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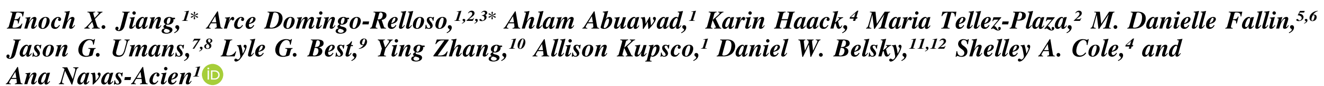
Arsenic Exposure and Epigenetic Aging: The Association with Cardiovascular Disease and All-Cause Mortality in the Strong Heart Study



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Background: Inorganic arsenic (As) may increase the risk of cardiovascular disease (CVD) and all-cause mortality through accelerated aging, which can be estimated using epigenetic-based measures.

Objectives: We evaluated three DNA methylation-based aging measures (PhenoAge, GrimAge, DunedinPACE) (epigenetic aging measures) as potential mediators of the previously reported association of As exposure with CVD incidence, CVD mortality, and all-cause mortality in the Strong Heart Study (SHS), an epidemiological cohort of American Indian adults.

Methods: Blood DNA methylation and urinary As levels were measured in 2,323 SHS participants (41.5% men, mean age of 55 years old). PhenoAge and GrimAge values were calculated using a residual-based method. We tested the association of urinary As with epigenetic aging measures using linear regression, the association of epigenetic aging measures with the three health outcomes using additive hazards models, and the mediation of As-related CVD incidence, CVD mortality, and all-cause mortality by epigenetic aging measures using the product of coefficients method.

Results: SHS participants with higher vs. lower urinary As levels had similar PhenoAge age, older GrimAge age, and faster DunedinPACE. An interquartile range increase in urinary As was associated with higher of PhenoAge age acceleration [mean difference (95% confidence interval) = 0.48 (0.17, 0.80) years], GrimAge age acceleration [0.80 (0.60, 1.00) years], and DunedinPACE [0.011 (0.005, 0.018)], after adjusting for age, sex, center location, genetic components, smoking status, and body mass index. Of the 347 incident CVD events per 100,000 person-years associated with a doubling in As exposure, 21.3% (9.1, 57.1) and 22.6% (9.5, 56.9), were attributable to differences in GrimAge and DunedinPACE, respectively.

Discussion: Arsenic exposure was associated with older GrimAge and faster DunedinPACE measures of biological age. Furthermore, accelerated biological aging measured from DNA methylation accounted for a relevant fraction of As-associated risk for CVD, CVD mortality, and all-cause mortality in the SHS, supporting the role of As in accelerated aging. Research of the biological underpinnings can contribute to a better understanding of the role of aging in arsenic-related disease. https://doi.org/10.1289/EHP11981

Introduction

Exposure to inorganic arsenic (As), a toxic and carcinogenic metalloid, is a pervasive global health problem. Arsenic exposure has been associated with a higher risk of numerous adverse health outcomes including cardiovascular disease (CVD)[1–3] and other age-related conditions such as diabetes and neurodegenerative disease.[4,5] Arsenic may contribute to multisystem decline by accelerating physiological aging processes.[4]

Physiological aging can be evaluated through modifications in the epigenome,[6,7] most commonly that of DNA methylation [the addition of a methyl group to a cytosine that precedes a guanine (CpG site)].[8] Arsenic exposure has been associated with differentially methylated CpGs in blood within various global populations.[9–13] Differential methylation of CpG sites has also been implicated in various forms of CVD[14–17] and underlying pathophysiological factors.[18–20] Whether As-related biological aging is related to CVD development is unknown.

Leading methods to quantify biological aging consist of a set of algorithms that are applied to blood DNA methylation, known as "epigenetic clocks." These algorithms output "epigenetic age" values that are correlated with chronological age and with age-related morbidity and mortality.[21] Initial epigenetic clocks (e.g., the Hannum and Horvath clocks), were developed to predict how long a person had lived until the time of sampling, i.e., their chronological age. These clocks, however, show weak and inconsistent associations with age-related morbidities.[22–24] A "second-generation" of epigenetic clocks (e.g., the PhenoAge[25] and GrimAge[26] clocks) were developed to predict the duration of survival from the time of sampling, i.e., time to death. PhenoAge and GrimAge included blood analytes in intermediate stages of

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algorithm development. These clocks more accurately predict age-related physical and cognitive decline and age-related morbidities.[24,27] In complement to these clocks, Belsky et al. introduced measures to quantify the pace of aging, defined as the rate of deterioration in physiological system integrity and measured from longitudinal analysis of change in a panel of blood analytes and organ function tests in the Dunedin Study birth cohort.[28,29] The current DNA methylation-based pace of aging measure is DunedinPACE (pace of aging computed from the epigenome).

The PhenoAge, GrimAge, and DunedinPACE aging measures are associated with CVD incidence and mortality as well as all-cause mortality in multiple cohorts, with some inconsistencies.[23,25,26,30–32] Few studies have evaluated the relation of As with biological aging, although a positive association has been suggested,[33] and, to the best of our knowledge, no studies have examined the intermediate role of DNA methylation-based aging measures in As-related diseases including CVD and all-cause mortality. We analyzed blood DNA methylation, urinary As, and CVD incidence and mortality data from the Strong Heart Study (SHS) to test the hypothesis that As exposure accelerates biological aging and that this accelerated aging mediates As-induced CVD incidence and CVD mortality, as well as all-cause mortality. The SHS is the largest study of CVD in American Indian populations, who were historically exposed to low-to-moderate levels of As in drinking water until the late 2000s.[34] Previous studies in the SHS have identified urinary As levels as a good measure of long-term As exposure in drinking water and As exposure as a risk factor for CVD incidence and all-cause mortality.[2,35–39] Arsenic exposure is also associated with changes in locus-specific DNA methylation patterns,[2,13] which in turn have been related to incident coronary heart disease (CHD).[40] There is thus an empirical basis for investigating epigenetic aging as a mediator of the As-CVD association within the SHS.

Methods

Study Population

The SHS recruited 4,549 men and women 45–75 years of age who were members of 13 tribes based in Arizona, Oklahoma, North Dakota, and South Dakota to participate in the baseline visit (1989–1991). In 2016, a Tribal Nation from Arizona declined further participation, leaving 3,517 participants. DNA methylation was analyzed in blood samples collected from 2,351 participants at the baseline visit (1989–1991) who did not have CVD, had community agreement, and had sufficient remaining blood samples for epigenetic analyses.[41] We excluded 26 participants given previously published preprocessing exclusion criteria[41] and two participants who did not have complete data on relevant variables, leaving 2,323 participants for this study who had complete data.[40] We restricted follow-up data through the end of 2009 for analysis to account for the change in groundwater As exposure that resulted from the enactment of the US EPA's Final Arsenic Rule in 2006.[39,42,43]

Urinary Arsenic Measurements

Arsenic concentrations were measured in urine samples (collected at the baseline period of 1989–1991 along with the blood samples for DNA extraction) that had a sufficient volume for measurement.[44] Baseline spot urine samples were stored in polypropylene tubes, and frozen samples were shipped on dry ice to the MedStar Health Research Institute (Washington DC, USA), where they were stored at $< -70°C$. In the period of 2009–2010, samples were thawed, and an aliquot of up to 1.0 mL was transported on dry ice to the Trace Elements Laboratory, Graz University (Austria) for analysis of As species [inorganic As (iAs), monomethyl-As (MMA), and dimethyl-As (DMA) species] using high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) (Agilent HPLC and Agilent 7700× ICP-MS; Agilent Technologies).[44] Interassay coefficients of variation were 6.0%, 6.5%, and 5.9% for iAs, MMA, and DMA, respectively. The limit of detection (LOD) for iAs [arsenite $(As^{III}) +$ arsenate (As^{V})], MMA, and DMA was $0.1\ \mu g/L$.[44] Samples with As species concentrations beneath the LOD were replaced with $LOD/\sqrt{2}$ (iAs: $n = 128$, 5.5%; MMA: $n = 17$, 0.7%; DMA: $n = 0$). Concentrations of arsenobetaine, a nontoxic As species found in seafood,[45] were low (median $= 0.65\ \mu g/g$ creatinine), reflecting little seafood intake in the study population. Sum of inorganic and methylated arsenic was calculated as the sum of the concentrations of iAs, MMA, and DMA and is referred to herein as urinary As. Urinary As concentrations $(\mu g/L)$ were divided by urinary creatinine (g/L) to account for urine dilution.

Cardiovascular Disease Follow-up

The endpoints of this study are CVD mortality, CVD incidence, and all-cause mortality.[2] Outcomes in the follow-up period were assessed by annual contact, by annual mortality and morbidity surveillance of hospitalization and death records through 2009, and at two research clinic visits conducted in 1993–1995 and 1998–1999. Follow-up through 2009 was more than 99% complete for mortality and nonfatal events. When a possible cardiovascular event was identified, medical records were reviewed by a physician committee experienced in the adjudication of CVD events for population-based research. We defined incident cardiovascular disease as the first occurrence of coronary heart disease or stroke, definite nonfatal congestive heart failure, or other fatal cardiovascular disease. We defined incident coronary heart disease as the first occurrence of definite nonfatal coronary heart disease or definite and possible fatal coronary heart disease. Incident stroke was defined as the first occurrence of a definite nonfatal stroke or a definite or possible fatal stroke. Detailed definitions of the criteria used by the review committees have been described in detail previously.[2,34] Follow-up time is defined as the period from the initial drawing of blood used for DNA methylation analysis to the time of a CVD event, CVD death, or death due to any reason. Follow-up was censored at the time of any of these events, when a participant was lost to follow-up, or on 31 December 2009.

Microarray DNA Methylation Measurements and DNA Methylation-Based Aging Measures

Details of microarray DNA methylation measurements in the SHS have been previously described.[41] Briefly, the Illumina Infinium MethylationEPIC BeadChip microarray (850K) was used to measure DNA methylation using bisulfite-converted DNA from white blood cells of 2,351 participants at baseline (same time as urine collection). Exclusion criteria included low detection *p*-values, cross-hybridizing probes, probes located in sex chromosomes, and single nucleotide polymorphisms (SNPs) with minor allele frequency >0.05. Single sample noob normalization and regression on correlated probes normalization were conducted following Illumina's recommendations for preprocessing.[46] Blood cell proportions (CD8T, CD4T, NK cells, B cells, monocytes, and neutrophils) were estimated using the R package FlowSorted.Blood.EPIC, which uses the Houseman projection method.[47]

We computed epigenetic age values from DNA methylation data of the SHS cohort using the epigenetic clock algorithms created by Levine et al. (PhenoAge),[25] Lu et al. (GrimAge)[26] (primary

analysis), and Hannum et al.[22] and Horvath[23] (secondary analysis). GrimAge was calculated using the Horvath Lab's webtool at https://dnamage.genetics.ucla.edu/new. So-called epigenetic "age acceleration" values, with a unit in years, were computed for each measure by extracting the residuals from the regression of each participant's epigenetic age against their chronological age. We computed pace of aging values using the algorithm for DunedinPACE developed by Belsky et al.[28] Pace of aging values, which have a unit of years of biological aging per chronological year (i.e., no units as years cancel out), inherently represent age acceleration as a ratio vs. chronological age; thus, their calculation differs from the other epigenetic aging measures.

Other Relevant Variables

Trained and certified interviewers administered standardized questionnaires [age, sex, center, smoking status (current, former, never), medication use, education]; centrally trained nurses and medical assistants measured height, weight, and systolic and diastolic blood pressures and collected blood and urine samples according to standardized protocols. Methods for measuring blood pressure, lipids [including total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides], fasting glucose, oral glucose tolerance, hemoglobin A1c, and plasma creatinine have been previously described.[2] LDL cholesterol levels were calculated using the Friedewald equation for participants with triglyceride levels <400 mg/dL, with missing values replaced by measured LDL levels.[2] Diabetes was defined as a fasting glucose level ≥ 126 mg/dL, 2-h postload plasma glucose level of ≥ 200 mg/dL, hemoglobin A1c $\geq 6.5\%$, or self-reported use of diabetes-related medications (insulin or an oral hypoglycemic agent). We used the Chronic Kidney Disease Epidemiology Collaboration formula to estimate glomerular filtration rate (eGFR), using plasma creatinine, age, and sex.[48] Urinary creatinine levels were measured by an automated alkaline picrate method.

Statistical Methods

All analyses were conducted using R software version 4.1.0 (R Development Core Team). The findings for PhenoAge and GrimAge age acceleration and DunedinPACE are presented within tables in the main manuscript, while the findings for Hannum and Horvath age acceleration are shown in supplementary tables. Arsenic has been previously associated with increased risk of CVD incidence, CVD mortality, and all-cause mortality in the SHS.[2,38] We thus focused on the association of As with epigenetic aging measures, the association of epigenetic measures of aging with the three study outcomes, and the potential mediation of As-related outcomes by epigenetic aging measures.

Linear regression for the association of arsenic with aging measures. We used linear regression models to assess the association between As exposure and each epigenetic measure of aging. Urinary As was adjusted for urinary creatinine levels and $\log_2$-transformed. Epigenetic age acceleration values for PhenoAge and GrimAge (and Hannum and Horvath) clocks were used in linear regression models. DunedinPACE values were directly used in linear regression models.

Models were progressively adjusted as follows. Model 1 was adjusted for several baseline covariates: chronological age (continuous), sex (categorical—male or female), center (categorical—Arizona, Oklahoma, or North Dakota and South Dakota), five genetic principal components[49] (PCs) (continuous) to account for potential ancestry-related genetic artifacts in the DNA methylation data[50] (see Domingo-Relloso et al.[49] for further detail regarding calculation of genetic PCs in the SHS), and eGFR (continuous) to account for the impact of kidney function in urinary As excretion.[51] Model 2 was further adjusted for body mass index (BMI) (continuous) and smoking status (categorical—never, former, or current). Model 3 was additionally adjusted for estimated Houseman cell proportions (CD8T, CD4T, NK, B cells, and monocytes) (continuous), and it was considered a sensitivity analysis.

Effect estimates were reported as mean epigenetic age acceleration (in years) or the increase in pace of aging, comparing each of the three highest quartiles of urinary As to the lowest quartile, as well as comparing an interquartile range increase of urinary As (in separate models).

Survival analysis for the association of DNA methylation-based aging measures with health outcomes. We generated Cox proportional-hazards models comparing the interquartile range of epigenetic age acceleration or pace of aging values for three outcomes: CVD incidence, CVD mortality, and all-cause mortality. We used the R package survival.[52] Models were progressively adjusted as follows. Model 1 was adjusted for chronological age (continuous), sex (categorical—male or female), center (categorical—Arizona, Oklahoma, or North Dakota and South Dakota), and the aforementioned five genetic PCs (continuous). Model 2 was further adjusted for BMI (continuous) and smoking status (categorical—never, former, or current). Model 3 was further adjusted for eGFR (continuous), LDL cholesterol (continuous), HDL cholesterol (continuous), systolic blood pressure (continuous), hypertension treatment (categorical—yes or no medication use), and diabetes status (categorical—yes or no diagnosis) to control for classical determinants of CVD. Model 4 was additionally adjusted for estimated Houseman cell proportions and was considered a sensitivity analysis. We conducted a competing risks analysis considering non-CVD mortality as a potential competing risk. We used the Fine-Gray model as implemented by the R package survival.[53] Fine-Gray models were adjusted for the full set of confounders of Model 4.

Mediation analysis. To evaluate whether DNA methylation-based aging measures have a statistically mediating role in As-related CVD incidence, CVD mortality, or all-cause mortality, we conducted a mediation analysis using the product of coefficients method with an approach that is suitable for time-to-event models as adapted by Lange and Hansen.[54] We used additive hazards models for mediation analysis as these models quantify the effects on an additive rate scale, which is collapsible, as opposed to the hazard ratio, which is non-collapsible.[54–56] However, we also present the total effects in a multiplicative scale using Cox proportional hazards models in the supplementary material for comparative purposes with other studies. Our outcome model was an additive hazards model with CVD incidence, CVD mortality, or all-cause mortality as the outcome, $\log_2$-transformed urinary As at baseline as the exposure, and epigenetic age acceleration or pace of aging as the mediator, additionally adjusted for two different sets of confounders as follows:

Model 1 is adjusted for chronological age, sex, center, genetic PCs, BMI, and smoking status. These covariates were selected based on knowledge from previous studies of As, DNA methylation, and CVD in the SHS. We did not adjust for education as our previous study showed a similar association between arsenic and incident CVD before and after adjustment for education.

Model 2 is additionally adjusted for eGFR, LDL cholesterol, HDL cholesterol, systolic blood pressure, hypertension treatment, and diabetes status. These variables were not included in the main model, as previous studies have suggested that they might be mediators in the association between As and CVD, and adjusting the models for mediators might lead to an underestimation of the effect estimates. However, prior research has demonstrated that As affects CVD risk factors,[57] including these covariates in our models would allow us to isolate the independent mediating

effect of biological aging on the As-CVD pathway beyond those established risk factors. We thus provide the mediation models with and without CVD risk factors for transparency and comparison to the same model without those adjustments.

Model 3 is a sensitivity analysis which is adjusted for Houseman cell proportions.

Thus, our outcome model was as follows:

$$\text{CVD} \sim \beta_1 \log \text{As} + \beta_2 \text{DNAm}_{\text{age}} + \sum \beta_i \text{confounder}_i.$$

Our mediator model was a linear model with epigenetic age acceleration or pace of aging as the outcome and As as the exposure, additionally adjusted for the same sets of confounders, as follows:

$$\text{DNAm}_{\text{age}} \sim \alpha_1 \log \text{As} + \sum \alpha_i \text{confounder}_i.$$

The product of coefficients method calculated the effects of interest in the following ways:

- Indirect effect $= \alpha_1 \times \beta_2$
- Direct effect $= \beta_1$
- Total effect = indirect effect + direct effect
- Relative indirect effect $= \frac{\text{indirect effect}}{\text{total effect}} \times 100$

Lange and Hansen[54] adapted the definition of those effects to a survival context. We calculated confidence intervals (CIs) for each effect of interest by resampling random values from a multivariate normal distribution of the estimated effects.

We reported total effects, natural indirect effects (IEs), and natural direct effects (DEs). The total effect refers to the number of events of incident CVD, fatal CVD, or death per 100,000 person-years attributable to a doubling in urinary As concentration. The natural DE refers to the effect of a doubling of urinary As concentration on the outcome when DNA methylation (DNAm) aging is fixed to the reference value of 0. The natural IE refers to the expected change in the outcome when As is fixed to the reference value of 0, and DNAm changes from the value it would take when As is fixed to the reference value of 0 to the value it would take under a doubling of urinary As concentration. To account for the withdrawal of one of the Tribal Nations from the study (see the "Study Population" section[41]), the primary mediation analysis computed an inverse probability weighting of participants to reduce selection bias.[58] We divided our data into

Table 1. Baseline characteristics by urinary arsenic concentration (μg/g creatinine) (1989–1991) and outcomes through 2009 among participants in the Strong Heart Study. Epigenetic age acceleration values (reported in years) were computed as the residuals of regressing epigenetic age values (calculated according to their respective algorithms) against participants' corresponding chronological age. Epigenetic pace of aging values (reported in years of biological aging per chronological year) were computed according to their respective algorithms as described in the main text.

	Quartiles of total urinary arsenic (μg/g creatinine)				
Parameter	<5.24 (3.82) ($n=582$)	5.24 – 8.56 (6.74) ($n=580$)	8.57 – 14.42 (11.05) ($n=580$)	>14.42 (20.66) ($n=581$)	Total ($n=2,323$)
Age [years (mean ± SD)]	56.2 (8.13)	56.4 (8.32)	55.8 (7.92)	56.2 (8.01)	56.2 (8.10)
Sex [n (%)]					
Male	293 (50.3%)	234 (40.3%)	229 (39.5%)	207 (35.6%)	963 (41.5%)
Female	289 (49.7%)	346 (59.7%)	351 (60.5%)	374 (64.4%)	1,360 (58.5%)
Study center [n (%)]					
Arizona	7 (1.2%)	35 (6.0%)	84 (14.5%)	186 (32.0%)	312 (13.4%)
Oklahoma	446 (76.6%)	312 (53.8%)	162 (27.9%)	61 (10.5%)	981 (42.2%)
North Dakota/South Dakota	129 (22.2%)	233 (40.2%)	334 (57.6%)	334 (57.5%)	1,030 (44.3%)
BMI [kg/m^2 (mean ± SD)]	30.5 (5.75)	30.5 (5.99)	30.2 (5.94)	30.0 (6.66)	30.3 (6.09)
Smoking status [n (%)]					
Never	168 (28.9%)	172 (29.7%)	165 (28.4%)	179 (30.8%)	684 (29.4%)
Ever	206 (35.4%)	192 (33.1%)	185 (31.9%)	164 (28.2%)	747 (32.2%)
Current	208 (35.7%)	216 (37.2%)	230 (39.7%)	238 (41.0%)	892 (38.4%)
LDL cholesterol [mg/dL (mean ± SD)]	123 (31.1)	122 (32.6)	120 (34.1)	116 (34.4)	120 (33.2)
HDL cholesterol [mg/dL (mean ± SD)]	44.2 (12.9)	45.1 (12.2)	46.7 (14.1)	49.4 (15.7)	46.4 (13.9)
Systolic blood pressure (mean ± SD)	126 (16.4)	127 (19.6)	125 (18.6)	126 (20.2)	126 (18.8)
Hypertension treatment [n (%)]					
No	446 (76.6%)	465 (80.2%)	468 (80.7%)	480 (82.6%)	1,859 (80.0%)
Yes	136 (23.4%)	115 (19.8%)	112 (19.3%)	101 (17.4%)	464 (20.0%)
Diabetes diagnosis [n (%)]					
No	377 (64.8%)	350 (60.3%)	348 (60.0%)	281 (48.4%)	1,356 (58.4%)
Yes	205 (35.2%)	230 (39.7%)	232 (40.0%)	300 (51.6%)	967 (41.6%)
Epigenetic age acceleration [years (mean ± SD)]					
PhenoAge	−0.76 (6.82)	−0.12 (6.78)	−0.26 (6.27)	1.12 (6.97)	0.00 (6.75)
GrimAge	−0.19 (4.57)	−0.19 (4.48)	0.00 (4.63)	0.38 (4.56)	0.00 (4.56)
Hannum	−0.33 (5.02)	−0.40 (5.19)	0.13 (4.63)	0.61 (5.33)	0.00 (5.06)
Horvath	−0.03 (5.09)	−0.40 (5.26)	0.13 (4.93)	0.29 (5.35)	0.00 (5.16)
Pace of aging estimate [years of biological aging/chronological years (mean ± SD)]					
DunedinPACE	1.11 (0.130)	1.12 (0.134)	1.12 (0.135)	1.14 (0.129)	1.12 (0.133)
Incident CVD [n (%)]					
No	339 (58.2%)	305 (52.6%)	325 (56.0%)	331 (57.0%)	1,300 (56.0%)
Yes	243 (41.8%)	275 (47.4%)	255 (44.0%)	250 (43.0%)	1,023 (44.0%)
CVD mortality [n (%)]					
No	520 (89.3%)	498 (85.9%)	493 (85.0%)	496 (85.4%)	2,007 (86.4%)
Yes	62 (10.7%)	82 (14.1%)	87 (15.0%)	85 (14.6%)	316 (13.6%)
All-cause mortality [n (%)]					
No	368 (63.2%)	328 (56.6%)	316 (54.5%)	256 (44.1%)	1,268 (54.6%)
Yes	214 (36.8%)	252 (43.4%)	264 (45.5%)	325 (55.9%)	1,055 (45.4%)

Note: No missing values. BMI, body mass index; CVD, cardiovascular disease; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; SD, standard deviation.

six different strata (one per sex and study center) and stored the quantity of individuals in each of the strata in the original population in the finite population correction (fpc) parameter of the svydesign function from the R package survey,[59] which is used to account for complex study designs. Then, the weights were calculated as 1 divided by the probability of each individual to belong to each stratum. Those weights were subsequently added to the regression models.

Three assumptions need to hold for causal effects to be validly estimated in observational studies.[60] First, the positivity assumption refers to each exposure level happening in all strata of different confounders. As all different participant subgroups for the variables used in the adjustment analysis are exposed to different levels of arsenic (Table 1), we assume positivity holds. Conversely, consistency refers to the exposure being uniquely defined, which holds as the exposure is defined as doubling of As exposure. Last, the exchangeability assumption refers to no unmeasured confounding. In particular, for mediation analysis, four assumptions related to confounding need to hold for direct and indirect effects to be interpreted as causal.[61] First, there is no unmeasured confounding on the exposure-outcome relationship. Second, there is no unmeasured confounding on the mediator-outcome relationship. Third, there is no unmeasured confounding on the exposure-mediator relationship. Finally, there should be no confounder that affects the mediator-outcome relationship and is itself affected by the exposure. Potential violations of these assumptions are discussed in the "Discussion" section. We evaluated potential exposure-mediator interactions for the model containing the full set of confounders (Model 4) by adding an interaction term to the outcome model for each outcome and each DNA methylation clock. The code to reproduce our mediation analysis can be found in the Supplementary Material (mediation_analysis_code.R).

Results

Participant Characteristics

The median [interquartile range (IQR)] urinary As of the participants in this study was 8.56 (5.24, 14.4) $\mu g/g$ creatinine. Participant characteristics stratified by quartile of As exposure are displayed in Table 1, while participant characteristics by CVD incidence, CVD mortality, and all-cause mortality outcome are displayed in Table 2. From a total of 2,323 participants, 1,023 developed incident CVD, 316 died of CVD, and 1,055 died of any cause in the follow-up period until the end of 2009 (Table 1). Compared to participants who did not develop CVD, those with incident CVD were older and more likely to be male and current smokers (Table 2). On average, participants who developed CVD also had higher BMI, LDL cholesterol levels, and systolic blood pressure and a greater frequency of hypertension and diabetes (Table 2).

Compared to the mean $\pm$ standard deviation (SD) chronological age of 56.2 (8.10) years, the mean $\pm$ SD epigenetic age values (estimated from epigenetic acceleration corrected for the mean epigenetic age) were younger for the PhenoAge, GrimAge, and Hannum clocks [50.1 (6.8), 40.7 (4.6), and 50.6 (5.1) years, respectively], and older for Horvath [58.3 (5.2) years] (Figure S1), although all measures were strongly correlated with chronological age (Figure 1). The mean $\pm$ SD value of DunedinPACE (years of epigenetic aging per chronological year) was 1.12 (0.13), indicating faster biological aging than expected, but this measure had a weak positive correlation with chronological age ($r = 0.08$; $p < 0.01$) (Figure 1). The associations of the epigenetic aging measures with other participant characteristics are shown in Figure S2.

Arsenic and DNA Methylation-Based Aging Measures

Participants with urinary As levels in the highest quartile had older PhenoAge and GrimAge age and faster DunedinPACE compared to those in the lowest quartile (Figure 2). In linear regression models adjusted for chronological age, sex, center, genetic PCs, and eGFR, an IQR increase in urinary As was associated with higher levels of PhenoAge age acceleration [mean difference (95% CI) = 0.48 (0.17, 0.80); $p = 0.04$], GrimAge age acceleration [0.80 (0.60, 1.00); $p < 0.001$], and DunedinPACE [mean difference (95% CI) = 0.011 (0.005, 0.018); $p = 0.01$] (Table 3, Model 1). After further adjustment for BMI and smoking status, all associations between As and the three aging measures remained significant, although the effect size for GrimAge age acceleration was attenuated (Table 3, Model 2). A sensitivity analysis that adjusted models for Houseman cell proportions attenuated the effect sizes of As on all three biological aging measures, although the associations between As and GrimAge age acceleration [mean difference (95% CI) = 0.44 (0.28, 0.61); $p < 0.001$] and DunedinPACE [mean difference (95% CI) = 0.009 (0.004, 0.015); $p = 0.02$] remained significant (Table 3, Model 3). Hannum and Horvath age acceleration were not significantly associated with urinary As in any adjusted models (Table S1).

Mediation Analysis

In initial mediation models adjusting for chronological age, sex, center, genetic PCs, BMI, and smoking status, the IEs of PhenoAge, GrimAge, and DunedinPACE on As-related CVD incidence and CVD mortality were statistically significant (Table 4). Of the 347 incident CVD events per 100,000 person-years associated with a doubling in As exposure, 33.8 (95% CI: 3.7, 74.5) events [8.9% (1.0%, 26.3%) of the total effect of As] could be attributed to As-related changes in PhenoAge. The relative IEs (95% CI) of GrimAge and DunedinPACE were 21.3% (9.1%, 57.1%) and 22.6% (9.5%, 56.9%), respectively (Table 4). The relative IEs of PhenoAge, GrimAge, and DunedinPACE for As-related CVD mortality were 9.2% (0.7%, 31.5%), 28.3% (12.0%, 87.7%), and 20.0% (6.9%, 65.6%), respectively.

In mediation models additionally adjusted for CVD risk factors, the indirect effects (IEs) of PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE on As-related CVD incidence remained statistically significant, contributing relative IEs of 6.8% (0.3%, 22.4%), 17.2% (6.7%, 50.7%), and 15.0% (4.9%, 42.8%), respectively (Table 5). The IEs of GrimAge age acceleration and DunedinPACE for As-related CVD mortality also remained statistically significant, with each measure contributing a relative IE of 23.0% (8.9%, 80.5%) and 13.1% (3.2%, 48.9%), respectively. The total effects of As and corresponding IEs of PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE on all outcomes are depicted in Figure 3. The relative IEs of all three measures of aging for As-related all-cause mortality remained statistically significant. A sensitivity analysis that further adjusted the mediation models in Table 5 for Houseman cell proportions attenuated the indirect effect sizes for all DNA methylation-based aging measures, although the IEs of GrimAge age acceleration and DunedinPACE remained significant for all outcomes (Table S2).

The results of the exposure-mediator interaction analysis can be found in Table S3. No exposure-mediator interactions were present for GrimAge for any outcome. For DunedinPACE, the interaction term was statistically significant for all-cause mortality. However, the coefficient of DunedinPACE itself was not significant in that model. Thus, we did not consider this as an exposure-mediator interaction. For PhenoAge, exposure-mediator interaction was significant for the three outcomes. Thus, we also calculated

Table 2. Baseline characteristics by CVD incidence, CVD mortality, and all-cause mortality status among participants in the Strong Heart Study. Epigenetic age acceleration values (reported in years) were computed as the residuals of regressing epigenetic age values (calculated according to their respective algorithms) against participants' corresponding chronological age. Epigenetic pace of aging values (reported in years of biological aging per chronological year) were computed according to their respective algorithms as described in the main text.

Parameter	Incident CVD ($n=1{,}023$)	No CVD incidence ($n=1{,}300$)	CVD mortality ($n=316$)	No CVD mortality ($n=2{,}007$)	All-cause mortality ($n=1{,}055$)	No mortality ($n=1{,}268$)	Total ($n=2{,}323$)
Age [years (mean ± SD)]	57.5 (8.13)	55.1 (7.91)	59.5 (8.25)	55.7 (7.95)	58.8 (8.47)	54.0 (7.05)	56.2 (8.10)
Sex [*n* (%)]							
Male	445 (43.5%)	518 (39.8%)	141 (44.6%)	822 (41.0%)	492 (46.6%)	471 (37.1%)	963 (41.5%)
Female	578 (56.5%)	782 (60.2%)	175 (55.4%)	1,185 (59.0%)	563 (53.4%)	797 (62.9%)	1,360 (58.5%)
Study center [*n* (%)]							
Arizona	99 (9.7%)	213 (16.4%)	41 (13.0%)	271 (13.5%)	149 (14.1%)	163 (12.9%)	312 (13.4%)
Oklahoma	395 (38.6%)	586 (45.1%)	122 (38.6%)	859 (42.8%)	399 (37.8%)	582 (45.9%)	981 (42.2%)
North Dakota/South Dakota	529 (51.7%)	501 (38.5%)	153 (48.4%)	877 (43.7%)	507 (48.1%)	523 (41.2%)	1,030 (44.3%)
BMI [kg/m^2 (mean ± SD)]	31.0 (5.92)	29.7 (6.17)	31.2 (6.46)	30.2 (6.02)	30.1 (6.51)	30.5 (5.72)	30.3 (6.09)
Smoking status [*n* (%)]							
Never	282 (27.6%)	402 (30.9%)	101 (32.0%)	583 (29.0%)	291 (27.6%)	393 (31.0%)	684 (29.4%)
Ever	331 (32.4%)	416 (32.0%)	91 (28.8%)	656 (32.7%)	328 (31.1%)	419 (33.0%)	747 (32.2%)
Current	410 (40.1%)	482 (37.1%)	124 (39.2%)	768 (38.3%)	436 (41.3%)	456 (36.0%)	892 (38.4%)
LDL cholesterol [mg/dL (mean ± SD)]	124 (33.7)	117 (32.5)	125 (33.9)	120 (33.0)	116 (33.2)	124 (32.7)	120 (33.2)
HDL cholesterol [mg/dL (mean ± SD)]	44.6 (13.3)	47.8 (14.2)	44.0 (12.7)	46.7 (14.1)	46.5 (14.5)	46.2 (13.4)	46.4 (13.9)
Systolic blood pressure (mean ± SD)	129 (19.8)	123 (17.5)	134 (21.0)	125 (18.1)	129 (20.6)	123 (16.6)	126 (18.8)
Hypertension treatment [*n* (%)]							
No	746 (72.9%)	1,113 (85.6%)	220 (69.6%)	1,639 (81.7%)	807 (76.5%)	1,052 (83.0%)	1,859 (80.0%)
Yes	277 (27.1%)	187 (14.4%)	96 (30.4%)	368 (18.3%)	248 (23.5%)	216 (17.0%)	464 (20.0%)
Diabetes diagnosis [*n* (%)]							
No	485 (47.4%)	871 (67.0%)	112 (35.4%)	1,244 (62.0%)	492 (46.6%)	864 (68.1%)	1,356 (58.4%)
Yes	538 (52.6%)	429 (33.0%)	204 (64.6%)	763 (38.0%)	563 (53.4%)	404 (31.9%)	967 (41.6%)
Urinary As [μg/g creatinine (median Q1, Q3)]	8.39 (5.40, 14.2)	8.69 (5.13, 14.5)	9.26 (5.75, 15.0)	8.39 (5.15, 14.3)	9.87 (5.77, 15.9)	7.72 (4.86, 12.8)	8.56 (5.24, 14.4)
Epigenetic age acceleration [years (mean ± SD)]							
PhenoAge	−0.13 (6.56)	0.09 (6.89)	0.55 (6.70)	−0.09 (6.75)	1.36 (6.93)	−1.14 (6.38)	0.00 (6.75)
GrimAge	0.24 (4.40)	−0.19 (4.68)	0.99 (4.50)	−0.16 (4.55)	1.27 (4.58)	−1.06 (4.27)	0.00 (4.56)
Hannum	−0.13 (4.97)	0.10 (5.13)	−0.16 (4.94)	0.03 (5.08)	0.72 (5.24)	−0.60 (4.83)	0.00 (5.06)
Horvath	−0.03 (5.14)	0.02 (5.18)	−0.10 (5.50)	0.01 (5.11)	0.50 (5.34)	−0.42 (4.97)	0.00 (5.16)
Pace of aging estimate [years of biological aging/chronological years (mean ± SD)]							
DunedinPACE	1.14 (0.13)	1.11 (0.13)	1.16 (0.13)	1.12 (0.13)	1.16 (0.13)	1.09 (0.13)	1.12 (0.13)

Note: No missing values. BMI, Body Mass Index; CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

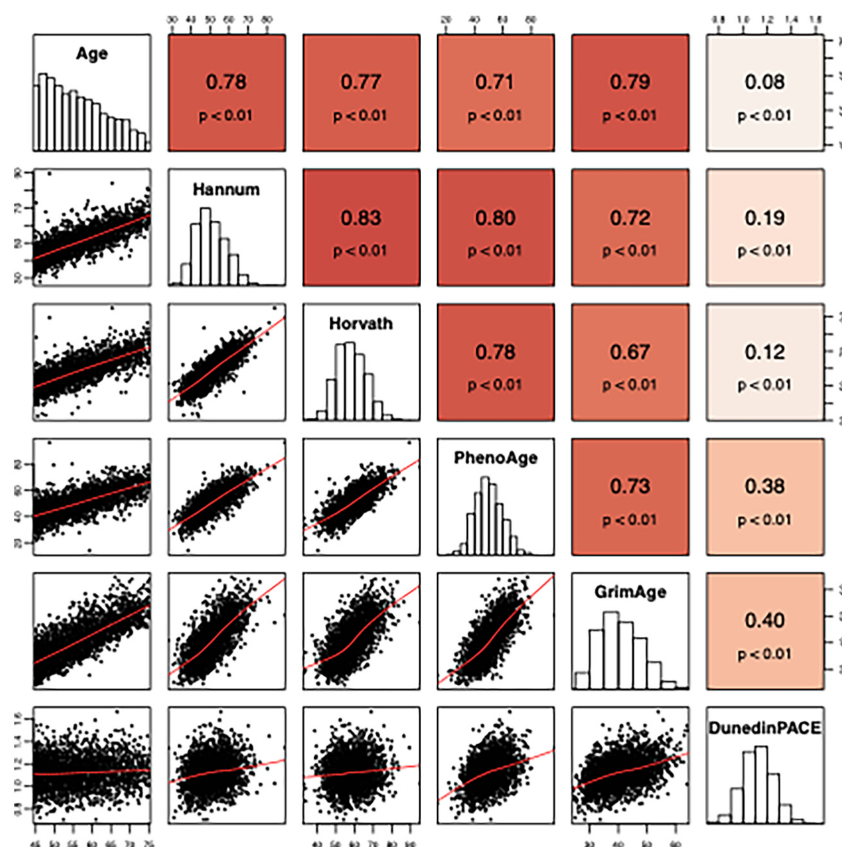

Figure 1. (Left) Scatter plots of participants' epigenetic age against their chronological age. (Middle) Distribution of participants' chronological and epigenetic ages. (Right) Simple correlations (Spearman's ρ) between the chronological/epigenetic ages for each participant ($n = 2{,}323$) in the Strong Heart Study. Corresponding data is in Excel Table S1.

natural direct and indirect effects using the formulas provided in Valeri and Vanderweele[62] adapted to the additive hazards model setting. The results are shown in Table S4. When considering an interaction term, the indirect effect was negative for both CVD incidence and CVD mortality.

Hannum and Horvath age acceleration did not contribute a significant IE to As-related CVD incidence, CVD mortality, or all-cause mortality in initial or adjusted mediation models (Table S5 and Table S6). A directed acyclic graph depicting the relationships between all variables used in our mediation analysis can be found in Figure S2.

DNA Methylation-Based Aging Measures and Health Outcomes in Multiplicative Models

Although our mediation analysis was focused on additive models, we ran multiplicative Cox proportional hazards models for comparative purposes with other studies. PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE were associated with increased risk of CVD incidence and CVD mortality in initial models adjusted for chronological age, sex, center, and genetic PCs (Table S7, Model 1). In models additionally adjusted for eGFR, BMI, smoking status, LDL cholesterol, HDL cholesterol, systolic blood pressure, hypertension treatment, and diabetes status, the hazard ratio (HR) (95% CI) for CVD incidence was similar for the interquartile range in GrimAge age acceleration and DunedinPACE, but the association between PhenoAge age acceleration and CVD incidence was not statistically significant. A sensitivity analysis that further adjusted models for Houseman cell proportions did not significantly change the HRs of the associations of GrimAge age acceleration or DunedinPACE with either CVD incidence or CVD mortality (Table S7, Model 4). Hannum and Horvath age acceleration were not associated with CVD incidence nor CVD mortality in any adjusted models (Table S8).

Results from the competing risks analysis can be found in Table S9. The subdistribution HRs obtained from the Fine-Gray model were slightly attenuated, but generally still significant, for both CVD incidence and CVD mortality. Thus, we considered competing risks not to be an issue in this model.

Discussion

In this population-based study of American Indian adults with a history of chronic exposure to As in drinking water, we found a positive cross-sectional association between urinary As exposure and increased epigenetic age acceleration or pace of aging through several different DNA methylation-based measures of aging, with the strongest associations for GrimAge age acceleration and DunedinPACE. Under the assumption that urinary As levels reflect long-term exposure and precede the changes in DNA methylation, this study showed that DNA methylation-based aging measures mediate the associations of As exposure with incident CVD, fatal CVD, and all-cause mortality. In mediation models fully adjusted for sociodemographic and CVD risk factors, both GrimAge age acceleration and DunedinPACE mediated a relevant portion of the total effects of As exposure on CVD. These results support a role for As exposure in accelerated physiological aging, which consequently could be a mechanism by which As contributes to increased CVD risk and all-cause mortality independent of its known effects on CVD risk factors.

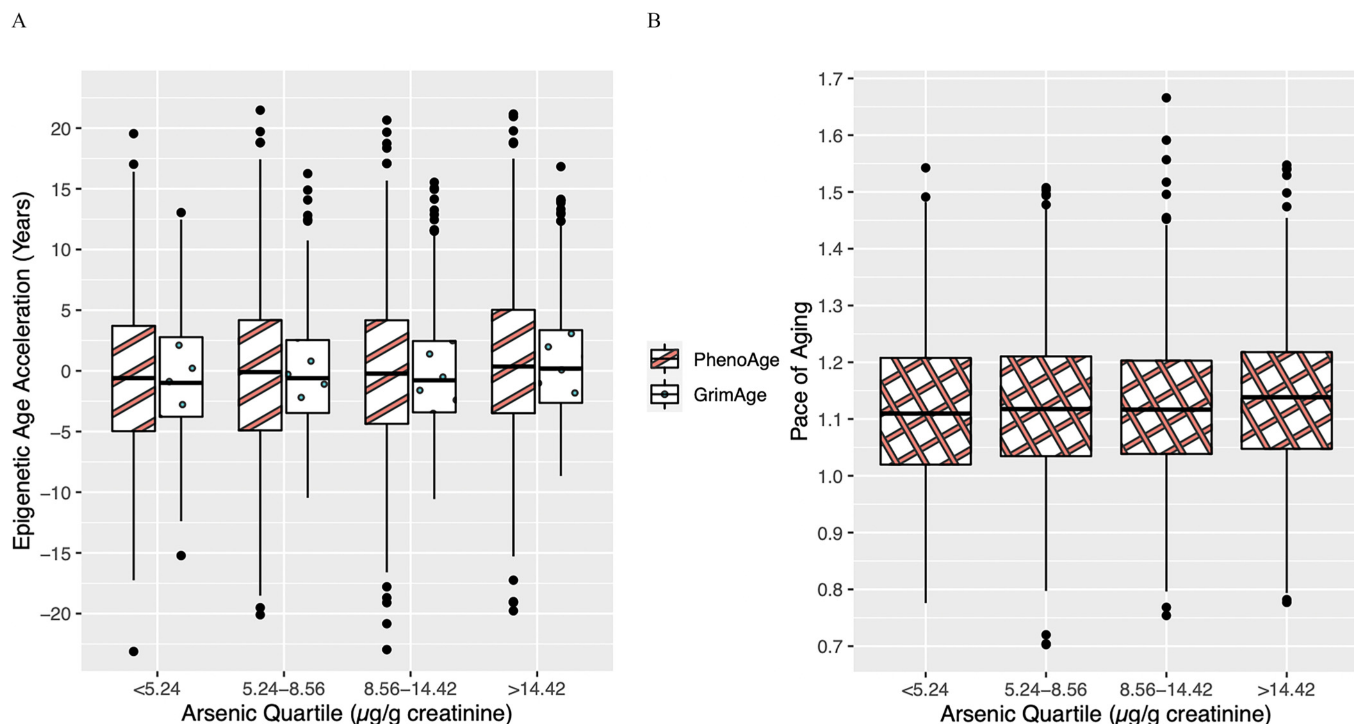

Figure 2. Distribution of participants' (A) PhenoAge and GrimAge age acceleration values and (B) DunedinPACE pace of aging values by quartile of urinary arsenic levels (μg/g creatinine). Epigenetic age acceleration is reported in years based on the residual method, and pace of aging is reported in years of biological aging per chronological year. $n = 2{,}323$ in the Strong Heart Study. Corresponding data is in Excel Table S2. Note: In panel A, four participants with PhenoAge age acceleration values out of the graph's range are not displayed (their data is still included in all analyses). Each box represents the interquartile range (IQR) of the epigenetic age acceleration or pace of aging value, and each whisker represents values up to 1.5 IQRs away from the first or third quartile; outlier values are beyond that.

We assessed the associations of epigenetic clocks with As exposure and CVD incidence, CVD mortality, and all-cause mortality. Previous studies have shown that first-generation DNA methylation-based aging measures such as the Hannum and Horvath clocks, which were trained based on chronological age, tend to have weaker associations with aging-related morbidity and mortality than second-generation measures, which were explicitly trained on aging-related biomarkers.[24,27,29]

Table 3. Effect estimates (95% CI) of the association between urinary arsenic and epigenetic aging measures (PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE) using multivariate linear regression in the Strong Heart Study ($n = 2{,}323$). In the model per IQR, arsenic was modeled as $\log_2$ transformed and reported comparing an interquartile range (p25 vs. p75). The effect estimates for PhenoAge and GrimAge compare epigenetic age acceleration values, reported in years based on the residual method. The effect estimate for DunedinPACE compares pace of aging values, which have a unit of years of biological aging per chronological year.

	Model 1		Model 2		Model 3	
Mean difference	Effect estimate (95% CI)	*p*-Value	Effect estimate (95% CI)	*p*-Value	Effect estimate (95% CI)	*p*-Value
PhenoAge						
IQR (5.24 vs. 14.42 μg/g creatinine)	0.48 (0.17, 0.80)	0.04	0.48 (0.17, 0.80)	0.04	0.26 (−0.031, 0.55)	0.23
Q1 (<5.24)	0.00 (Ref)	—	0.00 (Ref)	—	0.00 (Ref)	—
Q2 (5.24–8.56)	0.29 (−0.48, 1.10)	0.46	0.29 (−0.49, 1.10)	0.47	0.08 (−0.64, 0.79)	0.83
Q3 (8.56–14.42)	0.04 (−0.78, 0.86)	0.93	0.04 (−0.78, 0.86)	0.92	−0.25 (−1.00, 0.52)	0.53
Q4 (>14.42)	0.89 (0.00, 1.80)	0.05	0.88 (−0.01, 1.80)	0.05	0.38 (−0.45, 1.20)	0.37
GrimAge						
IQR (5.24 vs. 14.42 μg/g creatinine)	0.80 (0.60, 1.00)	9.51×10^{-8}	0.59 (0.41, 0.77)	9.53×10^{-6}	0.44 (0.28, 0.61)	3.19×10^{-4}
Q1 (<5.24)	0.00 (Ref)	—	0.00 (Ref)	—	0.00 (Ref)	—
Q2 (5.24–8.56)	0.44 (−0.06, 0.93)	0.09	0.39 (−0.05, 0.83)	0.08	0.27 (−0.13, 0.68)	0.19
Q3 (8.56–14.42)	0.83 (0.30, 1.40)	0.002	0.70 (0.23, 1.20)	0.003	0.57 (0.14, 1.00)	0.009
Q4 (>14.42)	1.60 (1.00, 2.20)	4.33×10^{-8}	1.20 (0.74, 1.80)	1.70×10^{-6}	0.91 (0.43, 1.40)	1.73×10^{-4}
DunedinPACE						
IQR (5.24 vs. 14.42 μg/g creatinine)	0.011 (0.005, 0.018)	0.01	0.012 (0.006, 0.018)	0.005	0.009 (0.004, 0.015)	0.02
Q1 (<5.24)	0.00 (Ref)	—	0.00 (Ref)	—	0.00 (Ref)	—
Q2 (5.24–8.56)	0.006 (−0.009, 0.021)	0.42	0.006 (−0.008, 0.021)	0.39	0.005 (−0.009, 0.018)	0.50
Q3 (8.56–14.42)	0.015 (0.001, 0.031)	0.06	0.016 (0.001, 0.031)	0.04	0.014 (0.000, 0.028)	0.04
Q4 (>14.42)	0.025 (0.0076, 0.042)	0.005	0.026 (0.009, 0.042)	0.003	0.019 (0.004, 0.034)	0.01

Note: Model 1 is adjusted for chronological age, eGFR, sex, center (Arizona, Oklahoma, and North Dakota and South Dakota), genetic PCs. Model 2 is additionally adjusted for smoking status (current/former/never) and BMI. Model 3 is additionally adjusted for Houseman cell proportions. *p*-Values were taken from linear regression and compare epigenetic age acceleration means per one interquartile range increase on arsenic. BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; IQR, interquartile range; PC, principal component; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile; Ref, reference.

Table 4. Number of incident CVD, fatal CVD, or all-cause death cases per 100,000 person-years attributable to a doubling in urinary arsenic levels through (indirect effect) and not through (direct effect) changes in epigenetic age acceleration or pace of aging for each epigenetic aging measure (PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE) using mediation analysis by the product of coefficients approach as adapted by Lange and Hansen. $n = 2,323$ in the Strong Heart Study.

Outcome and measure	Direct effect (95% CI)	Indirect effect (95% CI)	Total effect (95% CI)	Relative IE (%)
CVD incidence ($n = 1,023$)				
PhenoAge	347 (114, 580)	33.8 (3.7, 74.5)	381 (144, 617)	8.9 (1.0, 26.3)
GrimAge	299 (65, 533)	80.8 (38.5, 133.0)	380 (144, 615)	21.3 (9.1, 57.1)
DunedinPACE	300 (68, 530)	87.6 (37.3, 147.5)	387 (149, 626)	22.6 (9.5, 56.9)
CVD mortality ($n = 316$)				
PhenoAge	163 (38, 286)	16.5 (1.3, 38.3)	179 (53, 305)	9.2 (0.7, 31.5)
GrimAge	130 (5, 255)	51.2 (24.8, 83.6)	181 (55, 307)	28.3 (12.0, 87.7)
DunedinPACE	142 (17, 267)	35.6 (13.5, 63.9)	178 (51, 305)	20.0 (6.9, 65.6)
Total mortality ($n = 1,055$)				
PhenoAge	712 (460, 963)	89.7 (11.7, 176.9)	801 (536; 1,067)	11.2 (1.6, 21.9)
GrimAge	610 (360, 860)	178.1 (98.4, 266.8)	788 (525; 1,052)	22.6 (12.8, 36.1)
DunedinPACE	661 (408, 914)	112.5 (49.4, 184.6)	774 (513; 1,035)	14.5 (6.6, 25.3)

Note: Models adjusted for chronological age, sex, center (Arizona, Oklahoma, and North Dakota and South Dakota), genetic PCs, smoking status (current/former/never), and BMI. Epigenetic age acceleration is reported in years based on the residual method, and pace of aging is reported in years of biological aging per chronological year. BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; IE, indirect effect; PC, principal component.

Consistently, our study found that second-generation measures were more strongly associated with an increased risk of both CVD incidence and mortality than first-generation measures such as Hannum and Horvath. Higher As exposure was also more strongly associated with increases in second-generation biological aging measures, suggesting that these measures are more sensitive than their first-generation counterparts to As-related DNA methylation changes.

GrimAge and DunedinPACE were the strongest mediators of the association between As exposure and CVD outcomes in our study. Of note, both measures incorporated CVD-related biomarkers into their design. GrimAge was trained on biomarkers that have been associated with CVD risk, such as cystatin C, leptin, and plasminogen activator inhibitor-1 (PAI-1),[63–65] and DunedinPACE was trained on the rate of change of biomarkers such as BMI, blood pressure, glycated hemoglobin, total cholesterol, and eGFR, which are known risk factors for CVD in the SHS cohort.[66–68] Nevertheless, the effect of As on CVD incidence and mortality, as well as the mediating effect of these DNA methylation-based aging measures, remained significant even after adjusting for CVD risk factors in mediation models. It is unclear from our analyses whether As may act on biological processes of aging on a molecular level that then affects physiology or whether this relationship reflects known effects of As on multisystem physiological integrity. Nevertheless, our results suggest that the association between As and CVD incidence and mortality may include mechanisms not captured by traditional CVD physiological parameters. The potential mediating role of PhenoAge on the association between arsenic and CVD was less clear. In addition, we found evidence of an exposure-mediator interaction for this clock, which might lead to invalid mediation estimates. Additional research is needed to identify the biological reasons that might drive the different behavior of PhenoAge on the association with As exposure and CVD as compared to GrimAge and DunedinPACE.

Our mediation model results support that DunedinPACE can still capture some of the CVD incidence and all-cause mortality risk associated with As even after controlling for these factors. This could be related to DunedinPACE capturing the trajectory of risk factors over time or from measuring something beyond these risk factors.

Strengths of this study include the community-engaged and participatory nature of the SHS, which facilitated a large prospective cohort, a long follow-up period to follow participant outcomes, and robust DNA methylation data for a large amount of CpGs with current microarray technology (Illumina Infinium MethylationEPIC BeadChip). This is the first time that DNA methylation-based aging measures have been used in Native

Table 5. Results of further adjustment of mediation models in Table 4; number of incident CVD, fatal CVD, or all-cause death cases per 100,000 person-years attributable to a doubling in urinary arsenic levels through (indirect effect) and not through (direct effect) changes in epigenetic age acceleration or pace of aging for each epigenetic aging measure (PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE) using mediation analysis by the product of coefficients approach as adapted by Lange and Hansen. $n = 2,323$ in the Strong Heart Study. Figure 3 is based on the data in this table.

Outcome and measure	Direct effect (95% CI)	Indirect effect (95% CI)	Total effect (95% CI)	Relative IE (%)
CVD incidence ($n = 1,023$)				
PhenoAge	348 (108, 588)	25.4 (1.1, 61.0)	374 (131, 616)	6.8 (0.3, 22.4)
GrimAge	307 (66, 548)	63.8 (28, 108.8)	371 (129, 612)	17.2 (6.7, 50.7)
DunedinPACE	319 (79, 559)	56.3 (18.7, 103.5)	376 (131, 620)	15.0 (4.9, 42.8)
CVD mortality ($n = 316$)				
PhenoAge	169 (36, 303)	12.2 (−0.2, 31.5)	182 (47, 316)	6.7 (−0.3, 26.8)
GrimAge	141 (6, 275)	42 (19.1, 70.5)	183 (48, 318)	23.0 (8.9, 80.5)
DunedinPACE	156 (22, 291)	23.7 (6.4, 46.9)	180 (44, 316)	13.1 (3.2, 48.9)
Total mortality ($n = 1,055$)				
PhenoAge	678 (423, 931)	74.4 (6.6, 152.0)	752 (487; 1,017)	9.9 (1.0, 20.4)
GrimAge	597 (342, 851)	139.3 (72.1, 215.0)	736 (473, 999)	18.9 (9.9, 32.0)
DunedinPACE	651 (394, 908)	74.8 (26.5, 132.3)	726 (464, 987)	10.3 (3.7, 19.7)

Note: Models adjusted for chronological age, sex, center (Arizona, Oklahoma, and North Dakota and South Dakota), genetic PCs, smoking status (current/former/never), BMI, eGFR, LDL cholesterol, HDL cholesterol, systolic blood pressure, hypertension treatment, diabetes status. Epigenetic age acceleration is reported in years based on the residual method, and pace of aging is reported in years of biological aging per chronological year. BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IE, indirect effect; LDL, low-density lipoprotein; PC, principal component.

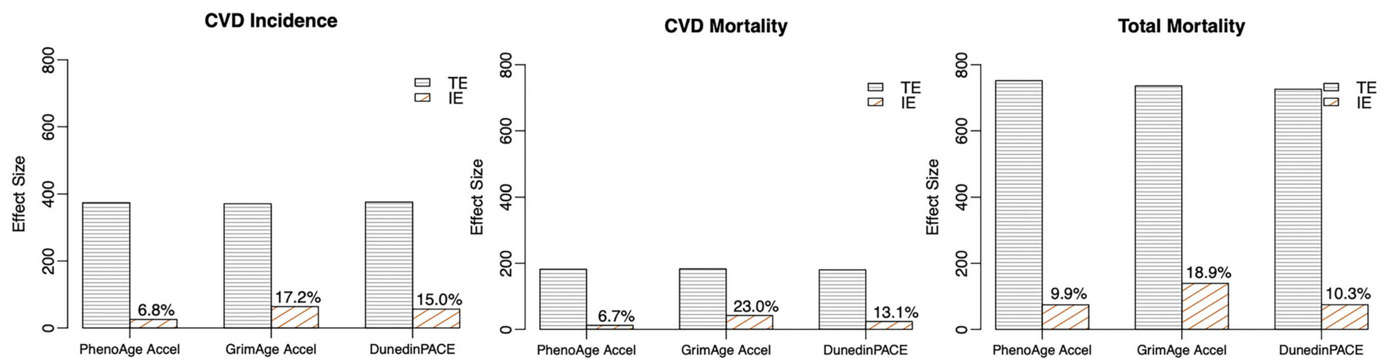

Figure 3. Contribution of PhenoAge age acceleration, GrimAge age acceleration, and DunedinPACE to total effect of urinary arsenic exposure on incident CVD (A), fatal CVD (B), and all-cause mortality (C) in fully adjusted mediation models. Effect size is reported as cases per 100,000 person-years attributable to a doubling in urinary arsenic levels (TE) or percent attributable to the mediating effect of the epigenetic aging measure (IE). $n = 2,323$, Missing $= 0$. Corresponding data is in Table 5. Note: CVD, cardiovascular disease; IE, indirect effect; TE, total effect.

American populations. DNA methylation-based aging measures need to be validated within diverse cohorts to assess their generalizability across different populations, as existing research and clock design has mainly focused on cohorts with predominantly white European ancestry.[21,28,69] Hence, the SHS adds novel and valuable results to the overall understanding of epigenetic aging.

Our study has several limitations. We restricted CVD outcomes to 31 December 2009, as drinking water As levels underwent a change in the SHS populations after the US EPA's Final Arsenic Rule was implemented in the years following 2006.[43] The SHS does not have information on urinary As levels in those years, so we are unable to assess any consequent changes in CVD incidence or mortality or any corresponding epigenetic mediation of these changes following changes in As exposure. We only have one measure of urinary As; however, based on our previous studies on repeated measurements of urinary As levels over a 10-year period,[39] the constant high levels of As in drinking water in the absence of an intervention,[70] and the relatively low contribution of diet to urinary arsenic in the SHS population,[71] we believe that As exposure was constant for decades in the SHS and hypothesize that exposure preceded DNA methylation. However, the possibility that DNA methylation influences As metabolism and excretion cannot be ruled out. In addition, the possible mechanisms by which As affects CVD risk remain an issue of ongoing research, which makes it difficult to draw conclusions from our mediation analyses. Finally, as discussed earlier, the DNA methylation-based aging measures used in this study were trained on predominantly white cohorts, and thus may not be able to predict epigenetic aging in the SHS cohort as accurately or comprehensively as in the original cohorts. Nevertheless, GrimAge and DunedinPACE maintained statistically significant associations with As exposure, as well as with CVD outcomes and all-cause mortality in all analyses.

Last, mediation analysis only provides valid causal estimates on the absence of unmeasured confounding, an assumption that is impossible to verify in practice in epidemiological studies. We fitted several different models adjusting for sets of variables that might be potential confounders as typically done in studies of arsenic exposure and CVD. We even went one step further by adding factors that might not be confounders but potential mediators of the association between arsenic and CVD such as diabetes and blood pressure, as our goal was to assess the potential mediating role of the epigenetic biomarkers tested beyond those well-established risk factors for CVD. Also, the existence of exposure-induced mediator-outcome confounders cannot be ruled out. For example, because arsenic exposure is known to affect DNA methylation, dysregulations of biological pathways influenced by As might induce confounding of associations between DNA methylation measures of aging and CVD. A weighting approach was proposed to deal with effect estimation in the presence of this kind of confounding.[72] However, to our knowledge, no R package has been developed to implement this approach. Experimental studies are ultimately needed to assess the potential causal role of DNA methylation aging on the association between As and CVD incidence and mortality.

In conclusion, we found that the second-generation epigenetic clock GrimAge and the pace of aging measure DunedinPACE were each statistically significant mediators of the relationships between As exposure and risk of CVD incidence, CVD mortality, and all-cause mortality in the SHS cohort, even after adjusting for sociodemographic confounders and CVD risk factors. Our study brings epidemiological evidence to the hypothesis that As accelerates biological aging, thereby affecting the risk of age-related morbidity and mortality. It also contributes valuable information to the field of epigenetic aging research as the first study to assess the predictive utility of several prevailing epigenetic clocks in an American Indian cohort. Further investigation can help to underpin the potential key role of biological aging in the pathway of As exposure to CVD, all-cause mortality, and other health outcomes, including the potential aging benefits of the reductions in arsenic levels in drinking water that have taken place in recent years.

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