Lead-Related Genetic Loci, Cumulative Lead Exposure and Incident Coronary Heart Disease: The Normative Aging Study

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Abstract

Background

Cumulative exposure to lead is associated with cardiovascular outcomes. Polymorphisms in the δ-aminolevulinic acid dehydratase (*ALAD*), hemochromatosis (*HFE*), heme oxygenase-1 (*HMOX1*), vitamin D receptor (*VDR*), glutathione S-transferase (*GST*) supergene family (*GSTP1*, *GSTT1*, *GSTM1*), apolipoprotein E (*APOE*), angiotensin II receptor-1 (*AGTR1*) and angiotensinogen (*AGT*) genes, are believed to alter toxicokinetics and/or toxicodynamics of lead.

Objectives

We assessed possible effect modification by genetic polymorphisms in *ALAD*, *HFE*, *HMOX1*, *VDR*, *GSTP1*, *GSTT1*, *GSTM1*, *APOE*, *AGTR1* and *AGT* individually and as the genetic risk score (GRS) on the association between cumulative lead exposure and incident coronary heart disease (CHD) events.

Methods

We used K-shell-X-ray fluorescence to measure bone lead levels. GRS was calculated on the basis of 22 lead-related loci. We constructed Cox proportional hazard models to compute adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for incident CHD. We applied inverse probability weighting to account for potential selection bias due to recruitment into the bone lead sub-study.

Results

Significant effect modification was found by *VDR*, *HMOX1*, *GSTP1*, *APOE*, and *AGT* genetic polymorphisms when evaluated individually. Further, the bone lead-CHD associations became larger as GRS increases. After adjusting for potential confounders, a HR of CHD was 2.27 (95%CI: 1.50–3.42) with 2-fold increase in patella lead levels, among participants in the top tertile of GRS. We also detected an increasing trend in HRs across tertiles of GRS (p-trend = 0.0063).

Conclusions

Our findings suggest that lead-related loci as a whole may play an important role in susceptibility to lead-related CHD risk. These findings need to be validated in a separate cohort containing bone lead, lead-related genetic loci and incident CHD data.

Introduction

Incident coronary heart disease (CHD) is a major health problem, and the leading cause of mortality worldwide [1]. Environmental toxicants, such as lead (Pb) and other heavy metals, are known to be associated with cardiovascular disease [2]. Weisskopf et al. reported an increased risk for future ischemic heart disease (IHD, also known as CHD) mortality with higher blood and bone lead levels in the VA Normative Aging Study (NAS) [3]. Several mechanisms could potentially explain lead's association with cardiovascular outcomes, including reduction in renal function, induction of oxidative stress and inflammation, stimulation of the renin-angiotensin system, as well as endothelial dysfunction [4–6].

Blood lead levels have been profoundly reduced in the U.S population over the last 30 years [6]. Although health-based guidelines limiting environmental and occupational exposures to lead have become more stringent to protect the population against adverse health effects, long-term lead exposure is still responsible for potential health risks [7,8]. In addition, genetically susceptible individuals may not be fully protected by current regulatory standards. It has been increasingly clear that individual genetic backgrounds influence predisposition to lead toxicity [2]. These genes can be divided into two groups: genes that influence lead uptake and retention (known as toxicokinetics), and genes that alter toxic effects of lead (known as toxicodynamics). A number of genes and gene encoding proteins have been identified to play important roles in lead toxicokinetics and/or toxicodynamics: δ-aminolevulinic acid dehydratase (ALAD), an enzyme on the heme-biosynthetic pathway that binds over 80% of lead in erythrocytes [9]; hemochromatosis (HFE), a membrane protein that regulates uptake of cellular iron and other divalent metals including lead [10]; heme oxygenase-1 (HMOX1), a heme-degrading enzyme that plays an important role in the inflammation and oxidative stress induced by lead [11–14]; vitamin D receptor (VDR) that plays a role in calcium homeostasis that influences the absorption and retention of lead into blood and bone [15]; apolipoprotein E (APOE) that affects lipid metabolism, down-regulates blood lead concentrations, and possesses antioxidative property [16–18]; glutathione S-transferases (GSTs), a family of phase-II detoxification isozymes involved in catalyzing the conjugation of lead and glutathione to form a thermodynamically stable complex, which decreases lead bioavailability and protects against lead toxicity through reduced oxidative stress [19–22]; and the renin-angiotensin system involved in the development of hypertension where lead exposure may enhance blood angiotensin I levels by

increasing plasma renin activity, which in turn contribute to production and activation of sub-strate-induced angiotensin converting enzyme [23,24]. Epidemiologic studies have identified that these genes may modify the association between lead exposure and cardiovascular and other health outcomes [13,18,25–27]. However, these previous studies focused on one or a few of the lead-related genetic polymorphisms. No study integrating multiple genetic polymorphisms in lead toxicokinetics and toxicodynamics has been conducted.

The purpose of this study was to evaluate effect modification of the association between cumulative exposure to lead and incident CHD events by genes related to lead toxicokinetics and toxicodynamics. We hypothesized that in the Normative Aging Study (NAS), the lead-CHD association would be stronger among participants with higher allelic risk profiles. The genes under consideration in this analysis are the *ALAD* gene, *HFE* gene, *HMOX1* gene, *VDR* gene, *GST* supergene family (*GSTT1* and *GSTM1*), *APOE* gene, angiotensin II receptor-1 (*AGTR1*) gene, and angiotensinogen (*AGT*) gene. In this study, we tested whether not only single nucleotide polymorphisms (SNPs) in each of those lead-related genes, but also integrated measures of all those polymorphisms and genetic risk score (GRS), modified the association between bone lead and incident CHD.

Materials and Methods

Ethics statement

All participants provided written informed consent. This study was reviewed and approved by the Institutional Review Boards of each participating institute, the University of Michigan School of Public Health, the Harvard School of Public Health and the Department of Veterans Affairs Boston Healthcare System.

Study population

The NAS is a longitudinal study of aging initiated by the Veterans Administration in Boston, Massachusetts in 1963. Participants were 2280 healthy males, mostly white, ranging from 21 to 80 years of age. All subjects enrolled in the study were free of past or present chronic medical conditions, including heart disease, cancer, diabetes, peptic ulcer, gout, bronchitis, sinusitis, recurrent asthma, or hypertension. Each participant returned for examination every 3 to 5 years.

Starting from August 1991, NAS participants who gave informed consent were invited to undergo K-shell-X-ray fluorescence (KXFR) bone lead measurements at the Ambulatory Clinical Research Center of the Brigham and Women's Hospital in Boston, Massachusetts. Of the 1283 participants who actively attended regular NAS examinations between August 1st 1991 and August 8th 2002, 878 participants had their bone lead measured. After excluding participants with high bone lead uncertainty (3 for patella and 6 for tibia lead), participants with negative lead levels (3 for patella and 6 for tibia lead), participants with missing values of the potential confounding factors (29 for patella and 30 for tibia lead), as well as participants with missing values of variables used in the prediction of enrollment into the bone lead study (8 for both patella and tibia lead, see more details of constructing inverse probability weighting in Statistical Analysis), 604 participants for patella lead and 607 participants for tibia lead, without a history of CHD (angina pectoris, myocardial infarction or CHD death) at the time of bone lead measurement visit, were included. The final analyses considered 589 participants (583 for patella lead, and 588 for tibia lead), with at least one SNP in *ALAD, HFE, HMOX1, VDR, APOE, GSTP1, GSTT1, GSTM1, AGTR1* and *AGT*.

Case ascertainment

Coronary heart disease (CHD) cases were defined as myocardial infarction, angina pectoris, or CHD deaths. We asked participants their history of heart disease since last visit. Board-certified cardiologists review each report of CHD events. Myocardial infarction was defined according to pathologic Q wave changes, increases in serum glutamic-oxaloacetic transaminase and lactic dehydrogenase levels, as well as concurrent chest discomfort consistent with myocardial infarction, or by autopsy. Angina pectoris was defined by recurrent chest discomfort lasting up to 15 min and related to exertion and relieved by rest or nitroglycerin. CHD deaths were ascertained from death certificates based on the 9th revision of the International Classification of Disease (ICD-9) codes 410 to 414 and 429.2.

Bone lead measurement

NAS participants had their bone lead levels measured at both the patella and mid-tibial shaft by using ABIOMED K-shell-X-ray instrument, as described in detail previously [28]. The measurement of bone lead, with unit of μg/g bone mineral, consists of an unbiased estimate and an estimate of uncertainty. 3 and 6 participants with uncertainty for patella or tibia lead, higher than 10 or 15 μg/g, respectively, were excluded since high levels of uncertainties likely represented less than optimal measurements due to excessive motion during the measurement, or a degraded signal of X-ray [29]. Both patella and tibia lead measurements were used as indices of cumulative lead levels in humans. Patella is predominantly made up of trabecular-type bone; while, for tibia, the major component is cortical-type bone. Lead in the patella is more mobilizable than that in the tibia, representing a half-life of decades for tibia lead and a half-life of a few years for lead in the patella [30].

Selection of gene polymorphisms

Candidate gene selection was based on functionality, which had been found in biological and epidemiological studies evaluating associations between lead and genetic variants. The next step was to identify 'functional' polymorphisms (i.e. non-synonymous and splice junction SNPs), synonymous and noncoding SNPs. Presently known polymorphisms in the lead-related genes are shown in Table 1.

Genotyping

Multiplex polymerase chain reaction (PCR) assays were designed with Sequenom SpectroDE-SIGNER software (Sequenon, Inc, San Diego, CA) by inputting sequences containing the SNP site and 100 base pairs of flanking sequence on either side of the SNP. A total of 24 SNPs were included: *ALAD* rs1800435; *VDR* rs1544410, rs731236, rs7975232, rs1073581, rs757343; *HFE* rs1799945, rs1800562; *HMOX1* rs2071746, rs2071747, rs2071749, rs5995098; *APOE* rs440446, rs405509, rs449647, rs7412, rs429358, rs769446; *GSTP1* rs1695; *AGTR1* rs12695908; and *AGT* rs699, rs5046, rs5050, rs2493137. *GSTM1* and *GSTT1* were deletion polymorphisms. The assay consists of PCR amplification of exons 4 and 5 of the *GSTM1* allele and concomitant amplification of the cytochrome P450 1A1 (*CYP1A1*) gene as a positive control. PCR products indicate the presence of one or more copies of the gene. The *HMOX1* gene was also genotyped for length polymorphism. Microsatellite (GT)n-length assay was designed as described by Yamada et al. [53]. PCR products at the 5'-flanking region of *HMOX1* were analyzed with a laser-based automated DNA sequencer, and the numbers of (GT)n repeats were classified according to the previous study [13], as class S (<27 (GT)n repeats), class M (27–32 (GT)n repeats), and class L

Table 1. Gene polymorphisms affecting lead toxico-kinetics and toxico-dynamics.

Gene	SNP	Minor Frequency Allele	SNP Location	Reported Functions	References
δ-aminolevulinic acid dehydratase gene (ALAD)	rs1800435	C	Exon 4[a]	The presence of ALAD2 allele was associated with higher blood lead levels in some occupational cohorts with high exposure, whereas others have not. Blood lead levels at background exposure levels show no clear association with ALAD genotype, although a small study showed ALAD2 was associated with lower blood lead levels in Japanese population.	Wetmur et al., 1994 [31]; Alexander et al., 1998 [32]; Schwartz et al., 1995 [33]; Hu et al., 2001 [34]; Pawlas et al., 2012 [35]; Miyaki et al., 2009 [36];
Vitamin D receptor (VDR)	rs1544410	A	Intron 8	Bsm1 (rs1544410) and Fok1 (rs10735810) affects bone mineral density and both blood/tibia lead levels; Apa1 (rs7975232) affects serum lead levels for the haplotype combined with Bsm1 and Fok1; Significant effect modification by Bsm1 or Taq1 (rs731236) found in the association between bone lead and pulse pressure.	Hurwitz et al., 1996 [37]; Haynes et al., 2003 [38]; Cooper et al., 1996 [39]; Rezende et al., 2007 [40]; Jhun et al., 2015 [25];
	rs731236	C	Exon 9[a]		
	rs7975232	C	Intron 8		
	rs1073581	A	Exon 2[a]		
	rs757343	A	Intron 8		
Hemochromatosis gene (HFE)	rs1799945	G	Exon 2[a]	C282Y (rs1799945) and H63D (rs1800562) are associated with hemochromatosis, affecting iron uptake; They are also found to influence bone lead absorption and oxidative stress.	Waheed et al., 1997 [41]; Wright et al., 2004 [42];
	rs1800562	A	Exon 4[a]		
Heme oxygenase-1 gene (HMOX1)	rs2071746	T	Promoter	rs2071746, rs2071747 and rs2071749 are associated with regulation of expression of antioxidant enzymes HMOX1; Long (GT)n repeats in the HMOX1 promoter may reduce heme oxygenase inducibility by reactive oxygen species.	Tanaka et al., 2011 [14]; Ashrafunnisa et al., 2014 [11]; Park et al., 2009 [13];
	rs2071747	C	Exon 1[a]		
	rs2071749	A	Intron 3		
	rs5995098	G	Intron 4		
	(GT)n repeats	Long[b]	Promoter		
Apolipoprotein E gene (APOE)	rs440446	C	Intron 1	APOE ε4 carriers would have depressed nitric oxide bioavailability, and contribute to lead-related hypertension; APOE ε4 also possesses reduced antioxidant abilities, bound metal ions, and magnified their cytotoxicity; rs405509 and rs440446 could be associated with cortical bone strength, which is associated with tibia lead levels; rs769446 is associated with the plasma APOE concentration.	Colton et al., 2002 [43]; Miyata et al., 1996 [44]; Tolonen et al., 2011 [45]; Theppeang et al., 2008 [46]; Mannila et al., 2013 [47];
	rs405509	C	Promoter		
	rs449647	A	Promoter		
	rs7412	T	Exon 4[a]		
	rs429358	C	Exon 4[a]		
	rs769446	C	Promoter		
Glutathione S-transferase pi 1 gene (GSTP1)	rs1695	G	Exon 5[a]	GSTM1, GSTP1 and GSTT1 may alter the responses to oxidative stress and inflammatory process caused by lead exposure; GSTT1 deletion, GSTM1 deletion, or at least one risk allele of GSTP1 rs1695 are associated with strengthened oxidative stress and inflammation.	Ercal et al., 2001 [48]; Sirivarasai et al., 2013 [22]; Coral-Vazquez et al., 2013 [49];
Glutathione S-transferase theta 1 gene (GSTT1)[c]	–	Gene Deletion	–		
Glutathione S-transferase mu 1 gene (GSTM1)[c]	–	Gene Deletion	–		
Angiotensinogen gene (AGT)	rs699	C	Exon 2[a]	AGT M268T (rs699), rs5046, rs5050 and rs2493137 are associated with lead-induced hypertension; AGTR1 is associated with hypertension.	Kim et al., 2014 [26]; Warrell et al., 2010 [50]; Li et al., 2014 [51]; Nie et al., 2009 [52];
	rs5046	T	Promoter		
	rs5050	G	Promoter		
	rs2493137	C	Promoter		
Angiotensin II receptor-1 gene (AGTR1)	rs12695908	G	Intron 3		

[a] Amino acid substitution in exons: rs1800435 Lys59Asn, rs731236 Ile352Ile, rs1799945 His63Asp, rs1800562 Cys282Try, rs2071747 Asp7His, rs7412 Arg176Cys, rs429358 Cys130Arg, rs1695 Ile105Val, and rs699 Met268Thr.

[b] The numbers of (GT)n repeats were classified as class Short (<27 (GT)n repeats), class Medium (27–32 (GT)n repeats), and class Large (≥33 (GT)n repeats).

[c] GSTT1 and GSTM1 polymorphisms are considered as deletion vs. no-deletion in our study.

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($\geq$33 (GT)n repeats). The pass rate of genotyping for these SNPs varied between 94% and 98.6%.

Genetic risk scores

We selected polymorphisms with plausible biological relationships to the pathophysiology of lead-induced CHD. The genetic risk score 1 was constructed by summing up all 22 available SNPs. The genetic risk score 2 was constructed by using 9 SNPs which we found significantly modified the relationship between bone lead and incident CHD events in our study. The 9 SNPs were *VDR* (rs1544410, rs7975232, rs757343), *HMOX1* (rs2071746, rs2071749, rs5995098), *APOE* (rs429358), and *AGT* (rs699, rs5046).

Covariates

Data on all covariates were obtained from regular NAS visits since the time of the baseline bone lead measurements. Body mass index (BMI) was calculated based on height (cm) and weight (kg) assessment. We considered cigarette smoking as both status indicators (never/ever) and pack-years. We categorized education as less than high school, high school, some college and no less than 4 years graduate study. Blood samples were analyzed for serum high-density lipoprotein (HDL) and total cholesterol levels. Hypertension were defined as systolic blood pressure greater than 140 mmHg and diastolic blood pressure greater than 90 mmHg, any medications before, or ever diagnosed with hypertension by a physician. Diabetes were defined as fasting glucose levels greater than 126 mg/dL, or ever diagnosed with diabetes by a physician.

Statistical analysis

Univariate statistics were calculated and examined for cases and non-cases of CHD. We used Chi-square or exact Fisher tests for categorical variables, and t test for continuous variables. For SNPs, we calculated allele frequencies and assessed Hardy-Weinberg equilibrium for each genotype.

We computed person time for each individual from baseline to the date of first CHD event (angina pectoris or myocardial infarction) or CHD death, whichever came first; and for non-cases, from the date of bone lead measurement to the last active follow-up visit until June, 2011. We used Cox proportional hazard regression models to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for the development of new CHD in relation to lead exposure. Bone lead variables were $\log_2$-transformed, modeled as linear continuous variables. HRs and 95% CIs were, therefore, presented for a two-fold increase in bone lead levels to facilitate interpretation of the effect estimates.

To assess effect modification by different genes, we included log-transformed lead levels, indicators for genotype classification, and cross product terms between lead biomarker and genotype. We also controlled for age, smoking status, BMI and the ratio of total cholesterol to HDL cholesterol level. Hypertension is a well-known risk factor for CHD. However, sufficient evidence revealed a causal relationship between lead exposure and hypertension. We, therefore, decided not to include hypertension or blood pressure in our models because hypertension could be in the causal pathway (i.e. overadjustment bias) [54]. In addition, we constructed an unweighted multi-locus GRS (GRS 1) for each individual by simply summing up the number of risk alleles (0/1/2) for all 22 available SNPs. We found *VDR* rs1544410 and rs731236 are in high linkage disequilibrium ($r^2 = 0.97$), also similar for *VDR* rs7975343 and rs1073581 ($r^2 = 0.99$) (linkage disequilibrium results are shown in Tables A-D in S1 File). For this reason we exclude rs731236 and rs1073581 in our GRS. We also created another GRS (GRS 2) using 9

and 8 SNPs that significantly modified the patella lead-CHD and tibia lead-CHD associations, respectively. We computed false discovery rates (FDRs) from p-values using the Benjamini-Hochberg method to adjust for multiple comparisons [55].

In our study population, collider stratification bias might exist and produce misleading results, since selection into the KXRF bone lead sub-study was linked to both cardiovascular diseases and potential confounders at the time of enrollment [3]. To mitigate the bias, we assigned weights to the subjects based on inverse probability weighting (IPW) as per Weisskopf et al. [3]. Briefly, a single logistic regression model was constructed to predict the probability of sub-study entrance, with one record per study visit at the time of bone lead measurements for those participants in the sub-study; for those without bone lead measured, we utilized the last visit before 2002 (the last year of the bone lead measurements used in our study). The C statistic from this model was 0.87. All the predictors incorporated in the logistic regression model are presented in S1 Table.

We conducted sensitivity analyses to verify analytical consistency. We investigated possible intermediates by incorporating blood pressure into the models. We replaced ever/never smoking indicator with smoking in pack-years in our models. We also tried to mitigate the potential selection bias by restricting our study population to those who were 45 years or younger at the entry into the NAS study, combined with IPW [3]. All the above analyses were performed using SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina) and R version 3.2.2 for the multiple comparison correction (R Core Team 2014).

Power Calculations

We calculated post-hoc powers for the gene-environment interaction terms using Quanto 1.2.4, which is a web-based software program to compute either sample size or power in studies of gene-gene or gene-environment interaction (http://biostats.usc.edu/Quanto.html). Since the Quanto software can only allow five types of study designs (i.e., "unmatched case-control", "matched case-control", "case-sibling", "case-parent", and "case-only"), we assumed that our study design was a standard unmatched case-control (not a prospective cohort) consisting of a random sample of unrelated subjects. Under this setting, we assumed our observed HRs to approximate odds ratios. In addition, we also assumed the dominant inheritance model (i.e. risk allele carriers vs. participants with no risk allele) and a two-sided test with type-1 error of 0.05.

Results

S2 Table shows that genotype frequencies, and incident CHD events by *ALAD*, *HFE*, *HMOX1*, *VDR*, *GSTP1*, *GSTT1*, *GSTM1*, *APOE*, *AGTR1*, and *AGT* genotypes. All the 24 SNPs were in Hardy-Weinberg equilibrium with p-value>0.10, except for *HFE* rs1799945 (p = 0.03), and *AGT* rs5046 (p = 0.003) (S3 Table). Data are not shown in the table for length polymorphisms and deletion polymorphisms. 72 (13.3%) subjects were *HMOX1* L-allele carriers; 75 (19.4%) subjects were with *GSTT1* deleted; 283 (54.5%) subjects were with *GSTM1* deleted. CHD cases and non-cases did not differ by minor allele profiles, except for *APOE* rs449647 (χ^2 = 8.97, p-value = 0.01). There was no significant difference in bone lead levels by genotypes (S4 Table).

Of 589 subjects in our study, 136 subjects had been diagnosed as CHD cases during the follow-up periods (Table 2). Participants were followed for up to 20 years. The mean age at the time of bone lead measurement among the study population was 66 years (range: 48–96 years). Although cholesterol and BMI were significantly higher, the distribution of other covariates, including known risk factors for CHD such as smoking, socioeconomic status

Table 2. Baseline Characteristics of CHD cases and non-cases, Normative Aging Study (n = 589).

	No CHD (n = 453)			CHD (n = 136)		
Main Exposure						
	Total[c]	**Mean ± SD**	**Total**	**Mean ± SD**		**P value[a]**
Patella lead	449	29.2 ± 18.1	135	32.1 ± 18.8		0.12
Tibia lead	452	20.2 ± 12.5	136	22.6 ± 13.5		0.03
Continuous variables						
Age (years)	453	66.1 ± 7.4	136	65.8 ± 6.6		0.63
Follow-up time (years)	453	9.7 ± 5.8	136	9.0 ± 5.1		0.10
Body mass index (kg/meter^2)	453	27.5 ± 3.5	136	28.6 ± 3.9		<0.01
Smoking (pack-years)	444	21.0 ± 25.6	133	22.3 ± 25.3		0.35
Total cholesterol (mg/dL)	453	226.8 ± 35.6	136	234.2 ± 39.5		0.03
High-density lipoprotein (mg/dL)	453	49.2 ± 13.3	136	47.8 ± 11.1		0.21
Total cholesterol/HDL ratio	453	4.9 ± 1.4	136	5.1 ± 1.2		0.13
Categorical variables						
	Total[c]	**N (%)**	**Total**	**N (%)**		**P value[b]**
Smoking	453		136			0.19
Never smokers		147 (32.5)		36 (26.5)		
Ever smokers		306 (67.5)		100 (73.5)		
Education	433		131			0.60
<high school		42 (9.7)		18 (13.7)		
high school		149 (34.4)		45 (34.4)		
some college		110 (25.4)		32 (24.4)		
≥ 4 year college graduate		132 (30.5)		36 (27.5)		
Hypertension	453	201 (44.4)	136	92 (47.8)		0.48
Diabetes	453	51 (11.3)	136	17 (12.5)		0.69

[a] P value from t-test or Wilcoxon rank-sum test (patella and tibia lead, total cholesterol, and total cholesterol to HDL ratio).

[b] P value from Chi-square test.

[c] Totals vary because of missing data.

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(i.e. education), systolic and diastolic blood pressure, and diabetes status were not significantly different between cases and non-cases.

After incorporating interactions between bone lead and genotypes into the models, we found that 5 and 4 polymorphisms reached statistical significance (p<0.05) and borderline significance (p<0.1), respectively, when genotypes were modeled as additive (Table 3). Using the dominant genetic model, 4 and 3 polymorphisms reached statistical significance and borderline significance (S5 Table).

For *VDR* rs1544410 (*Bsm1*), rs731236 (*Taq1*), rs757343 (*Fok1*), rs757343 (*Tru91*) and *HMOX1* rs2071749, a 2-fold increase in patella lead was associated with HRs of incident CHD by 1.65 (95%CI: 1.31–2.08), 1.61 (95%CI: 1.29–2.02), 1.47 (95%CI: 1.17–1.83), 1.48 (95%CI: 1.18–1.85), and 1.51 (95%CI: 1.22–1.86), respectively, among subjects with at least one minor allele; whereas no significant associations were found among those with homozygous major alleles. For VDR rs7975232 (*Apa1*), a 2-fold increase in patella lead was associated with a HR of CHD by 1.48 (95%CI: 1.11–1.97) among subjects with no minor allele, and a HR of CHD by 1.71 (95%CI: 1.30–2.25) among subjects with only one minor allele; however, no significant association was found among those with two minor alleles. On the contrary, among subjects without any minor allele in *HMOX1* rs2071746, a 2-fold increase in patella lead was significantly associated with elevated CHD risk (HR: 1.51, 95%CI: 1.07–2.13); while, the toxic effects

Table 3. Adjusted estimates[a] of CHD per 2-fold increase in patella lead levels, stratified by different gene polymorphisms.

Associations in the entire population					
			95%Confidence Interval		
	N	Hazard Ratio[b]	Lower	Upper	P value[c]
Crude model	583	1.42	1.20	1.68	< .0001
Adjusted model	583	1.36	1.15	1.61	0.0004
Associations by genotype of each lead-related SNPs					
Vitamin D (1,25-dihydroxyvitamin D3) receptor gene (VDR gene)					
VDR rs1544410 (Bsm1)	494				0.004
G/G		0.97	0.73	1.28	
G/A		1.59	1.20	2.10	
A/A		1.77	1.18	2.68	
VDR rs731236 (Taq1)	521				0.009
T/T		1.00	0.76	1.33	
T/C		1.56	1.19	2.04	
C/C		1.70	1.15	2.52	
VDR rs7975232 (Apa1)	520				0.007
A/A		1.48	1.11	1.97	
A/C		1.71	1.30	2.25	
C/C		0.84	0.62	1.13	
VDR rs1073581 (Fok1)	509				0.09
G/G		1.19	0.91	1.56	
G/A		1.28	1.01	1.63	
A/A		2.11	1.19	3.74	
VDR rs757343 (Tru91)	516				0.07
G/G		1.17	0.89	1.52	
G/A		1.30	1.02	1.64	
A/A		2.09	1.18	3.73	
δ-aminolevulinic acid dehydratase gene (ALAD gene)					
ALAD rs1833435	545				0.84
A/A		1.34	1.20	1.60	
A/T		1.36	0.74	2.50	
T/T		1.19	0.24	5.93	
Hemochromatosis gene (HFE gene)					
HFE rs1799945 (H63D)	509				0.25
C/C		1.41	1.15	1.72	
G/C		1.10	0.79	1.56	
G/G		1.67	0.26	10.78	
HFE rs1800562 (C282Y)	510				0.22
G/G		1.36	1.13	1.64	
G/A		0.99	0.58	1.69	
A/A		—[d]	—	—	
Heme oxygenase 1 gene (HMOX1 gene)					
HMOX1 rs2071746	516				0.09
A/A		1.51	1.07	2.13	
A/T		1.44	1.13	1.83	
T/T		1.02	0.74	1.41	
HMOX1 rs2071749	514				0.10
G/G		1.02	0.77	1.35	

(Continued)

Table 3. (*Continued*)

Associations in the entire population	N	Hazard Ratio[b]	95%Confidence Interval		P value[c]
			Lower	Upper	
G/A		1.54	1.21	1.98	
A/A		1.34	0.91	1.99	
HMOX1 rs5995098	519				0.15
C/C		1.62	1.23	2.14	
C/G		1.18	0.94	1.49	
G/G		1.40	0.78	2.51	
HMOX1 rs2071747	510				0.82
G/G		1.36	1.13	1.63	
G/C		1.45	0.81	2.61	
C/C		–	–	–	
HMOX1 length polymorphisms	541				0.18
S or M alleles		1.46	1.20	1.77	
Any L allele		1.09	0.74	1.60	
Alipoprotein E gene (APOE gene)					
APOE rs429358	500				0.10
T/T		1.43	1.17	1.75	
T/C		1.05	0.75	1.48	
C/C		0.87	0.39	1.95	
APOE rs440446	521				0.64
G/G		1.25	0.95	1.63	
G/C		1.42	1.09	1.86	
C/C		1.34	0.83	2.16	
APOE rs405509	534				0.33
A/A		1.22	0.86	1.74	
A/C		1.26	0.99	1.60	
C/C		1.58	1.10	2.28	
APOE rs449647	513				0.60
T/T		1.30	1.05	1.61	
T/A		1.60	1.15	2.21	
A/A		1.19	0.45	3.16	
APOE rs7412	540				0.30
C/C		1.34	1.10	1.64	
C/T		1.45	0.98	2.16	
T/T		2.42	0.57	10.25	
APOE rs769446	509				0.46
T/T		1.32	1.08	1.61	
T/C		1.87	1.20	2.91	
C/C		0.47	0.11	2.05	
Angiotensinogen gene (AGT gene)					
AGT rs699	485				0.02
T/T		2.17	1.51	3.12	
T/C		1.29	1.01	1.64	
C/C		1.18	0.82	1.7	
AGT rs5046	487				0.05
C/C		1.57	1.27	1.94	

(*Continued*)

Table 3. (*Continued*)

Associations in the entire population					
			95%Confidence Interval		
	N	**Hazard Ratio**[b]	**Lower**	**Upper**	**P value**[c]
C/T		1.13	0.83	1.53	
T/T		1.04	0.35	3.09	
AGT rs5050	483				0.75
T/T		1.36	1.09	1.69	
T/G		1.39	1.02	1.91	
G/G		–	–	–	
AGT 2493137	485				0.25
T/T		1.61	1.20	2.15	
T/C		1.37	1.08	1.74	
C/C		1.07	0.53	2.18	
Angiotensin II receptor 1 gene (AGTR1 gene)					
AGTR1 rs12695908	486				0.72
C/C		1.44	1.20	1.74	
C/T		1.25	0.63	2.50	
T/T		–	–	–	
Glutathione S-transferase pi 1 gene (GSTP1 gene)					
GSTP1 rs1695	496				0.31
A/A		1.39	1.10	1.77	
A/G		1.23	0.93	1.62	
G/G		0.78	0.31	2.00	
Glutathione S-Transferase Theta 1 (GSTT1 gene)					
GSTT1	386				0.16
Deletion		1.21	0.84	1.76	
No deletion		1.65	1.32	2.08	
Glutathione S-Transferase Mu 1 (GSTM1 gene)					
GSTM1	519				0.15
Deletion		1.56	1.21	2.01	
No deletion		1.21	0.95	1.54	

[a] Hazard ratio and 95% confidence interval with adjustment for age, BMI, smoking status (ever/never) and total cholesterol to HDL cholesterol ratio for all models.

[b] Hazard ratio indicates HR of CHD events for 2-fold increase in lead levels.

[c] P value from Wald test in the adjusted Cox proportional hazard models, when genotype indicators treated as a continuous variable.

[d] Null values are due to the absence of subjects or a small number of subjects.

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of lead tended to decrease among subjects with one or no minor allele. Similarly, we detected a HR of CHD, i.e. 1.62 (95%CI: 1.23–2.14), 1.43 (95%CI: 1.17–1.75), 2.17 (95%CI: 1.51–3.12) and 1.57 (95%CI: 1.27–1.94), respectively, for *HMOX1* rs5995098, *APOE* rs429358, *AGT* rs699 and rs5046, as well as a decreasing trend. In the Benjamini-Hochberg FDR tests, all of the observed significant interactions were substantially reduced and we found borderline significant interactions for *VDR* rs1544410 (*Bsm1*, p = 0.06), rs731236 (*Taq1*, p = 0.06), rs7975232 (*Apa1*, p = 0.06) and *AGT* rs699 (p = 0.06).

For tibia lead, we detected 5 polymorphisms with statistical significance and 2 with borderline significance in additive genetic models (data not shown). Using the dominant genetic model, 4 polymorphisms reached statistical significance and 3 reached borderline significance.

Table 4. Adjusted effect estimates[a] of patella lead, genetic score and interaction between patella lead and genetic risk score.

	N	Patella lead			
Genetic Risk Score 1[b,d]	**393**	**Hazard ratio**	**95% CI**		**P value[e]**
8–16	113	0.97	0.69	1.36	0.006
17–19	143	1.21	0.85	1.72	
20–27	137	2.27	1.50	3.42	
Genetic Risk Score 2[c,d]	444				
4–9	130	0.82	0.60	1.13	<0.0001
10–12	194	1.58	1.19	2.10	
13–17	120	2.77	1.78	4.31	

[a] Adjusted for age, BMI, smoking status (ever/never) and total cholesterol to HDL cholesterol ratio for all models;

[b] Genetic risk score1 was constructed by using all 22 available SNPs in our study;

[c] Genetic risk score2 was constructed by using 9 SNPs we found significantly modified the relationship between lead and incident CHD events. The 9 SNPs were *VDR* (rs1544410, rs7975232, rs757343), *HMOX1* (rs2071746, rs2071749, rs5995098), *APOE* (rs429358), and *AGT* (rs699, rs5046).

[d] Tertiles of genetic risk score with its range in each tertile. Unit for genetic risk score is one risk allele.

[e] P value for interaction term between genetic risk score and patella lead levels from Wald test in the adjusted Cox proportional hazard models.

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The results are similar with regard to *VDR* rs1073581 (*Fok1*), *VDR* rs757343 (*Tru91*), *HMOX1* rs2071749 and *AGT* rs699. We detected 5 different SNPs that significantly altered the relationship between lead exposure and CHD. Among subjects with at least one minor frequency allele, HRs of CHD were 2.04 (95%CI: 1.47, 2.84) for *GSTP1* rs1695, 2.24 (95%CI: 1.45–3.44) for *APOE* rs7412 and 2.81 (95%CI: 1.51, 5.23) for *APOE* rs769446. On the other hand, we found HRs of CHD among subjects with no minor allele for *AGT* rs2493137 (2.06; 95%CI: 1.48–2.87) and *APOE* rs449647 (1.70; 95%CI: 1.36–2.12); whereas, the toxic effects of lead tended to decrease among subjects with one or two minor alleles.

The GRS constructed using significant polymorphisms (GRS 2) ranged from 4 to 17, with a mean of 10. We observed a significant interaction between patella lead levels and GRS 2 (p = <0.0001) (Table 4). For subjects possessing 10 to 12 risk alleles, a HR of CHD was 1.58 (95% CI: 1.19–2.10) times higher with a 2-fold increase in patella bone lead levels; and for subjects possessing 13 to 17 risk alleles, a HR of CHD was 2.77 (95%CI: 1.78, 4.31). However, no significant results were found with people having 4 to 9 risk alleles. Significant effect modification was also detected when we constructed GRS using all the SNPs (GRS 1) (p = 0.006). We also found similar results with tibia lead (S6 Table).

In the sensitivity analysis, we did not find significant changes in association between bone lead and incident CHD when incorporating blood pressure into the models. However, this analysis cannot rule out the possibility of blood pressure as an intermediate on the causal pathway from bone lead to CHD events. After replacing the smoking indicator with pack-years, we detected little change in the point estimates. In analyses restricted to participants whose enrollment age was ≤ 45 years along with IPW, we found only slight differences in HRs (data not shown).

Using the observed HRs, we computed post-hoc powers for the gene-lead interaction terms (S7 Table). As expected, our study was underpowered given the sample size of 371 to 545 (power ranged from 0.05 to 0.57). For example, our sample provided 57% power to detect a HR of 2.56 given the allele frequency of 0.55 (*VDR* rs7975232) and the sample size of 520. Because the power calculation was based on unmatched case-control design, actual powers would be higher given our prospective design.

Discussion

In our study, we systematically analyzed 27 genetic variations in 10 lead-related genes and their modification of the association between cumulative lead and incident CHD. We found significant effect modification by *VDR*, *HMOX1*, *APOE*, *GSTP1* and *AGT* genotype, suggesting that these genes may alter susceptibility to lead-related future development of CHD. We also found significant interactions between GRS and bone lead levels in relation to incident CHD, suggesting that having more risk alleles in lead toxicokinetics and toxicodynamics may enhance cumulative effects of lead on the development of CHD.

This is the first study to construct a GRS using genetic polymorphisms biologically related to lead toxicokinetics and toxicodynamics, and evaluate its interaction with bone lead in relation to incident CHD. Previous studies focused on genetic susceptibility by analyzing SNPs individually. Although the effect of a single locus is found to be relatively small, the combination of relevant SNPs may additively contribute to the lead-related CHD risk. The stronger association found with GRS compared with individual SNPs suggests that GRS has better risk stratification/discrimination power with regards to cardiovascular toxicity of lead. This finding also suggests important clinical and public health implications: genetic profiles can also be considered a useful tool for risk prediction. In addition, lead exposure standards based on average effects in the general population should be revised to protect genetically vulnerable subjects.

Genetic variations related to lead transport might account for the modifications between bone lead levels and CHD risk. Vitamin D receptor (*VDR*) gene is a calcium homeostasis gene and its product serves as a modulator that regulates bone mineral transport [37]. The blood-borne hormonal form of vitamin D, i.e. calcitriol (1α25(OH)2D3), activates VDR. The high affinity VDR interacts with calcitriol to constitute a calcitriol-VDR complex, stimulating the production of calcium-binding proteins such as calbindin-D, which influences calcium transport [56]. During calcium deficiency, calbindin-D is highly expressed. Other divalent cations, especially lead, will bind to the calbindin-D, giving rise to absorption and retention of lead in blood and bone [15]. This supports lead transportation, mediated by *VDR* SNPs, and might explain variation in the lead-CHD association.

Oxidative stress is one of the mechanisms linking genetic variations to lead toxicity. Nitric oxide (NO) plays a central role in regulation of blood pressure by promoting vasodilation and vascular remodeling [57]. Elevation in lead exposure is related to NO inactivation. A mechanistic experiment with two mouse models suggested that *APOE ε4* carriers would have depressed NO bioavailability [43]. In addition, *APOE ε4* might also contribute to lead-related hypertension [58]. Significant results in *APOE* rs429358 are consistent with the underlying theory.

Alleviation of oxidative stress by antioxidants ameliorates the cytotoxicity of ROS and enhances NO bioavailability. It has been recently shown that depletion of glutathione might result in hypertension, with NO inactivation and reduction [59]. Glutathione S-transferase supergene family encodes isoforms modifying lead-induced hypertension, and also contributing to the increased ROS activity and down-regulated NO production in rats [60]. This is also supported by effect modification by *GSTP1* rs1695 in our study.

Heme oxygenase-1 (HMOX1) serves as a protector by reducing free iron levels in cells against oxidative stress. In our study, we found elevated adverse effects of cumulative lead among A-G or A-A genotypes of rs2071749, compared with G-G carriers. However, *HMOX1* length polymorphism was not identified as an effect modifier and interestingly we observed that the cumulative lead effect on CHD risk was increased among subjects with A allele of rs2071746. A single genetic polymorphism may not represent the whole promoter region, unless we incorporate all tagged SNPs, and it is still unknown what function of genetic variations of *HMOX1* promoter might modify associations between lead exposure and CHD risk.

The occurrence of CHD with advancing age, is partially attributed to hypertension caused by the renin-angiotensin system. *AGT* M268T with hypertension was established in Caucasian hypertensive sibling pairs, where *TT* genotype conferred 31% higher risk compared with *MM* genotype [50]. This finding could plausibly be explained by the observation that *TT* genotype associated with higher plasma AGT levels, heightened the production of angiotensin I and II [61], which might also account for our results with regard to the *AGT* gene. Intriguingly, findings from mechanistic and clinical studies showed that calcitriol could serve as a negative endocrine regulator of renin production. Deletion polymorphisms or SNPs in *VDR* gene may affect VDR-vitamin D signaling, which will in turn upregulate activity of RAS. This was supported by augmentation in both renin mRNA and protein levels in the kidney, as well as plasma angiotensin II levels in *VDR* (-/-) mice [62]. Thus RAS over-stimulation mediated by *VDR* polymorphisms may account in part for an association between lead exposure and the development of CHD.

Polymorphisms in *HFE* were not identified as effect modifiers in our study. Although genetic hemochromatosis induced by *HFE* was reported to regulate bone lead absorption, *HFE*-related genetic hemochromatosis would remedy significant bone mineral loss in the elderly groups [63]. Of note, *HFE* C282Y and H63D variants were also linked to lower levels of HDL cholesterol, which can stimulate vascular inflammation, through reduction in bone mineral density [64]. It is reasonable to infer that the failure of *HFE* variants to alter lead levels and toxicity results from of perturbation in bone mineral and lipid metabolisms.

ALAD, *HMOX1* and *APOE* are hypothesized to alter the circulation of lead, as described above. However, given that bone lead levels were not much different by the genotypes of these genes (S4 Table), it is unlikely that toxicokinetics of bone lead account for the apparent *VDR*, *HMOX1* and *APOE* genotype effects.

Our study has several limitations. In the NAS, all participants were men and predominantly white, so the present findings may not be generalizable to women or other racial/ethnic groups. Moreover, the study sample was reduced somewhat by the requirements of non-missing KXRF bone lead measurements and genotyping. Thus, these prerequisites in our analysis limited the power of our statistical tests. In addition, our findings need to be replicated in other prospective studies with bone lead levels, genotypes and incident CHD data.

Conclusions

Our results suggest that *VDR*, *HMOX1*, *APOE*, *AGT* and *GSTP1* genes may modify the association between higher bone lead levels and CHD risk. Combined with other evidence that genetic polymorphisms can alter the relationship of CHD-related traits with cumulative exposure to lead, this body of research supports a joint effect of genetic biomarkers of susceptibility and environmental lead exposure on the development of CHD. However, we need to replicate our findings in a separate cohort.

Supporting Information

S1 File. R^2 of linkage disequilibrium between polymorphisms.
(DOC)

S1 Table. Predictors included in the inverse probability weighting model.
(DOC)

S2 Table. Genotype frequencies and incident CHD events by genotype.
(DOC)

S3 Table. Hardy-Weinberg equilibrium analysis for SNPs.
(DOC)

S4 Table. Genotype frequencies and bone lead concentrations by genotype.
(DOC)

S5 Table. Adjusted estimates of CHD per 2-fold increase in patella lead levels, stratified by different gene polymorphisms (no minor allele vs at least one minor allele).
(DOC)

S6 Table. Adjusted effect estimates of tibia lead, genetic score, and interaction between patella lead and genetic score.
(DOC)

S7 Table. Statistical powers of the gene-environment interaction terms.
(DOC)

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