ORIGINAL RESEARCH

Epigenetic Biomarkers of Lead Exposure and Cardiovascular Disease: Prospective Evidence in the Strong Heart Study

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BACKGROUND: Lead is a cardiotoxic metal with a variety of adverse health effects. In the absence of data on bone lead exposure, epigenetic biomarkers can serve as indicators of cumulative lead exposure and body burden. Herein, we leveraged novel epigenetic biomarkers of lead exposure to investigate their association with cardiovascular disease (CVD) incidence and mortality.

METHODS AND RESULTS: Blood DNA methylation was measured using the Illumina MethylationEPIC BeadChip among 2231 participants of the Strong Heart Study (SHS) at baseline (1989–1991). Epigenetic biomarkers of lead levels in blood, patella, and tibia were estimated using previously identified cytosine-guanine dinucleotide (CpG) sites. CVD incidence and mortality data were available through 2017. Median concentrations of lead epigenetic biomarkers were 13.8 µg/g, 21.3 µg/g, and 2.9 µg/dL in tibia, patella, and blood, respectively. In adjusted models, the hazard ratio (HR) (95% CI) of CVD mortality per doubling increase in lead epigenetic biomarkers were 1.42 (1.07–1.87) for tibia lead, 1.22 (0.93–1.60) for patella lead, and 1.57 (1.16–2.11) for blood lead. The corresponding HRs for incident CVD were 0.99 (0.83–1.19), 1.07 (0.89–1.29), and 1.06 (0.87–1.30). The association between the tibia lead epigenetic biomarker and CVD mortality was modified by sex (interaction *P* value: 0.014), with men at increased risk (HR, 1.42 [95% CI, 1.17–1.72]) compared with women (HR, 1.04 [95% CI, 0.89–1.22]).

CONCLUSIONS: Tibia and blood epigenetic biomarkers were associated with increased risk of CVD mortality, potentially reflecting the cardiovascular impact of cumulative and recent lead exposures. These findings support that epigenetic biomarkers of lead exposure may capture some of the disease risk associated with lead exposure.

Key Words: American Indian populations ■ DNA methylation ■ epigenetic biomarkers ■ lead

L ead (Pb) is a toxic metal associated with adverse cardiovascular, neurological, renal, hematological, immunological, reproductive, and developmental outcomes.[1-4] Before widespread bans in the 1970s, lead was included in gasoline, paint, water piping, and plumbing fixtures, resulting in extensive contamination of the air, soil, dust, and water.[5] Lead is still widely refined and processed in the United States,[6] where lead levels remain relatively high and individuals remain at risk of exposure. There is also evidence of racial and socioeconomic disparities concerning the burden of lead exposure,[7-9] with several racial and ethnic groups and low-income populations facing increased exposures compared with other groups. Because of the persistence of lead in the environment and continuous new exposures, lead and its associated adverse health effects remain relevant today.

Despite its importance as a potential cardiovascular risk factor, large cohort studies of cardiovascular disease (CVD) often lack data on lead exposure.

CLINICAL PERSPECTIVE

What Is New?
- Higher levels of epigenetic biomarkers of lead exposure estimated in tibia and blood were associated with increased risk of cardiovascular disease mortality.
- The association between the tibia lead epigenetic biomarker and cardiovascular disease mortality was modified by sex, with men at increased risk compared with women.

What Are the Clinical Implications?
- In the absence of data on bone lead exposure, epigenetic biomarkers can serve as indicators of cumulative lead exposure.
- Epigenetic lead biomarkers can capture some of the cardiovascular risk associated with lead exposure.

Nonstandard Abbreviations and Acronyms

DNAm	DNA methylation
eBlood	DNA methylation–based biomarker of lead exposure in blood
ePatella	DNA methylation–based biomarker of lead exposure in patella
eTibia	DNA methylation–based biomarker of lead exposure in tibia
NAS	Normative Aging Study
SHS	Strong Heart Study

Traditionally, biomarkers of lead exposure have been measured in blood, urine, plasma, and bone.[10] Lead accumulates in bone with a half-life of decades, and bone lead measures can be used to reflect cumulative lead exposure and long-term health effects.[11] In blood, lead reflects both endogenous sources from bone and exogenous sources from the environment, with a half-life of 1 to 2 months. Obtaining bone lead measures, however, can be challenging on a population scale, as the technology used requires exposure to radiation and is not widely available.[12]

Genome-wide DNA methylation (DNAm) data can serve as biomarkers of epigenetic signatures to estimate lead concentrations in tibia, patella, and blood,[12] by leveraging the knowledge that lead exposure induces sensitive and specific changes in whole blood DNAm.[13] These methylation-based biomarkers were well correlated with lead concentrations in tibia and patella,[12] and in a separate analysis, increasing levels of the tibia DNAm biomarker was associated with increased odds of Parkinson disease status.[14] These results highlight the potential of methylation-based biomarkers to provide estimates of lead exposure, and their relation to disease.

The Strong Heart Study (SHS), a study of CVD in American Indian adults across the Southwest and the Great Plains,[15] represents an opportunity to investigate the relationship of these epigenetic biomarkers with cardiovascular health. Lead exposure has been documented in American Indian communities,[16,17] where a legacy of environmental contamination remains a concern. Research has also identified that SHS communities have a high burden of CVD,[18] and that exposure to toxic metals, including cadmium and arsenic, contributes to this increased risk.[19-22] Because of the evidence highlighting the importance of metals on CVD,[23,24] there is further need to investigate the impact of lead in American Indian communities.

The objective of this study was to apply these recently developed epigenetic biomarkers of lead exposure and investigate their association with CVD incidence and mortality in the SHS. Given the accumulation of lead in bone, and consistent with previous findings,[25] we anticipated that epigenetic biomarkers of bone lead would be more strongly associated with cardiovascular outcomes than an epigenetic biomarker of blood lead. This work is a novel application of these epigenetic biomarkers to study cardiovascular outcomes.

METHODS

Study Population

The SHS is a prospective cohort of CVD and its risk factors among American Indians adults, funded by the National Heart, Lung, and Blood Institute and the National Institute of Environmental Health Sciences.[15] In 1989 to 1991, all adults aged 45 to 74 years across 13 tribes and communities in Arizona and Oklahoma, and random subsets in North Dakota and South Dakota, were eligible for recruitment.[15] The SHS protocol was approved by institutional review boards, participating tribes, and the respective area Indian Health Service institutional review boards. All participants provided informed consent. A total of 4549 adults were initially recruited. For this study, 1032 participants from one community were not included on their request. We also excluded 252 participants with CVD at baseline, 429 participants without sufficient urine for metal analyses, and 44 participants missing cardiovascular risk factors, resulting in 2792 participants eligible for analysis of blood DNAm. However, 445 participants had insufficient amounts of DNA for analysis, and 26 were further excluded in quality control, leaving

a final sample size of 2321 participants included in this study. The data underlying this article cannot be shared publicly in an unrestricted manner because of limitations in the consent forms and in the agreements between the SHS tribal communities and the SHS investigators. The data can be shared to external investigators following the procedures established by the SHS, available at https://strongheartstudy.org/.

All participants provided sociodemographic and medical history information, including age, sex, education, study center of recruitment, smoking status (never, former, or current), body mass index, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, blood pressure, hypertension treatment (yes/no), and diabetes (yes/no) via baseline questionnaires, physical examinations, and laboratory analyses, as described previously.[26] Estimated glomerular filtration rate was calculated using age, sex, and plasma creatinine via the Chronic Kidney Disease Epidemiology Collaboration formula.[27] Diabetes status at baseline was defined as fasting glucose $\geq$126 mg/dL, 2-hour plasma glucose $\geq$200 mg/dL, hemoglobin A1C level $\geq$6.5%, self-reported history of diagnosis, or current use of diabetic medication.

DNA Methylation

Buffy coat from fasting blood samples was collected in 1989 to 1991 on recruitment, and biological specimens were stored at <–70 °C. DNA from white blood cells was extracted and stored at the MedStar Health Research Institute under a strict quality control system. In 2015, blood DNA was shipped to the analytical laboratory at the Texas Biomedical Research Institute for DNAm analysis. DNA was bisulfite converted with the EZ DNAm kit (Zymo Research, Irvine, CA), according to the manufacturer's instructions. Bisulfite-converted DNA was measured using the Illumina MethylationEPIC BeadChip (850K; Illumina, San Diego, CA), which provides a measure of DNAm at a single-nucleotide resolution at >850 000 CpGs. Samples were randomized across and within plates to remove potential batch artifacts and confounding effects, and replicate and across-plate control samples were included on every plate. All the preprocessing was conducted using R version 3.6.1.[28] Data were read in 6 different batches (of ~400 individuals each) and combined using the R package minfi (version 1.18.4).[29] CpGs with a *P*-detection value of >0.01 in >5% of the individuals (6159 CpGs) were removed. Single sample normalization was conducted using the preprocess Noob function in minfi,[30,31] which includes a background correction with dye-bias normalization for Illumina Infinium methylation arrays. Regression on correlated probes normalization was applied to account for probe type bias.[32]

As a result of these preprocessing preliminary analyses, we had data from 2321 individuals and 860 079 CpGs. Cross-hybridizing probes, sex chromosomes, and single-nucleotide polymorphism probes with minor allele frequency >0.05[33] were removed from the analysis. The final number of CpGs for analysis was 790 026. Quality checks, data normalization, statistical preprocessing, and β-value calculation, which ranges from 0 to 1 and represents the proportion of unconverted cytosines in bisulfite-converted DNA at specific locations, were performed using the R package minfi.[30] We estimated Houseman cell proportions (CD8T cells, CD4T cells, natural killer cells, B cells, monocytes, and granulocytes)[34] using the R package minfi, to use them as adjustment variables in regression models. We detected and corrected for potential batch effects by sample plate, sample row, and DNA isolation time with the combat function (sva R package).[35] We annotated CpGs to the nearest gene, according to the Illumina Infinium MethylationEPIC Manifest File (version 1.0 B4).[30,36]

Lead Epigenetic Biomarkers

Epigenetic biomarkers of lead exposure reflecting lead in patella (ePatella) and tibia (eTibia) were calculated according to Colicino et al (2019).[12] To generate these biomarkers, Colicino et al used blood DNAm and bone lead measures available in a subset of 348 elderly men from the NAS (Normative Aging Study), a prospective cohort study of aging in adult men established in 1963 by the US Department of Veterans Affairs.[37] Blood DNAm was obtained with the Illumina Infinium HumanMethylation450 BeadChip (450k) array, and probes overlapping the 450k array and the Infinium MethylationEPIC BeadChip platform (395 005 CpG sites) were included in their analysis. Lead levels in tibia and patella were measured noninvasively with K-shell X-ray fluorescence spectroscopy.[38,39]

Epigenome-wide robust linear regressions were performed to select the most significant CpG sites associated with $\log_2$-transformed lead concentrations in each tissue separately, and then an elastic net regression approach, which takes into account the high-dimensional nature of CpG data,[40] was used to create the lead epigenetic biomarkers. CpGs from the epigenome-wide analysis were selected at $P<0.0001$. Elastic net with leave-one-out cross-validation was performed on the training data set, composed of 80% of participants. Lead biomarkers were then validated in the remaining test data set of the NAS cohort. ePatella and eTibia biomarkers were calculated as the linear combination of regression coefficients, and DNAm β values were calculated from the test data set. Biomarkers were originally only estimated for lead in bone; however, we further extended the epigenetic

biomarkers to estimate lead in blood (eBlood), to provide a marker of recent exposure, using the above approach in the NAS cohort (Data S1, Supplemental Methods). Lead levels in blood were measured using Zeeman background-corrected flameless atomic absorption (graphite furnace).[41,42] Estimation and validation of the blood lead epigenetic biomarker can be found in Tables S1 and S2 and Figures S1 through S5. In the current analysis, we used the same significant CpG sites associated with lead levels in blood (74 of 75 CpG sites), patella (58 CpG sites), and tibia (138 CpG sites) in NAS to estimate 3 lead DNAm-based biomarkers (eTibia, ePatella, and eBlood) in 2321 participants from the SHS. All CpG sites included in biomarker development in NAS, except one CpG used to calculate eBlood, were available for lead epigenetic biomarkers in SHS.

Cardiovascular Outcomes

Morbidity and mortality surveillance in the SHS is ongoing and has been previously described.[43-45] Briefly, all CVD outcomes and deaths are identified through coordination between Field Centers, the Coordinating Center, and Surveillance Reporting. For this study, all deaths and potential cardiovascular outcomes occurring through 2017 were reviewed by the Morbidity and Mortality Review Committee, which is composed of physicians with experience in reviewing medical records for the ascertainment of cardiovascular outcomes.[43,44] Incident CVD was defined as any definite or possible fatal or nonfatal coronary heart disease, stroke, or heart failure. Cardiovascular deaths were ascertained according to international diagnostic criteria, and events possibly meeting these criteria included the following: definite fatal myocardial infarction, definite sudden death attributable to coronary heart disease, definite fatal coronary heart disease, possible fatal coronary heart disease, definite fatal stroke, possible fatal stroke, definite fatal congestive heart failure, possible fatal congestive heart failure, and other fatal CVD.

Statistical Analysis

Distributions of eTibia, ePatella, and eBlood lead biomarkers were analyzed according to demographic and clinical covariates. Spearman correlations were performed among lead epigenetic biomarkers and urinary cadmium concentrations. We included urinary cadmium as this metal is moderately correlated with lead biomarkers in other studies.[46] Urinary lead biomarkers, unfortunately, are not available at the SHS examination 1 as the vials for sample collection were contaminated with lead.[47] Urinary cadmium concentrations were expressed in micrograms per gram of urine creatinine. The significance level in this analysis was $P=0.05$.

We used progressively adjusted multivariable Cox proportional hazards models to estimate the risk of incident CVD and CVD mortality according to each lead epigenetic biomarker. Lead epigenetic biomarkers were analyzed in tertiles, as continuous variables, and in a nonlinear manner using a restricted quadratic spline model with knots at the 10th, 50th, and 90th percentiles with the reference at the 10th percentile. Lead epigenetic biomarkers were $\log_2$ transformed to remove skewness (Figure S6). In all Cox models, center of recruitment was incorporated as a strata term, and age was used as the time metric. In progressively adjusted models, the first model (model 1) adjusted for sex, smoking status, body mass index, 5 genetic principal components (to account for population stratification[48]), and Houseman cell proportions (CD8T cells, CD4T cells, natural killer cells, B cells, and monocytes). The second model (model 2) further adjusted for low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and diabetes status. Model 3 further adjusted for systolic blood pressure, hypertension treatment, and estimated glomerular filtration rate.

Effect modification of the relationship between lead epigenetic biomarkers and CVD incidence and mortality was investigated according to the following subgroups: age (44.0–49.9, 50.0–64.9, and 65.0–75.4 years), sex (male/female), study center (Arizona, Oklahoma, and North Dakota/South Dakota), smoking status (never/former/current), urinary cadmium (<0.71, 0.71–1.26, and >1.26 µg/g), and diabetes (yes/no). Cox proportional hazards models included interaction terms between the subgroup and an increase in an interquartile range change in the respective lead epigenetic biomarker (eTibia, ePatella, and eBlood). Finally, as these biomarkers were estimated in men, we examined the differences in methylation levels between men and women for individual probes included in the biomarkers (Table S3). t-Tests were performed to examine statistically significant differences in these methylation levels. All analyses were conducted in R version 4.0.2.[28]

RESULTS

Median (interquartile range) concentrations of lead epigenetic biomarkers were 21.3 (18.5–24.8) µg/g in ePatella, 13.8 (11.7–16.1) µg/g in eTibia, and 2.9 (2.6–3.4) µg/dL in eBlood. The concentrations of each lead epigenetic biomarker were largely similar across participant characteristics and medical covariates (Table 1). Correlations among lead epigenetic biomarkers and urinary cadmium, adjusted for urine creatinine, revealed slight but significant positive associations between eTibia lead and urinary cadmium (Spearman $r=0.10$; $P<0.001$), ePatella lead and urinary cadmium

Table 1.   Lead Epigenetic Biomarkers by Participant Characteristics (N=2321)

	Variable	No. (%)	eTibia lead, μg/g	ePatella lead, μg/g	eBlood lead, μg/dL	Urinary cadmium, μg/g
Sex	Men	962 (41.5)	13.2 (11.1–15.3)	20.8 (18.1–24.5)	2.9 (2.5–3.4)	0.71 (0.47–1.10)
	Women	1359 (58.5)	14.2 (12.2–16.6)	21.6 (18.8–25.1)	2.9 (2.6–3.4)	1.16 (0.77–1.78)
Age, y	44.0–50.8	668 (28.8)	14.0 (12.0–16.4)	21.0 (18.6–24.3)	3.0 (2.6–3.4)	0.90 (0.56–1.31)
	50.9–59.5	1244 (53.6)	13.8 (11.6–16.1)	21.2 (18.3–24.9)	2.9 (2.6–3.4)	1.00 (0.64–1.55)
	59.6–75.4	409 (17.6)	13.7 (11.5–15.8)	21.6 (19.0–25.2)	2.9 (2.5–3.3)	1.03 (0.64–1.59)
Center	Arizona	311 (13.4)	13.9 (11.6–16.0)	20.5 (17.8–23.3)	2.9 (2.6–3.4)	0.76 (0.53–1.19)
	Oklahoma	981 (42.3)	13.9 (11.7–16.2)	21.0 (18.3–24.5)	3.0 (2.6–3.4)	0.86 (0.55–1.33)
	ND/SD	1029 (44.3)	13.8 (11.7–16.1)	21.7 (18.9–25.5)	2.9 (2.5–3.4)	1.11 (0.75–1.78)
Smoking status	Never	684 (29.5)	13.8 (11.8–15.8)	21.1 (18.3–24.8)	3.0 (2.6–3.5)	0.87 (0.55–1.36)
	Former	745 (32.1)	13.8 (11.8–16.0)	21.3 (18.4–25.1)	2.9 (2.6–3.3)	0.81 (0.55–1.25)
	Current	892 (38.4)	14.0 (11.7–16.5)	21.3 (18.7–24.6)	2.9 (2.6–3.4)	1.17 (0.77–1.81)
Urinary cadmium, μg/g	<0.71	758 (32.7)	13.4 (11.3–15.5)	20.8 (18.2–24.4)	2.9 (2.6–3.4)	0.51 (0.38–0.61)
	0.71–1.26	790 (34.0)	13.8 (11.8–16.2)	21.3 (18.4–25.0)	3.0 (2.6–3.5)	0.96 (0.83–1.10)
	>1.26	773 (33.3)	14.1 (12.0–16.7)	21.6 (18.8–25.2)	2.9 (2.5–3.3)	1.81 (1.50–2.33)
BMI, kg/m²	<25	406 (17.5)	14.1 (12.0–16.3)	21.6 (18.7–25.1)	3.0 (2.6–3.5)	1.21 (0.78–1.87)
	25–30	830 (35.8)	13.6 (11.5–16.2)	21.2 (18.5–24.8)	3.0 (2.6–3.4)	0.98 (0.62–1.51)
	≥30	1085 (46.7)	13.8 (11.7–16.0)	21.2 (18.5–24.8)	2.9 (2.5–3.3)	0.87 (0.57–1.35)
Diabetes	Yes	966 (41.6)	13.8 (11.5–15.9)	21.2 (18.4–24.7)	2.9 (2.5–3.4)	0.89 (0.58–1.41)
	No	1355 (58.4)	13.8 (11.8–16.3)	21.3 (18.6–24.9)	3.0 (2.6–3.4)	1.02 (0.65–1.54)
Hypertension treatment	Yes	464 (20.0)	14.0 (12.0–16.0)	20.8 (18.2–24.6)	3.0 (2.6–3.5)	0.86 (0.56–1.33)
	No	1857 (80.0)	13.7 (11.7–16.2)	21.4 (18.6–24.9)	2.9 (2.6–3.4)	1.00 (0.64–1.54)
SBP, mm Hg	<124	1146 (49.4)	13.7 (11.6–16.0)	21.2 (18.5–24.8)	2.9 (2.6–3.4)	1.01 (0.65–1.53)
	≥124	1175 (50.6)	13.9 (11.8–16.3)	21.3 (18.5–24.8)	2.9 (2.6–3.4)	0.92 (0.60–1.46)
LDL-C, mg/dL	<119	1159 (50.0)	14.0 (11.8–16.3)	21.4 (18.5–24.9)	3.0 (2.6–3.4)	0.97 (0.61–1.48)
	≥119	1162 (50.0)	13.7 (11.5–16.0)	21.1 (18.5–24.8)	2.9 (2.5–3.3)	0.96 (0.62–1.52)
HDL-C, mg/dL	<44	1140 (49.1)	13.6 (11.4–15.9)	21.0 (18.3–24.7)	2.9 (2.5–3.4)	0.91 (0.57–1.41)
	≥44	1181 (50.9)	14.1 (12.0–16.3)	21.5 (18.8–24.9)	2.9 (2.6–3.4)	1.03 (0.67–1.59)
eGFR, mL/min per 1.73 m²	<60	76 (3.3)	14.7 (11.9–17.3)	21.4 (18.9–25.6)	2.9 (2.5–3.4)	0.91 (0.53–1.51)
	≥60	2245 (96.7)	13.8 (11.7–16.1)	21.3 (18.5–24.8)	2.9 (2.6–3.4)	0.97 (0.62–1.50)
CVD mortality	Yes	452 (19.5)	14.1 (11.7–16.3)	21.4 (18.6–25.0)	3.0 (2.6–3.4)	1.00 (0.65–1.60)
	No	1869 (80.5)	13.7 (11.7–16.1)	21.2 (18.5–24.8)	2.9 (2.6–3.4)	0.96 (0.61–1.48)
CVD incidence	Yes	1023 (44.1)	13.7 (11.4–16.0)	21.3 (18.5–25.0)	2.9 (2.5–3.3)	0.99 (0.62–1.54)
	No	1298 (55.9)	13.9 (11.9–16.2)	21.2 (18.5–24.7)	2.9 (2.6–3.4)	0.95 (0.61–1.46)

Data are given as median (25th–75th percentile), unless otherwise indicated. Urinary cadmium concentrations were expressed in micrograms per gram of urine creatinine. BMI indicates body mass index; CVD, cardiovascular disease; eBlood, DNA methylation–based biomarker of lead exposure in blood; eGFR, estimated glomerular filtration rate; ePatella, DNA methylation–based biomarker of lead exposure in patella; eTibia, DNA methylation–based biomarker of lead exposure in tibia; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ND, North Dakota; SBP, systolic blood pressure; and SD, South Dakota.

(r=0.07; P<0.001), and eTibia and eBlood lead (r=0.09; P<0.001) (Table 2). eTibia and ePatella lead biomarkers (r=−0.07; P<0.001) and ePatella and eBlood lead biomarkers (r=−0.13; P<0.001) were negatively correlated. The median (interquartile range) age at follow-up among those who experienced a CVD event was 68.5 (62.4–74.9) years, whereas the median (interquartile range) age at follow-up among those who did not experience a CVD event was 75.1 (68.7–80.5) years.

For CVD mortality, the fully adjusted hazard ratio (HR) (95% CI) for a doubling increase in each lead epigenetic biomarker was 1.42 (1.07–1.87) for eTibia lead, 1.22 (0.93–1.60) for ePatella lead, and 1.57 (1.16–2.11) for eBlood lead (Table 3, model 3). Modeling the eBlood lead epigenetic biomarker in tertiles, the fully adjusted HR (95% CI) for CVD mortality comparing the highest with lowest tertile was 1.31 (1.03–1.67) in the partially adjusted model (model 2), and the association was similar after adjustment for systolic blood pressure, hypertension treatment, and estimated glomerular filtration rate (HR, 1.28 [95% CI, 1.00–1.64]; model 3). Flexible dose-response models supported a

Table 2. Spearman Correlation Coefficients (*P* Value) of Lead Epigenetic Biomarkers (eTibia, ePatella, and eBlood Lead) and Urinary Cadmium Concentrations (N=2321)

Variable	eTibia lead	ePatella lead	eBlood lead	Urinary cadmium
eTibia lead	1.00			
ePatella lead	−0.07 (<0.001)	1.00		
eBlood lead	0.09 (<0.001)	−0.13 (<0.001)	1.00	
Urinary cadmium	0.10 (<0.001)	0.07 (<0.001)	−0.01 (0.62)	1.00

Urinary cadmium concentrations were expressed in micrograms per gram of urine creatinine. eBlood indicates DNA methylation–based biomarker of lead exposure in blood; ePatella, DNA methylation–based biomarker of lead exposure in patella; and eTibia, DNA methylation–based biomarker of lead exposure in tibia.

linear relationship for the association of eTibia lead and eBlood lead epigenetic biomarkers (log$_2$ transformed) with CVD mortality, whereas the ePatella lead biomarker showed a linear but not statistically significant relationship (Figure S7).

None of the 3 lead epigenetic biomarkers was significantly associated with CVD incidence, either in models analyzing each epigenetic biomarker in tertiles or as continuous variables (Table 4) or in flexible dose-response models (Figure S7), and with different levels of adjustment. In additional models treating cadmium as a confounder, we found that our analyses are robust to urine cadmium adjustment (Table S4).

The associations between lead epigenetic biomarkers and CVD mortality were modified by sex (Figure 1). The HR (95% CI) of CVD mortality for an interquartile range increase in tibia lead was 1.42 (1.17–1.72) for men versus 1.04 (0.89–1.23) for women (*P* value for interaction=0.014). The corresponding HRs (95% CIs) for eBlood and ePatella lead for men were 1.27 (1.09–1.49) and 1.12 (0.95–1.31), respectively

(eBlood *P* value for interaction=0.231, and ePatella *P* value for interaction=0.976). Furthermore, 38% of probes (113 of 270) used in this analysis differed between men and women at a nominal *P*<0.05 after controlling for the false discovery rate (Table S2). Effect modification models for incident CVD were not significant for eTibia and ePatella lead by any participant characteristic evaluated, including sex, although the association for tibia lead was nonsignificantly stronger in men than women (Figure 2). For eBlood lead, the association with CVD incidence was nonsignificantly stronger for men versus women (HR, 1.13 [95% CI, 1.01–1.26] versus 1.04 [95% CI, 0.93–1.16]; *P* for interaction=0.281) and significantly modified by age, with oldest participants showing higher risk (*P* for interaction=0.007) and by study center (*P*=0.002), with participants from North Dakota and South Dakota showing an increased risk (HR, 1.27 [95% CI, 1.13–1.42]), whereas no association was found in Arizona (HR, 1.04 [95% CI, 0.78–1.38]) and Oklahoma (HR, 0.95 [95% CI, 0.84–1.07]).

Table 3. HRs (95% CIs) for CVD Mortality by Lead Epigenetic Biomarkers (N=2321)

Epigenetic biomarker	Tertile 1	Tertile 2	Tertile 3	Per double increase
eTibia lead, µg/g	<3.6	3.6–3.9	>3.9	
Model 1	1.00 (Reference)	1.19 (0.94–1.50)	1.16 (0.91–1.47)	1.36 (1.03–1.79)*
Model 2	1.00 (Reference)	1.22 (0.97–1.54)	1.22 (0.95–1.55)	1.47 (1.11–1.94)*
Model 3	1.00 (Reference)	1.18 (0.94–1.49)	1.19 (0.93–1.52)	1.42 (1.07–1.87)*
ePatella lead, µg/g	<4.3	4.3–4.5	>4.5	
Model 1	1.00 (Reference)	1.01 (0.80–1.28)	1.08 (0.85–1.37)	1.14 (0.87–1.48)
Model 2	1.00 (Reference)	0.99 (0.79–1.25)	1.13 (0.88–1.44)	1.22 (0.93–1.59)
Model 3	1.00 (Reference)	0.98 (0.78–1.24)	1.12 (0.88–1.43)	1.22 (0.93–1.60)
eBlood lead, µg/dL	<1.4	1.4–1.7	>1.7	
Model 1	1.00 (Reference)	1.14 (0.90–1.45)	1.23 (0.96–1.57)	1.51 (1.12–2.04)*
Model 2	1.00 (Reference)	1.20 (0.95–1.52)	1.31 (1.03–1.67)*	1.59 (1.19–2.15)*
Model 3	1.00 (Reference)	1.17 (0.92–1.48)	1.28 (1.00–1.64)*	1.57 (1.16–2.11)*

Model 1: adjusted for sex, smoking status (never, former, or current), body mass index (kg/m^2), genetic principal components, and immune cell types (CD8$^+$ cells, CD4$^+$ cells, natural killer cells, B cells, and monocytes). Model 2: further adjusted for low-density lipoprotein cholesterol (mg/dL), high-density lipoprotein cholesterol (mg/dL), and diabetes status (yes/no). Model 3: further adjusted for blood pressure (mmHg), hypertension treatment (yes/no), and estimated glomerular filtration rate (mL/min per 1.73 m^2). All models included center of recruitment as a strata term, and age was accounted for in the follow-up times of all models. Tertiles were calculated on log$_2$-transformed lead epigenetic biomarker concentrations. CVD indicates cardiovascular disease; eBlood, DNA methylation–based biomarker of lead exposure in blood; ePatella, DNA methylation–based biomarker of lead exposure in patella; eTibia, DNA methylation–based biomarker of lead exposure in tibia; and HR, hazard ratio.

*Represents statistically significant associations.

Table 4. HRs (95% CIs) for CVD Incidence by Lead Epigenetic Biomarkers (N=2321)

Epigenetic biomarker	Tertile 1	Tertile 2	Tertile 3	Per double increase
eTibia lead, μg/g	<3.6	3.6–3.9	>3.9	
Model 1	1.00 (Reference)	0.98 (0.84–1.13)	0.99 (0.84–1.16)	0.96 (0.80–1.15)
Model 2	1.00 (Reference)	0.99 (0.85–1.15)	1.04 (0.89–1.22)	1.02 (0.85–1.23)
Model 3	1.00 (Reference)	0.96 (0.83–1.12)	1.03 (0.88–1.21)	0.99 (0.83–1.19)
ePatella lead, μg/g	<4.3	4.3–4.5	>4.5	
Model 1	1.00 (Reference)	0.92 (0.79–1.07)	1.00 (0.85–1.17)	1.02 (0.85–1.22)
Model 2	1.00 (Reference)	0.91 (0.78–1.06)	1.04 (0.88–1.22)	1.08 (0.90–1.29)
Model 3	1.00 (Reference)	0.90 (0.77–1.05)	1.03 (0.88–1.21)	1.07 (0.89–1.29)
eBlood lead, μg/dL	<1.4	1.4–1.7	>1.7	
Model 1	1.00 (Reference)	1.04 (0.89–1.21)	0.95 (0.81–1.12)	1.02 (0.83–1.25)
Model 2	1.00 (Reference)	1.08 (0.92–1.26)	1.01 (0.86–1.18)	1.07 (0.87–1.30)
Model 3	1.00 (Reference)	1.07 (0.92–1.25)	1.00 (0.85–1.17)	1.06 (0.87–1.30)

Model 1: adjusted for sex, smoking status (never, former, or current), body mass index (kg/m^2), genetic principal components, and immune cell types (CD8$^+$ cells, CD4$^+$ cells, natural killer cells, B cells, and monocytes). Model 2: further adjusted for low-density lipoprotein cholesterol (mg/dL), high-density lipoprotein cholesterol (mg/dL), and diabetes status (yes/no). Model 3: further adjusted for systolic blood pressure (mm Hg), hypertension treatment (yes/no), and estimated glomerular filtration rate (mL/min per 1.73 m^2). All models included center of recruitment as a strata term, and age was accounted for in the follow-up times of all models. Tertiles were calculated on log$_2$-transformed lead epigenetic biomarker concentrations. CVD indicates cardiovascular disease; eBlood, DNA methylation–based biomarkers of lead exposure in blood; ePatella, DNA methylation–based biomarkers of lead exposure in patella; eTibia, DNA methylation–based biomarkers of lead exposure in tibia; and HR, hazard ratio.

DISCUSSION

eTibia and eBlood lead biomarkers were associated with increased risk of CVD mortality in the SHS. The association of eTibia and eBlood lead biomarkers with CVD mortality was modified by sex, with a positive association found in men and no association found in women. This finding is likely explained by the creation of these epigenetic biomarkers in the NAS, an all-male population. The biomarkers have yet to be validated in women. However, findings remained significant for the whole population. eTibia and eBlood lead biomarkers were not associated with CVD incidence overall, but a positive association, which was significant for eBlood and borderline significant for eTibia, was found among men. No significant association was observed in models incorporating the ePatella lead biomarker with either CVD mortality or incidence.

This analysis builds on the development of bone and blood lead epigenetic biomarkers,[12] and is the first study to evaluate their relation with cardiovascular outcomes. Compared with the NAS subset that was used to generate these lead biomarkers, SHS participants were younger, had similar proportions of ever smokers, and included both men and women (Table S1). Lead levels (mean±SD) measured in tibia (21.1±12.9 μg/g), patella (27.4±17.7 μg/g), and blood (4.0±2.3 μg/dL) among the NAS subset were higher than epigenetic biomarker concentrations estimated in the SHS subset (eTibia: 14.1±3.5 μg/g; ePatella: 22.4±6.7 μg/g; and eBlood: 3.1±0.8 μg/dL), which is a reasonable finding as the SHS population is younger and resides in rural areas and small towns that have been less historically affected by traffic and leaded gasoline compared with the Boston, MA, area.

Although these epigenetic biomarkers have been estimated in 2 cohorts to predict Parkinson disease,[14] further research is needed to determine their transportability across different cohorts with diverse study populations and different health outcomes. This aforementioned study observed that increased concentrations of the tibia epigenetic biomarker was associated with Parkinson disease status in the Parkinson's Environment and Genes cohort and the System Genomics of Parkinson's Disease cohort,[14] whereas patella epigenetic biomarker concentrations were inversely associated with Parkinson disease status in the System Genomics of Parkinson's Disease cohort.[14] In both these cohorts, DNAm estimated tibia and patella lead concentrations were lower compared with the present analysis in the SHS, and these cohorts consisted of participants who were older and had a higher proportion of male participants than the present analysis. Notably, smoking information was missing in the System Genomics of Parkinson's Disease cohort, which is an important confounder that was included in the present study. The results presented in the current analysis are consistent in identifying that the tibia epigenetic biomarker was most strongly associated with disease.

The observed associations with the eTibia biomarker could stem from the longer half-life of lead in tibia, a cortical bone, in comparison to patella, a trabecular bone.[4] Evidence suggests that lead in trabecular bone is more biologically active and that lead is exchanged into the bloodstream more readily than in cortical
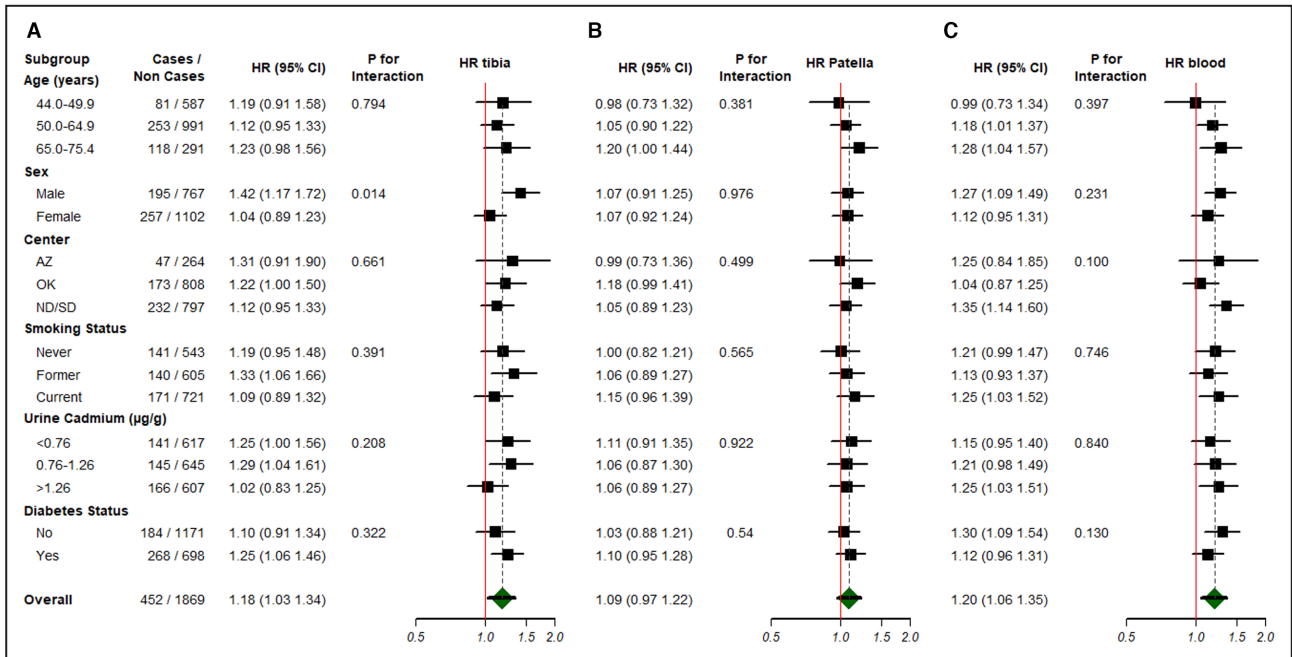
Figure 1. Hazard ratio (HR) (95% CI) of cardiovascular disease mortality by a doubling increase in epigenetic lead biomarker concentrations (log$_2$ transformed) of tibia (A), patella (B), and blood (C) biomarkers, corresponding to the interquartile range (75th–25th percentile) within subgroups.

AZ indicates Arizona; ND, North Dakota; OK, Oklahoma; and SD, South Dakota.

bones.[4,12] As the half-life of tibia lead is longer than patella lead, tibia lead is believed to be more representative of cumulative lead exposure. The K-shell X-ray

fluorescence spectroscopy measurement technique has also been cited as having greater measurement uncertainty for trabecular bones than cortical bones,

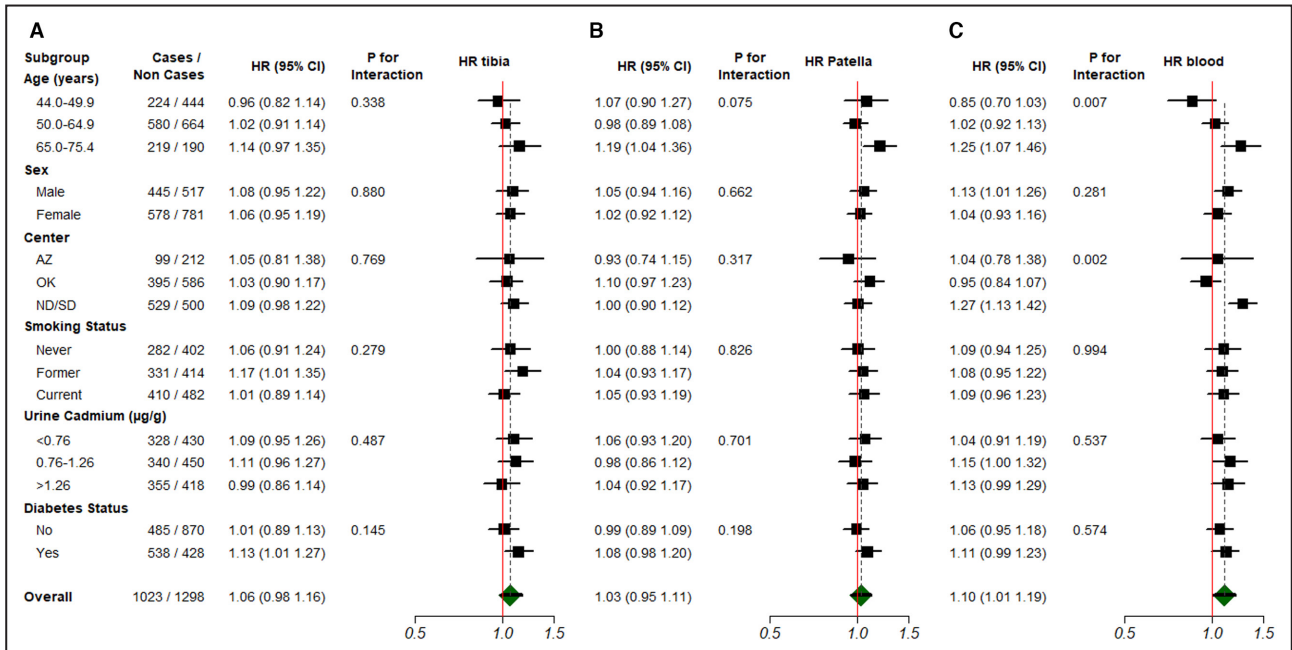**Figure 2.** Hazard ratio (HR) (95% CI) of cardiovascular disease incidence by a doubling increase in epigenetic lead biomarker concentrations (log$_2$ transformed) of tibia (A), patella (B), and blood (C) biomarkers, corresponding to the interquartile range (75th–25th percentile) within subgroups.

AZ indicates Arizona; ND, North Dakota; OK, Oklahoma; and SD, South Dakota.

attributable to a lower comparative mineral density in trabecular bones.[38] Thus, the greater precision of lead measurements in tibia could also explain the stronger associations reported between eTibia, rather than ePatella, biomarkers. Furthermore, the eBlood biomarker is a marker of short-term lead exposure, which creates potential challenges in its use as a predictive marker for disease, although blood lead reflects both endogenous and exogenous sources of exposure. We found significant associations between the eBlood biomarker and increased risk of CVD mortality, as well as between eBlood lead and CVD incidence among men, suggesting that this epigenetic biomarker is relevant in capturing disease risk from lead exposure. The stronger association with mortality has also been observed among other risk factors in the SHS, such as urinary arsenic[49] and urinary cadmium concentrations.[19] This relationship may also be related to measurement error and differences in clinical care across sites for morbid events, where mortality represents a more definitive and robust end point. An alternative explanation is that lead exposure results in more severe disease, which would reflect a stronger relationship with CVD mortality than incidence.

The present study adds to the weight of evidence of DNAm-based biomarkers to predict disease risk in the SHS. Prior research has identified that differential methylation at CpG sites and differentially methylated regions were associated with increased incidence of lymphatic-hematopoietic, solid, and overall cancers,[50] smoking,[51] arsenic[52] and cadmium,[51] and coronary heart disease.[53] One challenge of using these biomarkers, however, is replicating their ability to capture risk across diverse populations.[53] Although the present study implements 3 epigenetic biomarkers of lead exposure, these results would be strengthened through replication in different populations, including in other American Indian cohorts and other racial and ethnic groups.

One limitation of this analysis is that the SHS does not have bone and blood lead measured concurrently with DNAm to compare the accuracy of the epigenetic biomarkers. As a consequence, we cannot confirm that these biomarkers directly reflect lead exposure in the SHS. Another alternative explanation is that the biomarkers reflect DNAm pathways affected by lead that could also be affected by other exposures, but that nevertheless are part of the mechanisms by which lead induces CVD. Furthermore, the epigenetic biomarkers were originally created in the NAS, an elderly, all-male, and mainly White cohort, which could limit their generalizability to other cohorts like the SHS. In our analysis of sex-dependent effects, the epigenetic biomarkers of tibia lead and blood lead remained associated with cardiovascular mortality and cardiovascular incidence primarily in men, whereas the association was practically null in women, suggesting that these epigenetic lead biomarkers are primarily relevant for men. Given the sensitivity of DNA methylation biomarkers to the availability of specific probes, we recommend that future analyses estimating epigenetic biomarkers of lead exposure consider the availability of the probes used herein. Given the potential of epigenetic biomarkers to act as noninvasive approximations of lead exposure, it is vital to perform further validation of these epigenetic biomarkers in cohorts where bone lead measures are available, and determine their relation to disease risk in both men and women.

CONCLUSIONS

In the SHS, recently developed tibia and blood lead epigenetic biomarkers were associated with increased risk of cardiovascular mortality, potentially reflecting the cardiovascular impact of cumulative and ongoing lead exposures. Future work must perform further validation of these lead epigenetic biomarkers in different populations, given their potential to capture disease risk.

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Disclosures
None.

Supplemental Material
Data S1
Table S1–S4
Figures S1–S7

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SUPPLEMENTAL MATERIAL

Data S1. Supplemental Methods

Blood lead biomarker results from the Normative Aging Study (NAS)

An additional analysis was performed in a subset of the Normative Aging Study (N = 348) to examine the association between lead levels in blood with DNA methylation values (Table S1). Similar to the methodological approach for tibia and patella lead[12], an epigenome-wide robust linear regression was performed that accounted for outliers and heteroskedasticity in DNA methylation beta values. Covariates in the epigenome wide analysis were identified through principle-component analysis (Figure S1). Epigenome-wide analysis indicated no excess of false positive rates (Figure S2). An elastic net approach (Figure S3) was used to identify significant CpG sites ($p < 0.0001$), and 80% of participants were randomly assigned to the training dataset, while 20% were randomly selected for the test dataset. Elastic-net was performed on the training set with leave-one-out cross validation, and lead biomarkers for blood were estimated as the linear combination of regression coefficients and DNA methylation beta-values matrix of the test dataset (Table S2). There were 75 CpG sites associated with blood lead concentrations. Estimated blood lead concentrations were then compared to measured blood lead values in order to validate this biomarker in a subset of the NAS (Figures S4-S5). The Pearson's correlation coefficients between actual and estimated blood lead levels was moderate ($r = 0.49$), and the mean square error (MSE) was 0.40. Results from receiver operating characteristic (ROC) and area under the curve (AUC) indicated good accuracy (AUC: 0.82, 95% CI: 0.73-0.91). The difference in means between the estimated and measured blood lead concentrations were not significantly different ($p = 0.84$), and the Kolmogorov-Smirnov test ($p = 0.01$) indicated that these values likely came from the same distribution.

Table S1. Description of participants from the Normative Aging Study (NAS)

Variables	mean ± SD
Age (years)	76.25 ± 6.57
Education (years)	16 ± 3.02
Pack-years	33 ± 25.17
Smoking Status	
never	156 (0.3)
ever	360 (0.7)
Alcohol Consumption	
≤2 drinks per day	417 (0.81)
>2 drinks per day	99 (0.19)
Patella lead levels	27.36 ± 17.74
Tibia lead levels	21.05 ± 12.91
Blood lead levels	3.96 ± 2.32

Table S2. Identification of DNA methylation sites relative to blood lead exposure

CpG Name	Elastic Net Coeff	Chromosome	MAPINFO	UCSC_RefGene_Name	UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island
(Intercept)	-10.0414	NA	NA	NA	NA	NA
cg10442735	0.428246	1	3062633	PRDM16;PRDM16	Body;Body	S_Shelf
cg10442251	-1.20932	1	18700625	IGSF21	Body	N_Shelf
cg07031996	-0.39012	1	38324738	MTF1	5'UTR	N_Shore
cg17791651	0.078059	1	38513489	POU3F1	TSS1500	Island
cg06408034	-3.93669	1	45671987	ZSWIM5	1stExon	Island
cg16005939	0.034461	1	91191990			Island
cg08122338	23.79213	1	243419530	CEP170;CEP170;CEP170;SDCCAG8	TSS1500;TSS1500;TSS1500;1stExon	S_Shore
cg16596470	0.133058	1	246451744	SMYD3;SMYD3	Body;Body	
cg19407717	0.175645	2	1544120	TPO;TPO;TPO;TPO	Body;Body;Body;Body	N_Shore
cg12416637	-0.77252	2	154333650			N_Shore
cg14844194	-0.40731	2	200524259			S_Shore
cg12346504	-4.03666	2	214148862	SPAG16;SPAG16	TSS1500;TSS1500	N_Shore
cg22753768	-0.03878	3	52443424	BAP1;PHF7;PHF7	Body;TSS1500;TSS1500	N_Shore
cg12226306	1.55914	3	105087718	ALCAM	Body	N_Shore

cg03552688	0.985584	3	138822369	BPESC1	TSS1500	
cg12958778	-0.85696	4	6912474	TBC1D14;TBC1D14	5'UTR;5'UTR	S_Shore
cg01411468	-0.30947	4	36283588	DTHD1;DTHD1	5'UTR;1stExon	
cg11761728	0.322849	4	111536679			N_Shelf
cg07799386	-0.12272	5	122430821	PRDM6	Body	Island
cg12532966	0.453348	5	178854202			
cg25511429	0.092361	6	6008125	NRN1	TSS1500	N_Shore
cg23646360	-0.56428	6	10886130	SYCP2L	TSS1500	N_Shore
cg17779289	12.63892	6	33272148	TAPBP;TAPBP;TAPBP	Body;Body;Body	
cg20566286	-2.04254	6	168785923			
cg10884288	0.18269	7	4922196	RADIL	5'UTR	N_Shore
cg17469978	1.736623	7	116164704	CAV1	TSS200	Island
cg03917158	0.279592	8	1381012			
cg22883125	-0.31365	8	11540758			S_Shore
cg01546563	-0.22378	8	11567189	GATA4	Body	Island
cg09430976	3.464535	8	25907314			N_Shore
cg02504902	-0.53933	8	53326922			

cg17099568	-0.19174	8	65284438			N_Shore
cg20413392	-0.38521	9	131155013	MIR219-2	TSS200	Island
cg01240049	0.597319	9	138068091			Island
cg20395881	0.396681	10	1759215	ADARB2	Body	
cg05453333	-0.64092	10	49731375	ARHGAP22	Body	N_Shore
cg19880462	0.219686	10	61468548	SLC16A9	5'UTR	N_Shore
cg24591300	-0.41475	10	100993025	HPSE2;HPSE2;HPSE2;HPSE2	Body;Body;Body;Body	N_Shore
cg22391400	-12.7911	10	105727767	SLK	Body	S_Shore
cg20639805	0.816579	10	112404997	RBM20	Body	S_Shore
cg22773555	5.800023	11	830233	EFCAB4A	Body	Island
cg03922337	-0.21862	11	14380918	RRAS2;RRAS2	TSS200;TSS1500	S_Shore
cg09867084	-3.20354	11	63912367	MACROD1	Body	
cg14235783	-1.53413	11	65420518			Island
cg04800788	2.362246	11	123396571	GRAMD1B;GRAMD1B	1stExon;5'UTR	
cg22063247	-0.37862	11	133994642	JAM3	Body	N_Shore
cg14121142	-1.12071	12	49391363	DDN	Body	Island
cg09287190	-0.17873	13	100640914			N_Shore

cg24670552	-0.03639	13	110436780	IRS2	1stExon	Island
cg16851425	0.606277	13	110437759	IRS2	1stExon	Island
cg04393471	-0.37513	13	111010255	COL4A2	Body	
cg26053975	1.858657	14	54973785			N_Shelf
cg09298014	-0.40781	14	97058864			N_Shore
cg26471610	0.968717	15	34640893	C15orf55	Body	
cg24956533	-0.45115	15	37173638	LOC145845	Body	S_Shore
cg03603381	0.106808	15	38857474	RASGRP1;RASGRP1	TSS1500;TSS1500	Island
cg17865114	-0.65723	16	3067759	CLDN6	5'UTR	Island
cg09981030	-3.41555	16	3179796			
cg16631088	-0.46834	16	15528133	C16orf45	TSS200	N_Shore
cg10137837	0.063332	17	6926742	BCL6B	5'UTR	Island
cg21969795	-0.20118	17	7759140	TMEM88	3'UTR	N_Shore
cg06418907	-0.30942	17	7982510	ALOX12B	Body	N_Shore
cg13718961	-0.00948	17	27939261	ANKRD13B	Body	N_Shore
cg05478824	-0.10059	17	79970135	ASPSCR1	Body	S_Shelf
cg23928512	-0.19177	17	79970192	ASPSCR1	Body	S_Shelf

cg27226927	-0.09136	17	79993863	DCXR	Body	N_Shore
cg13214542	-0.94023	18	33552019	C18orf21	TSS1500	N_Shore
cg26901714	1.01678	19	376152	THEG;THEG	TSS200;TSS200	S_Shore
cg19399220	-0.30309	19	10527588			Island
cg01397939	-0.31035	19	14583568	PTGER1	Body	Island
cg11571942	-0.68779	19	55610938	PPP1R12C	Body	S_Shore
cg12014753	-0.32942	20	50384822	ATP9A	Body	Island
cg24875593	-0.12506	21	45153009	PDXK	Body	S_Shelf
cg06911679	0.764272	22	22877746			
cg03938598	-1.51491	22	50699868	MAPK12	1stExon	Island

Table S3. Average beta values for each probe used in the Strong Heart Study analysis according to men and women

CpG_Name	Mean Beta Values Males	Mean Beta values Females	p-value
cg00056541	0.58496843	0.582887355	0.7659865
cg00118365	0.42868554	0.418396093	0.007774756
cg00178249	0.20722654	0.210491875	0.2397211
cg00284153	0.6291812	0.645383531	8.45281E-08
cg00295339	0.94771763	0.94751594	0.7659865
cg00380835	0.74041844	0.779700844	3.6853E-48
cg00549219	0.4402119	0.444518224	0.2673082
cg00616922	0.95090747	0.951576707	0.1768622
cg00668034	0.22873846	0.239005099	0.05562816
cg00697358	0.84656619	0.849918297	0.1747424
cg00779216	0.75719151	0.759914162	0.2300414
cg00788177	0.12906659	0.134293886	0.1870585
cg00815440	0.01045778	0.01044907	0.9305097
cg00845862	0.02946731	0.029652953	0.5630539
cg00846121	0.86361652	0.865733048	0.2228284
cg00964109	0.01183793	0.011724312	0.4473895
cg01154573	0.01352264	0.013559338	0.7659865
cg01198591	0.04118068	0.04318176	0.1551233
cg01240049	0.88363526	0.888104586	0.01057448
cg01283863	0.95507173	0.955521172	0.4072994
cg01330312	0.77391814	0.774935805	0.7440688
cg01397939	0.38810596	0.396766654	0.00011298
cg01411468	0.92257617	0.933420123	1.26968E-12
cg01502872	0.26467011	0.264038505	0.811521
cg01546563	0.1292038	0.125979448	0.1434667
cg01815833	0.0254163	0.025306478	0.8024407
cg02012703	0.11240353	0.112911434	0.8023217
cg02021288	0.8237959	0.820060127	0.1534882

cg02033302	0.71107632	0.717584688	0.006621538
cg02440976	0.01595504	0.016007914	0.6585425
cg02493604	0.07910375	0.079218722	0.9517672
cg02504902	0.86003648	0.875486897	7.55121E-13
cg02613380	0.94954078	0.952041939	1.57038E-05
cg02631879	0.01528417	0.015650696	0.4563141
cg02806322	0.75089225	0.760056177	1.31511E-05
cg02830714	0.94905328	0.949217764	0.8320486
cg03206925	0.95450323	0.956274081	0.006776076
cg03318593	0.94517853	0.945546979	0.7659865
cg03454705	0.94211574	0.941637977	0.2289362
cg03523835	0.17066817	0.174034979	0.1747424
cg03552688	0.95985134	0.962409335	2.09537E-05
cg03591798	0.03447532	0.034326159	0.7952533
cg03603381	0.55829467	0.571340473	5.20594E-11
cg03612522	0.02987886	0.029775071	0.8469719
cg03764965	0.84014945	0.847386484	0.004543919
cg03791150	0.92590865	0.931427581	4.13411E-10
cg03897712	0.02738225	0.028314977	0.001660234
cg03917158	0.85410775	0.854560929	0.8291399
cg03922337	0.15495776	0.163709336	1.6947E-16
cg03938598	0.02645677	0.026315627	0.8024407
cg04276508	0.07283903	0.074651596	0.1932282
cg04300684	0.53469052	0.505786098	7.08008E-08
cg04338871	0.6741962	0.683706213	0.002612561
cg04379155	0.01805047	0.017804687	0.3354398
cg04393471	0.94511639	0.945218367	0.9305097
cg04427735	0.00864506	0.008458977	0.1100234
cg04456892	0.91369662	0.912503997	0.4936289
cg04458670	0.02299539	0.025273104	5.89511E-05
cg04730882	0.35418876	0.359704605	0.003470847

cg04800788	0.92392269	0.928567365	5.29813E-08
cg04804542	0.86811638	0.872293814	0.04984412
cg04827747	0.0120057	0.012142951	0.06104231
cg04929736	0.02632464	0.026226671	0.805865
cg05005659	0.94775253	0.951312908	0.000210125
cg05347216	0.61562419	0.607575812	0.000496259
cg05453333	0.31287689	0.310320094	0.4473895
cg05459971	0.35325474	0.372120463	2.5017E-06
cg05478824	0.62652694	0.625713873	0.837371
cg05499853	0.01782655	0.017866197	0.8214504
cg06100461	0.06666467	0.066981879	0.8024407
cg06155303	0.29759262	0.334689604	2.37168E-18
cg06376277	0.88733509	0.892669751	0.000400618
cg06382254	0.94982495	0.953266852	0.000006456
cg06408034	0.02705669	0.026694588	0.4473587
cg06418907	0.38295105	0.384082316	0.8024407
cg06436673	0.96246947	0.964520213	0.1363552
cg06773604	0.02771975	0.029220305	0.000217691
cg06890950	0.76827007	0.769505077	0.7783067
cg06911679	0.923253	0.926617845	0.0006456
cg07015663	0.84660682	0.848419866	0.2477632
cg07031996	0.1455926	0.14512796	0.8300571
cg07105947	0.13450664	0.147806192	0.000108698
cg07122529	0.72713874	0.731313517	0.2300414
cg07361385	0.96913929	0.970529982	5.89511E-05
cg07513561	0.96262694	0.963626463	0.007907952
cg07545743	0.30837788	0.320011077	0.02597441
cg07764113	0.84288543	0.840901313	0.5121869
cg07799386	0.09218271	0.094209988	0.1741885
cg07846297	0.14843267	0.150739046	0.12105
cg08069883	0.8084274	0.815907877	0.000402556

cg08122338	0.02539538	0.025325591	0.8811339
cg08220084	0.03895264	0.040035359	0.1845172
cg08421126	0.25305366	0.255224475	0.2585871
cg08570458	0.95446321	0.95664953	0.000235253
cg08615567	0.08744007	0.091646716	0.006008831
cg08693172	0.14543769	0.147709499	0.4473895
cg08790487	0.91713536	0.919099112	0.1119232
cg09062550	0.11324341	0.113864512	0.8024407
cg09135656	0.76687302	0.762111709	0.3354398
cg09178385	0.82794409	0.836735747	3.15403E-05
cg09264065	0.48855514	0.52139771	6.994E-23
cg09284949	0.22763095	0.19635569	1.27103E-16
cg09287190	0.41326462	0.416510742	0.24748
cg09298014	0.06879225	0.077160597	1.27711E-08
cg09430976	0.02326638	0.023384074	0.8214504
cg09436495	0.79545639	0.798029022	0.2603782
cg09463656	0.06354496	0.063660055	0.9326718
cg09741221	0.04794629	0.048743499	0.2857081
cg09867084	0.90759998	0.910090956	0.005631067
cg09981030	0.02118936	0.02118163	0.9863333
cg10042319	0.02970335	0.031895207	2.152E-07
cg10086141	0.02469616	0.024244482	0.2476225
cg10137837	0.32732382	0.338338996	5.68263E-08
cg10197862	0.01962838	0.019735399	0.4065568
cg10442251	0.52114124	0.535732908	7.12466E-06
cg10442735	0.89969056	0.902355832	0.1183124
cg10521014	0.53174386	0.533214412	0.7915947
cg10716862	0.90709192	0.908609051	0.53531
cg10845249	0.12378774	0.126648879	0.2300414
cg10884288	0.04859265	0.050792374	0.00160119
cg11352369	0.91263601	0.914790372	0.04890909

cg11571942	0.89022465	0.891443684	0.2567727
cg11728928	0.29951645	0.303285247	0.4434066
cg11753765	0.0274747	0.029409776	8.12604E-05
cg11761728	0.32048304	0.325056358	0.01069886
cg11888571	0.01762917	0.017541554	0.8193292
cg11990334	0.0733973	0.075752209	0.2399189
cg12063795	0.24392602	0.243865421	0.9826113
cg12153668	0.81991514	0.830428023	3.14394E-06
cg12226306	0.14751235	0.14949392	0.2289362
cg12346504	0.01551764	0.01568627	0.6181827
cg12416637	0.04044338	0.039561378	0.2289362
cg12532966	0.84560692	0.850668008	0.03147872
cg12841525	0.8517187	0.85660277	0.047075
cg12858300	0.0163708	0.016530306	0.329689
cg12903529	0.85671824	0.866156656	4.88683E-05
cg12920180	0.09328164	0.095989235	0.2289362
cg12958778	0.7257577	0.734699749	0.000178471
cg13214542	0.09717336	0.097668665	0.8024407
cg13251292	0.87232593	0.875138183	0.2566928
cg13257129	0.86239076	0.868595301	0.008351512
cg13415073	0.10183125	0.107537822	0.003673877
cg13446622	0.91100134	0.915060704	0.00267394
cg13448092	0.59668003	0.590516236	0.2300414
cg13463245	0.81078363	0.807887931	0.3623588
cg13580008	0.02582386	0.025374774	0.2231704
cg13718961	0.90134212	0.909583663	8.4466E-22
cg13807056	0.89878117	0.90485048	0.001336323
cg14074486	0.69496203	0.705761845	2.56941E-07
cg14121142	0.04100012	0.04490217	0.008351512
cg14133708	0.04817508	0.048690267	0.7388203
cg14235783	0.03490175	0.034300926	0.2673082

cg14312661	0.94709223	0.94740029	0.751726
cg14727952	0.02366804	0.02361159	0.8811339
cg14762973	0.06256058	0.063904141	0.5035128
cg14821507	0.02071654	0.020803245	0.6926121
cg14844194	0.1527787	0.158403321	0.02327435
cg14923295	0.01548794	0.015543837	0.6832347
cg15081825	0.76421339	0.768065598	0.4710984
cg15349474	0.94768242	0.948792569	0.04721224
cg15364504	0.84316391	0.850931172	0.002143723
cg15739944	0.02172167	0.021367435	0.5163719
cg15825186	0.0216944	0.021919112	0.7185787
cg15996769	0.32133988	0.337130398	2.97636E-06
cg16005939	0.18548533	0.190787977	0.01121337
cg16218705	0.88664416	0.890462077	0.3649591
cg16520800	0.02074738	0.021019908	0.2397211
cg16596470	0.97268657	0.972950518	0.341485
cg16619425	0.05512619	0.059504217	5.79247E-05
cg16631088	0.82325635	0.841250428	4.94288E-12
cg16851425	0.1020518	0.102712251	0.7867655
cg16937735	0.60400759	0.620827609	2.73483E-10
cg16944026	0.95926577	0.959454336	0.7783067
cg16961545	0.02480515	0.024711424	0.862952
cg16978871	0.01349794	0.013510411	0.9451073
cg17099568	0.14696479	0.151779387	0.048958
cg17331296	0.07388515	0.072352709	0.538
cg17469978	0.04744632	0.045549368	0.007229375
cg17531889	0.93830515	0.939070663	0.3858229
cg17584477	0.95487962	0.955672452	0.2604331
cg17779289	0.966167	0.966380222	0.5847826
cg17791651	0.57487655	0.595586109	1.6409E-15
cg17825384	0.02365016	0.023782885	0.6532857

cg17865114	0.95143862	0.953281848	0.000057835
cg17884698	0.95781925	0.958107423	0.5512512
cg17886028	0.01777533	0.017791895	0.9451073
cg18086761	0.06642791	0.068131302	0.4473895
cg18207091	0.02494037	0.025356759	0.5745631
cg18598900	0.07088687	0.072099249	0.5050612
cg18605120	0.93271021	0.932714301	0.997
cg18766080	0.02251333	0.022392814	0.7952533
cg18916055	0.87493137	0.874835272	0.9696274
cg19246761	0.08484054	0.082469422	0.175704
cg19399220	0.28103753	0.265107892	1.32507E-07
cg19407717	0.8723388	0.877941395	0.00060639
cg19531536	0.45638608	0.48506147	8.9039E-37
cg19615017	0.66761604	0.670326365	0.6512632
cg19880462	0.11121461	0.108836595	0.2289362
cg20100987	0.73623035	0.747374251	5.95939E-14
cg20326704	0.06693369	0.080600025	2.70537E-18
cg20395881	0.64511559	0.653942524	0.04714433
cg20413392	0.85533839	0.852730889	0.4186124
cg20506843	0.53583564	0.542926044	0.329689
cg20566286	0.92798539	0.928975282	0.2437248
cg20606555	0.76190802	0.764352055	0.538
cg20639805	0.32798408	0.372460976	3.29077E-31
cg20844771	0.01543032	0.015102398	0.1440746
cg20922251	0.03211064	0.032654739	0.01303615
cg21244880	0.01571983	0.0158056	0.5065939
cg21365094	0.04850061	0.046962078	0.2103459
cg21371809	0.26956099	0.265685871	0.4473895
cg21531679	0.94099427	0.94298578	0.1605107
cg21558508	0.01339452	0.013466776	0.4453934
cg21587066	0.09330338	0.092772035	0.8300571

cg21616420	0.95669641	0.958222857	0.01282233
cg21969795	0.74956769	0.757652125	0.002899662
cg21994822	0.66081969	0.655212663	0.1274826
cg21996068	0.54517711	0.556029723	0.003326507
cg22063247	0.37974464	0.371364492	0.003243371
cg22391400	0.011535	0.011303061	0.1085872
cg22691824	0.7133124	0.720137557	0.00298629
cg22753768	0.16210773	0.161696145	0.8755896
cg22773555	0.01821075	0.017697478	0.329689
cg22784964	0.01311743	0.013208519	0.3769128
cg22883125	0.02760152	0.02767862	0.8320486
cg22908581	0.42664251	0.426491765	0.975125
cg23028436	0.06785963	0.069812587	0.04984412
cg23087931	0.96034556	0.960969909	0.2235074
cg23483656	0.10846348	0.109980304	0.4434066
cg23497569	0.94859057	0.950157554	0.2058258
cg23528492	0.77355495	0.774005914	0.9240941
cg23646360	0.2809995	0.297255569	2.73828E-05
cg23832388	0.64937939	0.652115089	0.5745631
cg23928512	0.85352002	0.853044957	0.8193292
cg23931558	0.10526118	0.109975864	0.08198095
cg24036116	0.81175374	0.821037861	4.02844E-05
cg24362016	0.57194556	0.588801689	5.51137E-05
cg24526433	0.01472322	0.014601141	0.3818563
cg24591300	0.45479133	0.472695179	2.15424E-15
cg24612305	0.02026427	0.021223631	0.04077474
cg24670552	0.88373103	0.889265878	0.006008831
cg24731731	0.58343984	0.611364315	7.8279E-09
cg24792682	0.05809228	0.058568899	0.7031535
cg24875593	0.84606152	0.837244819	0.2841313
cg24915508	0.94497843	0.946390852	0.1857977

cg24956533	0.07698776	0.077341201	0.8811339
cg24958687	0.31670006	0.30776088	0.1100234
cg25055120	0.94731815	0.94354373	0.001142148
cg25511429	0.0931265	0.092088066	0.4637448
cg25566285	0.95055679	0.952678922	0.09578411
cg25774020	0.92609292	0.931161217	0.1001278
cg25884854	0.01495966	0.014253054	0.000113402
cg25926515	0.36105646	0.384927735	8.0969E-16
cg25987408	0.82365331	0.833793407	1.68736E-08
cg25994470	0.01603081	0.016155502	0.3485858
cg26053975	0.93120769	0.933078806	0.008351512
cg26070426	0.23242671	0.229498579	0.4434066
cg26234034	0.01852287	0.018515323	0.984985
cg26471610	0.94765203	0.949957743	7.12466E-06
cg26607429	0.95421099	0.955775788	0.1747424
cg26901714	0.86566625	0.86992686	0.007306173
cg26937798	0.01846308	0.017875041	0.08780566
cg26955337	0.98254318	0.982459595	0.3818563
cg27111925	0.35220067	0.355807776	0.344256
cg27226927	0.78542235	0.786589855	0.656564
cg27425146	0.96754543	0.967870615	0.4473895
cg27532331	0.96654603	0.966543031	0.9946978
cg27585878	0.9619612	0.962014053	0.9326718
cg27622405	0.01253221	0.012636875	0.4207821

Bonferroni corrected p-values are reported.

Table S4. Hazard ratios (95% confidence interval) for cardiovascular disease mortality and cardiovascular disease incidence by lead epigenetic biomarkers after adjustment for urinary cadmium (N = 2,321)

CVD Mortality

Epigenetic biomarker	Tertile 1	Tertile 2	Tertile 3	Per double increase
eTibia lead	< 3.6 µg/g	3.6 - 3.9 µg/g	> 3.9 µg/g	
	1.00 (Reference)	1.18 (0.94 - 1.49)	1.19 (0.94 - 1.52)	**1.42 (1.08- 1.88)**
ePatella lead	< 4.3µg/g	4.3 - 4.5 µg/g	> 4.5 µg/g	
	1.00 (Reference)	0.98 (0.77 - 1.23)	1.12 (0.88 - 1.43)	1.22 (0.93 - 1.59)
eBlood lead	< 1.4 µg/dL	1.4 - 1.7 µg/dL	> 1.7 µg/dL	
	1.00 (Reference)	1.17 (0.93 - 1.48)	**1.28 (1.00 – 1.64)**	**1.56 (1.16 - 2.11)**

CVD Incidence

Epigenetic biomarker	Tertile 1	Tertile 2	Tertile 3	Per double increase
eTibia lead	< 3.6 µg/g	3.6 - 3.9 µg/g	> 3.9 µg/g	
	1.00 (Reference)	0.96 (0.83 - 1.12)	1.03 (0.88 - 1.21)	0.99 (0.83 - 1.19)
ePatella lead	< 4.3µg/g	4.3 - 4.5 µg/g	> 4.5 µg/g	
	1.00 (Reference)	0.89 (0.77 - 1.05)	1.03 (0.88 - 1.21)	1.07 (0.89 - 1.28)
eBlood lead	< 1.4 µg/dL	1.4 - 1.7 µg/dL	> 1.7 µg/dL	
	1.00 (Reference)	1.07 (0.92 - 1.25)	1.00 (0.85 – 1.18)	1.06 (0.87 – 1.30)

All models adjusted for sex, smoking status (never, former, current), BMI (kg/m2), genetic PC's, immune cell types (CD8+, CD4+, NK, B cells, monocytes), LDL-cholesterol (mg/dL) HDL-cholesterol (mg/dL), diabetes status (yes/no), blood pressure (mmHg),

hypertension treatment (yes/no), and estimated glomerular filtration rate (ml/min/1.73m2) and urinary cadmium (expressed in micrograms per gram of urine creatinine). All models included center of recruitment as a strata term, and age was accounted for in the follow-up times of all models. Tertiles were calculated on log2-transformed lead epigenetic biomarker concentrations.

Figure S1. Principal Component Regression Analysis including blood lead exposure: univariate association *P*-values between covariates of interest and the top 25 principal components that explain 56% of the variance for the whole blood DNA methylation data.
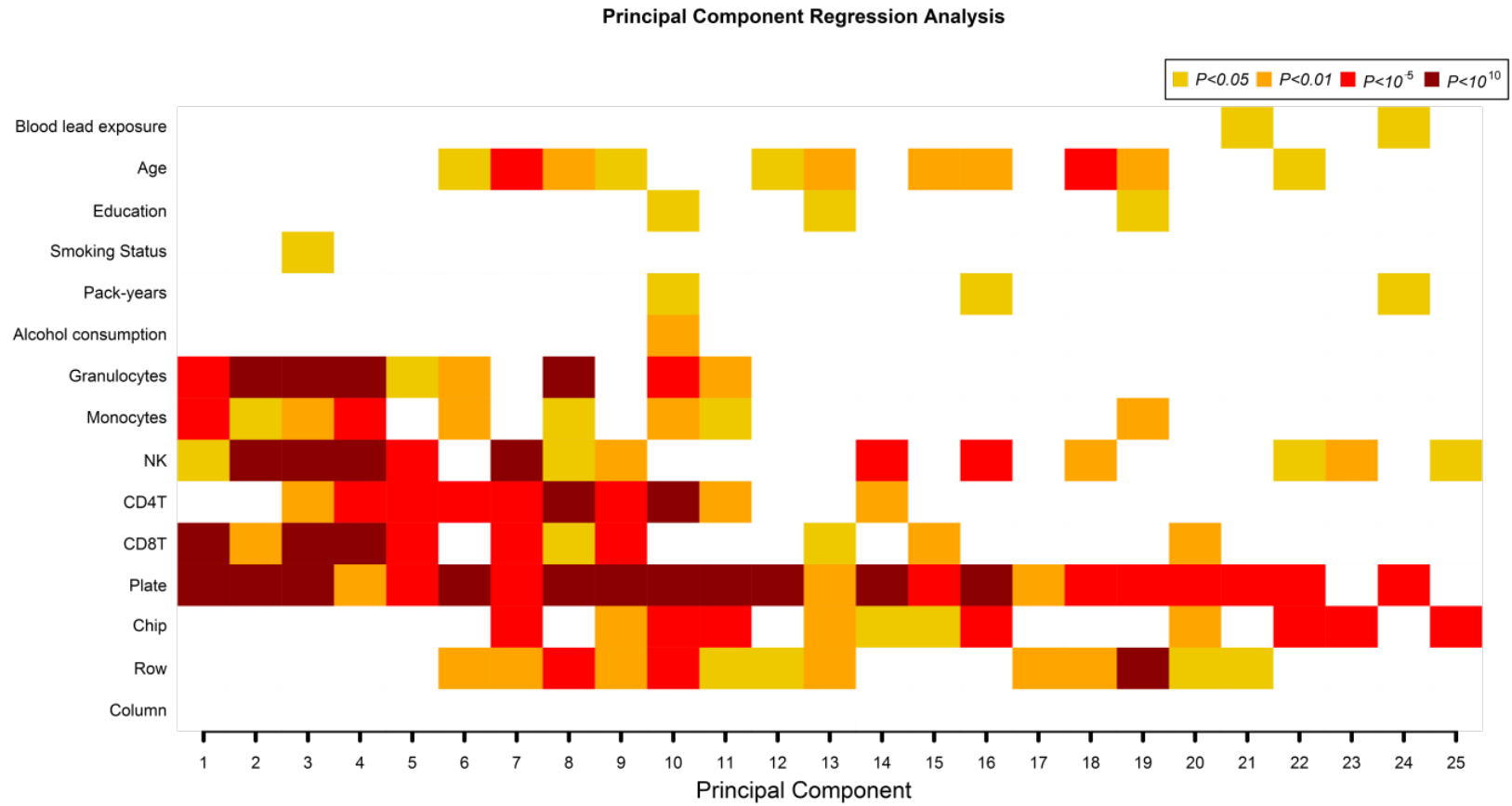
Figure S2. Quantile-Quantile plot and genomic inflation factor (lambda) for the Epigenome-Wide Association Analysis.
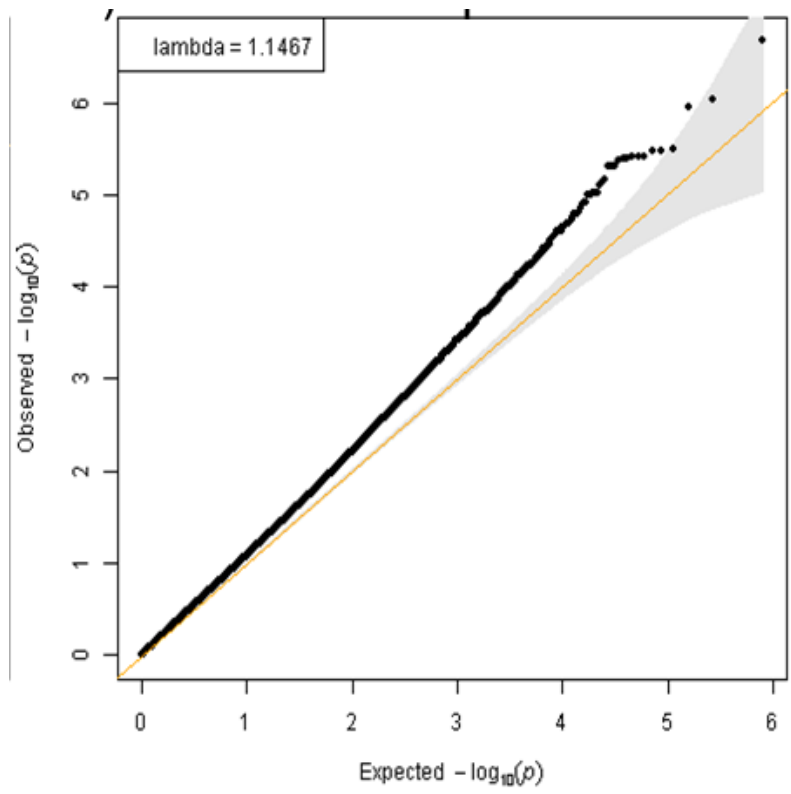
Figure S3. Alpha parameter selection (left) and Lambda parameter selection (right) of the elastic-net algorithm for methylation lead biomarkers in blood.
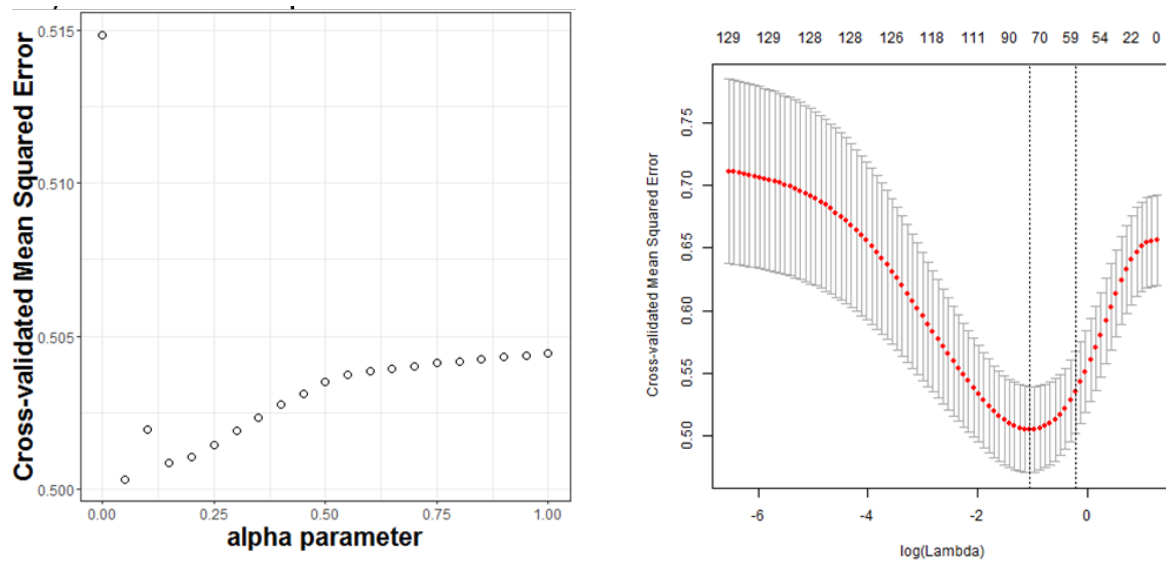
Figure S4. Relationship between actual and predicted (log$_2$-transformed) lead levels in whole blood (left), *Receiver Operating Characteristic, and* **Area Under the Curve (AUC)** *with* **95% Confidence Interval (95% CI)** *(middle), and* **box-plots and P-value for statistical difference between the means of actual and predicted (log$_2$-transformed) lead levels in blood (right).**
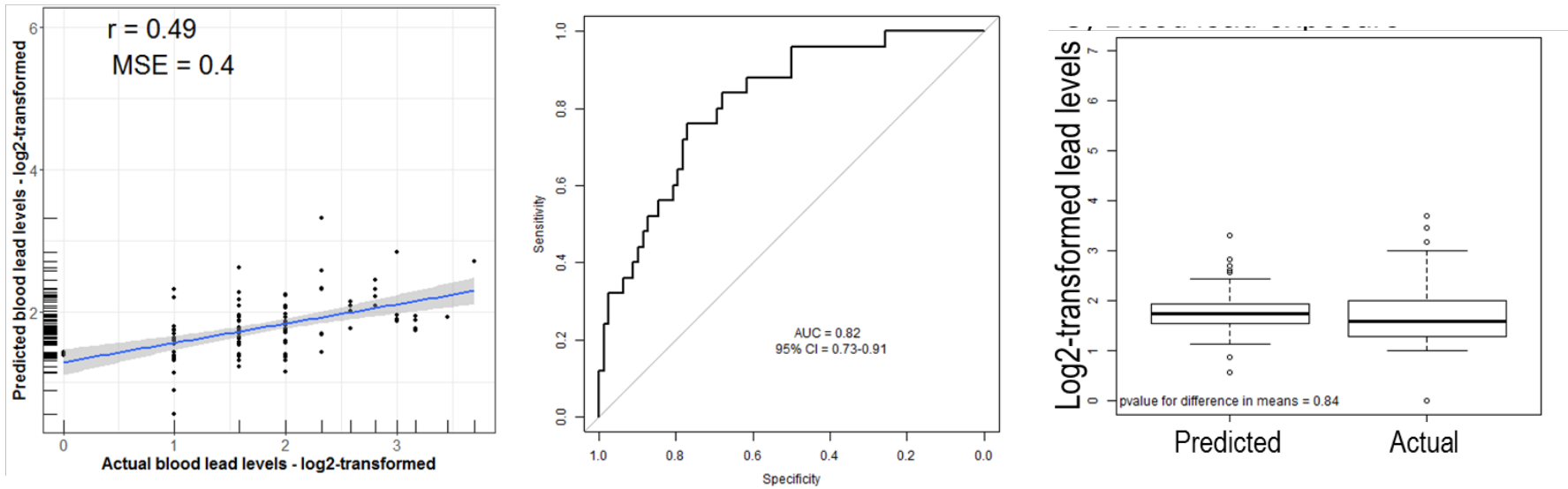
Figure S5. Empirical distribution function and Kolmogorov-Smirnov test of the actual and predicted lead levels in whole blood.
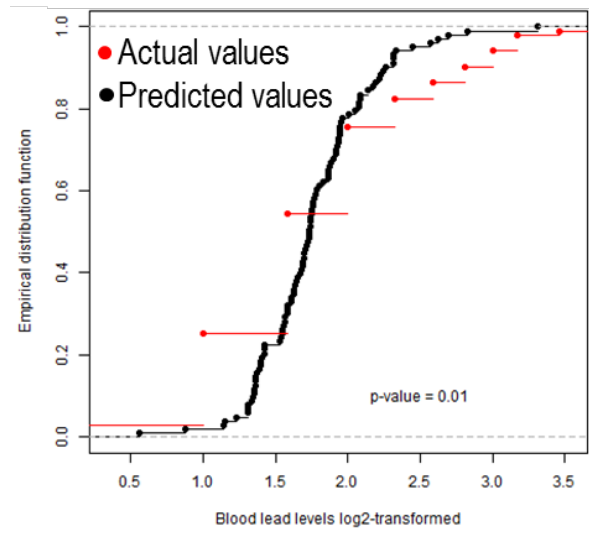
Figure S6. Distribution of tibia, patella, and blood epigenetic lead biomarkers in their native scale in the Strong Heart Study (n=2,231).
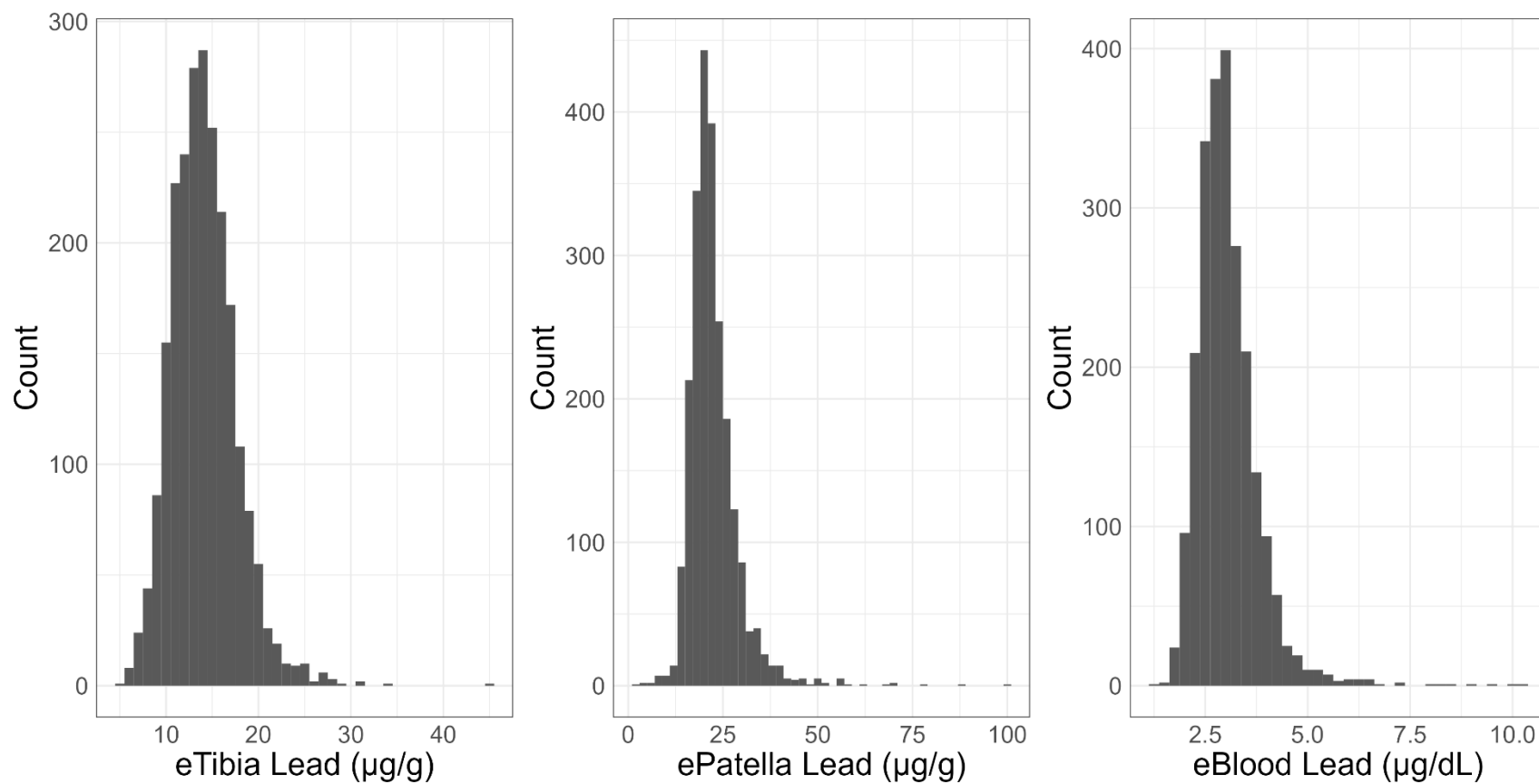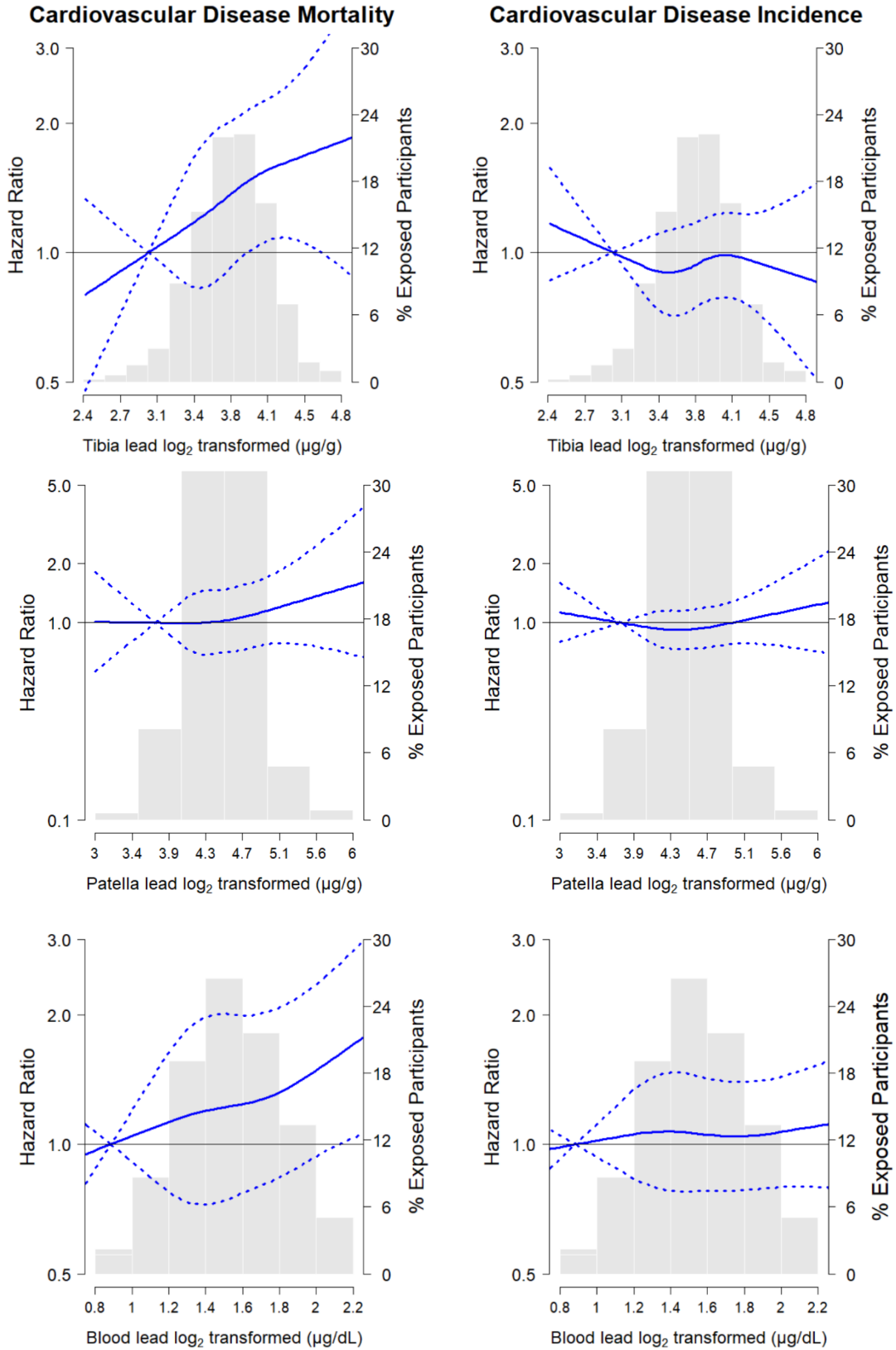
Figure S7. Associations between lead epigenetic biomarkers (eTibia, ePatella, eBlood) and cardiovascular disease mortality and incidence modeling lead epigenetic biomarkers using restricted quadratic splines (N = 2,321).

Hazard ratios incorporated restricted quadratic splines for epigenetic lead biomarkers with knots at the 10th, 50th, and 90th percentiles, where the 10th percentile was treated as the reference. Solid lines represent adjusted hazard ratios, and dotted lines represent 95% confidence intervals. All models were further adjusted for sex, smoking status (never, former, current), BMI (kg/m^2), genetic PCs, immune cell types (CD8+, CD4+, NK, B cells, Monocytes), LDL (mg/dL), HDL (mg/dL), diabetes status (yes/no), systolic blood pressure (mmHg), hypertension treatment (yes/no), and eGFR (ml/min/1.73m^2). All models included center of recruitment as a strata term, and age was accounted for in the follow-up times of all models.