	HR ^a (95% CI)	No. Deaths	HR ^a (95% CI)	No. Deaths	HR ^a (95% CI)
Blood lead level (ug/dL) ^b						
<5 ^c	359	1.00	178	1.00	66	1.00
5	195	1.19 (0.91 – 1.56)	95	1.11(0.69 – 1.78)	53	1.48 (0.92 – 2.38)
ALAD rs1800435 ^b						
GG ^c	499	1.00	245	1.00	106	1.00
CG/CC	55	0.68 (0.50 – 0.93)	28	0.89 (0.62 – 1.27)	13	0.62 (0.29 – 1.29)
Lead-gene interaction						
Lead<5 μg/dL ALAD ^{GG}	321	1.00	159	1.00	57	1.00
Lead 5 μg/dL ALAD ^{GG}	178	1.16 (0.92 – 1.45)	86	1.03 (0.66 – 1.60)	49	1.43 (0.93 – 2.37)
Lead<5 μg/dL ALAD ^{CG/CC}	38	1.00	19	1.00	NR ^d	
Lead 5 μg/dL ALAD ^{CG/CC}	17	1.53 (0.69 – 3.39)	9	1.78 (0.71 – 4.44)		
Test for interaction ^e	P = 0.37		P = 0.13		P = 0.95	

^a Adjusted for age, education, sex, smoking status, race/ethnicity, ALAD and blood lead levels using Cox proportional hazard regression.

^b Estimates are derived from the same model.

^c Reference category.

^d NR indicates not reportable. Due to confidentiality requirements with the NCHS, we cannot report data when the number of deaths is fewer than 5.

^e Interactions were tested using a T test and adjusting for age, education, sex, and smoking.

ALAD indicates 5-aminovulnic acid dehydratase; HR, hazard ratio; CI, confidence interval.