ehp ENVIRONMENTAL HEALTH PERSPECTIVES
http://www.ehponline.org

Association of Global DNA Methylation and Global DNA Hydroxymethylation with Metals and other Exposures in Human Blood DNA Samples

Maria Tellez-Plaza, Wan-yee Tang, Yan Shang, Jason G. Umans, Kevin A. Francesconi, Walter Goessler, Marta Ledesma, Montserrat Leon, Martin Laclaustra, Jonathan Pollak, Eliseo Guallar, Shelley A. Cole, M. Dani Fallin, and Ana Navas-Acien

http://dx.doi.org/10.1289/ehp.1306674
Received: 19 February 2013
Accepted: 22 April 2014
Advance Publication: 25 April 2014

NIH National Institute of Environmental Health Sciences

Association of Global DNA Methylation and Global DNA Hydroxymethylation with Metals and other Exposures in Human Blood DNA Samples

Maria Tellez-Plaza,[1,2,3] Wan-yee Tang,[2] Yan Shang,[2,4] Jason G. Umans,[5,6] Kevin A. Francesconi,[7] Walter Goessler,[7] Marta Ledesma,[8] Montserrat Leon,[8] Martin Laclaustra,[9] Jonathan Pollak,[2] Eliseo Guallar,[1,10] Shelley A. Cole,[11] M. Dani Fallin,[1] and Ana Navas-Acien[1,2]

[1]Department of Epidemiology and Welch Center for Prevention, Epidemiology and Clinical Research, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA; [2]Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA; [3]Fundacion de Investigacion del Hospital Clinico de Valencia-INCLIVA, Valencia, Spain; [4]Department of Respiratory Disease, Changhai Hospital, Second Military Medical University, Shanghai, China; [5]MedStar Health Research Institute, Hyattsville, Maryland, USA; [6]Georgetown-Howard Universities Center for Clinical and Translational Science, Washington DC, USA; [7]Institute of Chemistry – Analytical Chemistry, Karl-Franzens University Graz, Austria; [8]Aragon Health Sciences Institute, Zaragoza, Spain; [9]Department of Epidemiology, Atherothrombosis and Imaging, National Center of Cardiovascular Research-CNIC, Madrid, Spain; [10]Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA; [11]Department of Genetics, Texas Biomedical Research Institute, San Antonio, Texas, USA

Address correspondence to Maria Tellez-Plaza, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Room W7513D,

Baltimore, MD 21205 USA. Telephone: (410) 502-4267. Fax: (410) 955-1811. E-mail: mtellezp@jhsph.edu

Running title: DNA global methylation and hydroxymethylation

Acknowledgments: This work was supported by grants from the National Heart Lung and Blood Institute (R01HL090863 and by SHS grants HL41642, HL41652, HL41654 and HL65520), the National Institute for Occupational Safety and Health, (T42 OH0008428 from the Education and Research Center for Occupational Safety and Health at the Johns Hopkins Bloomberg School of Public Health) and the National Institute of Environmental Health Sciences (R01ES021367, R00ES016817 and P30ES03819). Dr. Tellez-Plaza was supported by the Strategic Action for Research in Health sciences from the Carlos the Third Health Institute at Spanish Ministry of Economy and Innovation (CP12/08030) and co-funded with European Funds for Regional Development (FEDER).

Competing financial interests: The authors declare they have no actual or potential competing financial interests.

Abstract

Introduction: The association between human blood DNA global methylation and global hydroxymethylation has not been evaluated in population-based studies. No studies have evaluated environmental determinants of global DNA hydroxymethylation, including exposure to metals.

Objective: We evaluated the association between global DNA methylation and global DNA hydroxymethylation in 48 Strong Heart Study participants who had selected metals measured in urine at baseline and DNA available in 1989-1991 and 1998-1999.

Methods: % 5-methylcytosine (5-mC) and % 5-hydroxymethyl-cytosine (5-hmC) levels were measured by capture and detection antibodies followed by colorimetric quantification. We explored the association of participant characteristics (i.e. age, adiposity, smoking, and metal exposure) with both global DNA methylation and global DNA hydroxymethylation.

Results: The Spearman's correlation coefficient for 5-mC and 5-hmC levels was 0.32 (p –value = 0.03) at visit 1 and 0.54 (p – value < 0.001) at visit 3. Trends for both epigenetic modifications were consistent across potential determinants. In cross-sectional analyses the odds ratios of methylated and hydroxymethylated DNA were 1.56 (95% CI: 0.95, 2.57) and 1.76 (95% CI: 1.07, 2.88), respectively, comparing participants above and below the median of % dimethylarsinate. The corresponding odds ratios were 1.64 (95% CI: 1.02, 2.65) and 1.16 (95% CI: 0.70, 1.94), respectively, comparing participants above and below median cadmium. Arsenic exposure and metabolism were consistently associated with both epigenetic markers in cross-sectional and prospective analyses. The positive correlation of 5-mC and 5-hmC levels was confirmed in an independent study population.

Conclusions: Our findings support that both epigenetic measures are related at the population level. The consistent trends in the associations between these two epigenetic modifications and the characteristics evaluated, specially arsenic exposure and metabolism, suggest the need for understanding which of the two measures is a better biomarker for environmental epigenetic effects in future large-scale epidemiologic studies.

Introduction

DNA 5-methylcytosine (5-mC) modifications are increasingly recognized as a key process in the pathogenesis of complex disorders, including cancer, diabetes and cardiovascular disease (Feinberg 2010; Ordovas and Smith 2010). 5-hydroxymethyl-cytosine (5-hmC), a hydroxylated and methylated form of cytosine, has been recently investigated for its epigenetic functions. The conversion of 5-mC to 5-hmC is a step prior to demethylation (Shen et al. 2013; Song et al. 2013) and seems to also play a direct role in the regulation of gene expression (Branco et al. 2012). While it has been recently described that 5-hmC can bind to DNA methylation binding domain (MBD)-3, (Baubec et al. 2013), it reduces the binding of other MBD proteins to methylated DNA and prevents DNA methyltransferase (DNMT)-mediated methylation of the target cytosine (Tahiliani et al. 2009). A number of studies have evaluated blood cell global DNA methylation and its determinants in population-based studies (Terry et al. 2011). Few human studies, however, have evaluated global DNA hydroxymethylation. Specifically, the association between human blood cell global DNA methylation and global DNA hydroxymethylation has not been previously evaluated in epidemiologic studies.

Environmental exposures, including arsenic and cadmium, disrupt gene expression (Andrew et al. 2008; Bourdonnay et al. 2009; Castillo et al. 2012; Cheng et al. 2012; Hossain et al. 2012; Su et al. 2006). These findings suggest that the health effect of environmental exposures, including metals, could be partly mediated by epigenetic mechanisms (Arita and Costa 2009; Reichard and Puga 2010; Ren et al. 2011; Smeester et al. 2011). Work conducted *in vitro* and in primary human tissue samples also support that metals can have epigenetic effects (Huang et al. 2008; Kile et al. 2012; Lambrou et al. 2012; Smeester et al. 2011). No studies have evaluated environmental determinants of global DNA hydroxymethylation, including exposure to metals.

In this study, we describe the association between global DNA methylation and global DNA hydroxymethylation in a subsample of Strong Heart Study (SHS) participants who had metal measured in urine as well as blood DNA available for measurements of DNA methylation (Lee et al. 1990; Scheer et al. 2012). In addition, we explored the association of participant characteristics (age, sex, education, adiposity, smoking, alcohol intake, metal exposure and arsenic metabolism) with both global DNA methylation and global DNA hydroxymethylation. We had no a priori hypothesis on the direction of the associations under study.

Methods

Study population

The SHS is a population-based cohort study funded by the US National Heart Lung and Blood Institute that recruited 4,549 participants from Arizona, Oklahoma, and North and South Dakota in 1989-1991 (overall response rate 62%) (Lee et al. 1990). Starting in 1998, an ancillary study to the SHS called the Strong Heart Family Study (SHFS) recruited extended family members of the original SHS participants who were ≥ 18 years of age to evaluate genetic determinants of cardiometabolic disease in American Indian populations (North et al. 2003). For the current study, the population was restricted to Strong Heart study participants with measurements of metals in urine at baseline (visit 1) (Scheer et al. 2012) who also participated in the SHFS (North et al. 2003) and had biological samples collected at 2 follow-up clinic visits conducted in 1993 to 1995 (visit 2) and 1997 to 1999 (visit 3). The total of participants meeting those criteria was 517. To maximize efficiency of this relatively small epigenetic study (Stuart and Hanna 2013; Zubizarreta et al. 2013), we used a stratified random sample to select eight participants with moderate arsenic exposure and eight participants with low arsenic exposure from each region (16 from Arizona, 16 from Oklahoma and 16 from North or South Dakota), resulting in a total of 48

participants. Based on the SHS distributions, relatively low and moderate arsenic exposures were defined as urine concentrations < 7.2 μg/g (tertile 1) and ≥ 14.0 μg/g (tertile 3), respectively, for the sum of inorganic and methylated arsenic species in 1989 – 1991. The study protocol was approved by the Institutional and Indian Health Service Review Boards and the participating American Indian communities. All the participants provided oral and written informed consent.

Epigenetic measurements

Global methylation and global hydroxymethylation in DNA isolated from frozen buffy coat from visit 1 and frozen whole blood from visit 3 was detected with Methylamp (currently known as MethylFlash) Methylated and Hydroxymethylated DNA Quantification Kits (Epigentek, NY), according to the manufacturer's instructions. In brief, 5-mC and 5-hmC are separately detected using an ELISA-like reaction (Epigentek 2014). Levels of 5-mC or 5-hmC in DNA of all biological samples were measured as the amount of methylated or hydroxymethylated cystosines relative to the cytosine genomic content (%). For global DNA methylation assay, the capture antibody for 5-mC had no or negligible cross-reactivity to 5-hmC and unmethylated cytosine. Global DNA hydroxymethylation was quantified by specifically measuring levels of 5-hmC without cross-reactivity to 5-mC and unmethylated cytosine. The input DNA for 5-mC and 5-hmC assays was 100 and 37.5 ng, respectively. All samples (or repeats) were loaded with the same amount of DNA in the assay plate.

All samples and methylated/hydroxymethylated standards were measured in triplicate, and the average was reported. The observed quality control data were excellent (median [interquartile range] intra-assay CV ranged between 0.25 [0.10, 0.51]% to 0.37 [0.20, 0.69]% and ICC >0.998, see Supplemental Material, Table S1), indicating that the variability of the determinations can be almost completely attributed to the between-subjects variation. Leukocyte composition of

peripheral blood can covary with patterns of DNA methylation, and also with participant characteristics, such as sex or age (Lam et al. 2012). It is thus important to account for blood cell heterogeneity in the statistical analysis of DNA methylation and hydroxymethylation data. Blood cell count data, however, was only available for the blood samples corresponding to the DNA extracted in visit 3. Available subtype cell counts included neutrophils, lymphocytes, monocytes, eosinophils and basophils (see distribution in Supplemental Material, Table S2).

Other variables

Participants were interviewed and physically examined by centrally trained and certified staff following a standard protocol (Lee et al. 1990). Baseline information on sociodemographic data (age, sex, education), smoking status (never/former/current), cumulative smoking (cigarette pack-years), and alcohol use (never/former/current) were obtained during the questionnaire. Measures of adiposity obtained during the physical exam included body mass index (kg/m^2), percent body fat estimated by bioelectric impedance (Impedance Meter Model #B1A101, RJL Equipment Company), and waist circumference measured supine in centimeters.

Spot urine samples were frozen without chemical additives within 1-2 hours of collection at baseline (Bornhorst et al. 2005). In 2009, urine samples were thawed and arsenic, cadmium, antimony and tungsten were measured using inductively coupled plasma-mass spectrometry (ICPMS), as previously described (Scheer et al. 2012). Arsenic species (inorganic arsenic [arsenite, arsenate], methylarsonate [MMA] and dimethylarsinate [DMA]) in the same urine samples were measured using anion-exchange high performance liquid chromatography coupled with ICPMS. The limits of detection were 0.1 µg/L for total arsenic and arsenic species, and 0.05 µg/L for cadmium, antimony and tungsten. None of the samples included in this study were below the limit of detection. The inter-assay coefficients of variation for total arsenic, arsenite,

arsenate, MMA, DMA, cadmium, antimony and tungsten were 4.4, 14.7, 6.9, 6.4, 6.0, 8.7, 30.0 and 14.5%, respectively. For every batch of 79 samples, 10 of the samples were analyzed in duplicate. The mean intra-assay coefficient of variation for total arsenic, arsenite, arsenate, MMA, DMA, cadmium, antimony and tungsten was, 1.53, 4.46, 4.23, 3.02, 1.49, 1.34, 3.29 and 0.57 %, respectively. The estimated intra-assay intraclass correlation in these samples for total arsenic, arsenite, arsenate, MMA, DMA, cadmium, antimony and tungsten were 0.997, 0.990, 0.996, 0.992, 0.987, 0.994, 0.966 and 0.990, respectively. To account for urine dilution, urine metal concentrations (µg/L) were divided by urine creatinine concentrations (g/L) and reported in µg/g creatinine. The spearman correlation coefficients for metal-by-metal levels ranged from 0.07 for the correlation between antimony and arsenic to 0.43 for the correlation between tungsten and antimony. To assess arsenic metabolism, we computed % inorganic arsenic (iAs), %MMA, and %DMA by dividing the concentration of each of them over the sum of the inorganic and methylated species.

Statistical analyses

We estimated medians (interquartile range) of %5-mC and % 5-hmC levels by participant characteristics. The levels of % 5-mC and % 5-hmC were not normally distributed and were logit-transformed for statistical analyses. Scatter plots, lowess models and Spearman correlation coefficients were used to descriptively display the association between global DNA methylation and global DNA hydroxymethylation at visits 1 and visit 3. We also displayed crude linear trends in the association of global DNA methylation and global DNA hydroxymethylation with continuous variables including age, body mass index, percent body fat, waist circumference, urine metal concentrations (arsenic, cadmium, tungsten and antimony) and arsenic metabolic profile (%iAs, %MMA and %DMA), and reported the corresponding linear correlation

coefficients estimated as the square root of the R^2 of the underlying simple linear regression models. In addition, we used linear regression models on logit-transformed measures of global DNA methylation and global DNA hydroxymethylation to compare relative differences in the odds of having methylation and hydroxymethylation levels comparing levels of categorical variables including: sex, education (<12/≥12 years), smoking status (ever/never), alcohol status (ever/never), BMI (<30/≥30 kg/m^2), and the following variables dichotomized at their corresponding medians: waist circumference, % body fat, urine arsenic concentrations and arsenic metabolism, and urine cadmium, antimony and tungsten concentrations.

Due to limited sample size and difficulties to conduct longitudinal evaluations of changes over time, the analysis was conducted separately for visits 1 and 3 and results from parsimonious models with no multivariable adjustment were considered as the main results. The association of baseline urine metal concentrations with baseline global DNA methylation and global DNA hydroxymethylation evaluate the hypothesis that metals are cross-sectionally associated with DNA methylation and hydroxymethylation levels. The association of baseline metal exposure biomarkers with visit 3 global DNA methylation and global DNA hydroxymethylation evaluates the hypothesis that metals are prospectively related to DNA methylation and hydroxymethylation levels. For arsenic, under constant conditions of exposure over time, urine concentrations and metabolism biomarkers are fairly constant over time, as it has been shown in our study population (Navas-Acien et al. 2009) and in previous studies measuring arsenic in private and public drinking water systems over long periods of time (Karagas et al. 2001; Ryan et al. 2000; Steinmaus et al. 2005). In this situation, evaluating the association with epigenetic measures in visit 1 and 3 allows to evaluate the consistency of the associations assuming constant arsenic exposure and metabolic processes.

In addition to crude regression models (Model 1), we also conducted multivariable regression models adjusting for age (years), sex (men, women), body mass index (kg/m^2, continuous) and smoking status (never, former, current smokers). In order to evaluate a potential effect of blood cell type heterogeneity in the associations observed by participant characteristics, we also adjusted visit 3 models for cell type heterogeneity (Models 2 and 3). We only had available information on white blood cell count and percent cell type in visit 3. As the power in our study is limited, and the neutrophils are the cell type more common in blood, we show results for models adjusted for log-transformed total cell count and neutrophils cell count only. In additional sensitivity analyses we adjusted for log-transformed total cell count, neutrophils count, basophils count, monocyte count and lymphocyte counts, with similar findings (data not shown). All statistical analyses were conducted in R-software.

Post-hoc analysis

The SHS population is a population with high rates of cardiovascular disease. To evaluate 5-mC and 5-hmC levels in a population with a low burden of disease, we detected global methylation and global hydroxymethylation in DNA isolated from frozen whole blood in 48 healthy men from Spain (24 among them were never smokers and 14 were obese) who participated in the Aragon Workers Health Study (AWHS). The AWHS is a large prospective cohort study that aims to characterize the factors associated with metabolic abnormalities and subclinical atherosclerosis in a middle aged population free of cardiovascular disease (Casasnovas et al. 2012). The AWHS design and baseline characteristics have been reported elsewhere (Casasnovas et al. 2012). To measure 5-mC and 5-hmC in the AWHS, we used an ELISA method (Zymo Research 5-mC and 5-hmC kits) following the manufacturer's instructions and after confirmation that the results were consistent in a subsample of the Strong Heart Study with duplicate

determinations (data not shown). Levels of 5-mC or 5-hmC in DNA were measured as the amount of methylated or hydroxymethylated cystosines relative to the total DNA content (%). The input DNA for 5-mC and 5-hmC assays was 50 ng. All samples (or repeats) were loaded with the same amount of DNA in the assay plate. The intra-assay CV (interquartile range) and ICC were 3.14 (3.05, 3.20)% and 0.86, respectively, for % 5-mC and 2.80 (1.40, 4.30)% and 0.61, respectively for %5-hmC (see Supplemental Material, Table S1).

Results

The mean (SD) age of the study sample was 54.9 (7.2) years, 68.7% were women and 58.3% were ever smokers (Table 1). The study population was representative of the SHS participants who also participated in the SHFS (Table 1). The median (interquartile range) level for global DNA methylation and global DNA hydroxymethylation was 0.32 (0.15, 0.58) % of 5-mC and 0.12 (0.07, 0.17) % of 5-hmC in visit 1 and 0.32 (0.13, 0.55) % of 5-mC and 0.15 (0.09, 0.25) % of 5-hmC in visit 3 (see Supplemental Material, Table S3). The Spearman's correlation coefficient for 5-mC and 5-hmC levels was 0.32 (p –value = 0.03) at visit 1 and 0.54 (p – value < 0.001) at visit 3 (Figure 1).

The direction of estimated linear trends in 5-mC and 5-hmC levels by levels of possible determinants was generally consistent for both epigenetic modification measures and at both visits (see Supplemental Material, Figures S1 and S2), with some exceptions. In crude cross-sectional analyses, baseline age, % inorganic arsenic and % MMA showed a trend towards an inverse association with 5-mC and 5-hmC measured in visit 1 (Table 2, see Supplemental Material, Figures S1 and S2). Baseline adiposity measures (especially BMI), %DMA, cadmium, antimony and tungsten showed mostly a trend towards a positive association with 5-mC and 5-hmC measured in visit 1 (Table 2, see Supplemental Material, Figures S1 and S2). Arsenic

metabolism markers (%iAs, %MMA and %DMA) showed similar direction and magnitude for the cross-sectional and prospective associations with %5-mC and %5-hmC levels (Tables 2 and 3, see Supplemental Material, Figures S1 and S2). Comparing participants with baseline %DMA above and below 78.3%, the odds ratios of % 5-hmC were 1.75 (95% CI: 1.07, 2.88) at visit 1 and 1.34 (95% CI: 0.79, 2.26) at visit 3 (Table 3). Comparing participants with urine cadmium concentrations above and below 0.87 μg/g, the odds ratio of % 5-mC was 1.64 (95% CI: 1.02, 2.65) at visit 1 but 0.86 (95% CI: 0.47, 1.56) at visit 3 (Table 3).

In general, adjustment for age, sex, body mass index and smoking status did not change the magnitude and direction of the observed associations. Actually, the correlation of % 5-mC and % 5-hmC became stronger after those adjustments (Table 2). In sensitivity analyses adjusting for cell heterogeneity in the subset of individuals with cell count data available in visit 3 (N=44) (Table 3), results were consistent, although some additional prospective trends became statistically significant. The odds ratio of % 5-mC comparing participants with urine arsenic above 14.0 μg/g and below 7.2 μg/g, and with urine antimony above and below 0.27 μg/g, were 0.54 (95% CI: 0.30, 0.97) and 1.93 (95% CI: 1.07, 3.47), respectively. The odds ratio of % 5-hmC comparing participants with baseline %MMA above and below 14.4% was 0.58 (95% CI: 0.35, 0.98).

In a post-hoc analysis in the AWHS, the median % 5-mC and %5-hmC levels in the AWHS were 0.90% and 0.09%, respectively. The Spearman coefficient of correlation for %5-mC and %5-mC was 0.16 (p-value = 0.29) (see Supplemental Material, Figure S3).

Discussion

Global DNA methylation and global DNA hydroxymethylation measured in blood were moderately and positively associated in this subsample of participants from the Strong Heart Study. The association was confirmed in two time points 10 years apart, and in an independent study population from Spain with a low burden of disease, and supports the close relationship between both epigenetic measures. While we had limited sample size to evaluate determinants of global DNA methylation and global DNA hydroxymethylation in this study, we found statistically significant associations between urine cadmium concentrations and global DNA methylation and between arsenic metabolism (measured as %DMA) and global DNA hydroxymethylation. The observed associations of arsenic metabolism and global DNA methylation and DNA hydroxymethylation showed consistent patterns across time. This study provided the opportunity to evaluate the consistency of potential association and direction of the relationship between these 2 measures of epigenetic modification in human blood DNA samples.

Methylation at the 5' position of cytosine in DNA plays a role in regulating gene expression (Feinberg 2010). In addition to DNA methylation, DNA hydroxymethylation has also been related to changes in gene expression (Branco et al. 2012). In the mammalian genome, ten to eleven translocation proteins (TETs) are responsible for catalyzing 5mC oxidation to 5hmC. (Branco et al. 2012; Tahiliani et al. 2009). Hydroxymethylation at 5' CpGs via TETs is shown to contribute to gene transcription by influencing DNA demethylation (Baubec et al. 2013; Shen et al. 2013; Song et al. 2013) and/or recruitment of transcription complexes to repress the gene transcription (Cimmino et al. 2011). Bisulfite conversion, a traditionally used method for the enrichment of 5-mC, cannot distinguish between 5-mC and 5-hmC (Huang et al. 2010). Before the development of 5-hmC profiling strategies, it was postulated that hypermethylation of

promoter regions blocks gene expression, whereas hypermethylation of gene bodies increases gene expression (Jones 2012). Recent studies evaluating genome-wide 5-hmC profiles in mouse and human embryonic stem cells and brain cells have observed an enrichment of 5-hmC at gene body regions, regulatory (promoter) regions and sites with intermediate CpG density (Branco et al. 2012). The presence of 5-hmC in gene bodies has been consistently associated with gene expression (Branco et al. 2012). Altogether, it is suggested that hydroxymethylation has dual functions in regulation of gene transcription and it is gene specific. It has been proposed that the balance between DNA methylation and DNA hydroxymethylation in the genome is involved in the balance between cellular pluripotency and lineage commitment (Ficz et al. 2011). The health implications of the relationship between DNA methylation and hydroxymethylation in differentiated tissues, however, are currently unknown. Advanced technology by using massive parallel sequencing on profiling degree of 5-hmC across the genome may help to understand the role of DNA hydroxymethylation.

In our study population the levels of 5-hmC are ~2.5-fold lower compared to 5-mC (see Supplemental Material, Table S3). In blood samples from 12 healthy individuals from the US (Figueroa-Romero et al. 2012) the mean % 5-mC and % 5-hmC, measured by an ELISA method, were 0.41% and 0.03% respectively (5-hmC was ~12-fold lower compared to 5-mC). The CV and ICC of the assays were not provided. In the AWHS the ratio of 5-mC and 5-hmC levels was intermediate compared to the SHS results and the previously mentioned data (Figueroa-Romero et al. 2012), as 5-mC was 10-fold more abundant that 5-hmC (see Results section). In the SHS data, with very low CV and high ICC (see Supplemental Material, Table S1), the correlation between 5-mC and 5-hmC was statistically significant at two time points (Figure 1). In the AWHS, the replication study sample, the correlation between global DNA methylation and

global DNA hydroxymethylation was positive, supporting consistency in the direction of the association in a human population with a different risk profile. The correlation, however, was weaker and non-statistically significant. Overall, random sampling variability due to the small sample size and technical variability/measurement error cannot be discarded as the main reason for the discrepancies in global DNA methylation and hydroxymethylation levels from human blood DNA samples in different study populations.

Changes in DNA methylation have been related to environmental exposures including metals, air pollution, benzene, bisphenol A, diethylstilbestrol, and dioxins, in experimental and small-size population-based studies, although the exact mechanisms remain unclear (Hou et al. 2012; Bailey and Fry 2014; Breton and Marutani 2014). In our study we found consistent trends for both global DNA methylation and global hydroxymethylation by different determinants. It is possible that DNA hydroxymethylation acts as a proxy for DNA methylation or viceversa (the more DNA methylation exist, the greater potential for DNA hydroxymethylation). Indeed, 5-hmC was reduced in TET 1/2 knockdown cells and Np95 2/2 cells, and eliminated in DNMT-1 2/2 / DNMT-3a 2/2 / DNMT-3b 2/2 triple knockout embryonic stem cells, suggesting that most 5-hmC in the genome depends on pre-existing 5-mC (Ficz et al. 2011). Alternatively, it is also possible that determinants for DNA methylation and DNA hydroxymethylation are somewhat common. For instance, based on *in vivo* and *in vitro* experimental findings, it has been hypothesized that oxidative stress can regulate both DNA methylation and DNA hydroxymethylation processes by impairing one-carbon (Lee et al. 2009) and citric acid metabolism pathways (Chia et al. 2011), respectively. Additional studies with larger sample sizes are needed to investigate the correlation between global and gene-specific DNA methylation and DNA hydroxymethylation in relation to environmental determinants and health outcomes.

In our study, we found some support for the relationship between some environmental exposures with global DNA methylation and global DNA hydroxymethylation. In particular, we found a change in the level of the global DNA methylation and hydroxymethylation associated with metals exposure. For antimony and tungsten, we found positive cross-sectional associations with global DNA methylation and hydroxymethylation, although the association with global DNA hydroxymethylation was weaker. The prospective association of antimony and global DNA methylation was statistically significant. The association of global DNA methylation and hydroxymethylation with these metals has not been evaluated before in human studies. Very few studies have evaluated the association of arsenic and cadmium exposure with global DNA methylation in humans. Contrary to our findings, low-level environmental cadmium exposure was associated with global DNA hypomethylation, as measured in repetitive elements (a proxy for global DNA methylation), in women from Argentina (N = 202) (Hossain et al. 2012). In a population from Spain (N=892) increasing arsenic toenail concentrations were significantly associated with decreasing LINE-1 methylation (Tajuddin et al. 2013). Toenail cadmium was not associated with LINE-1 methylation in this study population. In populations exposed to high arsenic levels in drinking water in West Bengal and Bangladesh, increasing arsenic exposure levels were associated with increasing global DNA methylation in peripheral blood cells (Majumdar et al. 2010; Pilsner et al. 2007). The association of maternal urine arsenic and global methylation as measured in Alu and LINE-1 repetitive elements and in the LUMA assay in cord blood DNA was positive among male newborns (N = 58) but inverse among female newborns (N = 43) from Bangladesh (Pilsner et al. 2012). At low-moderate levels of arsenic exposure, increasing arsenic levels in toenails have been associated with methylation changes in repetitive elements (increasing Alu and decreasing LINE-1 DNA methylation) in 581 elderly men from the

US (Lambrou et al. 2012). In our visit 3 data, after adjustment for cell heterogeneity, higher arsenic exposure levels were significantly associated with decreased global DNA methylation. Random sampling variability and differences in residual confounding, study designs and population exposure levels may underlie inconsistencies across studies evaluating metal-related global methylation.

Arsenic exposure has been associated with both hyper and hypomethylation of gene-specific promoter, with a trend towards hypermethylation (Gribble et al. 2014; Hossain et al. 2012; Kile et al. 2012; Koestler et al. 2013). In 202 Argentinean women urinary arsenic concentrations were positively associated with methylation of p16 and MLH1 genes (Hossain et al. 2012). CpG sites in gene p16 were also positively associated with arsenic exposure in 113 women from Bangladesh (Kile et al. 2012). In a genome-wide study of DNA methylation in cord blood samples from 134 infants, 75% of the 44 top statistical significant arsenic-associated CpG islands showed increased methylation with increase arsenic exposure levels (Koestler et al. 2013). A hypomethylated region in the *AS3MT* promoter was associated with higher arsenic exposure in our study population (Gribble et al. 2014). Large-sample size epidemiologic studies are needed to evaluate the relationship of metal exposures with global DNA and gene-specific methylation and hydroxymethylation in human populations.

Arsenic metabolism in humans is usually studied as the relative amount of inorganic and methylated arsenic metabolites in urine (Vahter 2000). Differences in arsenic methylation patterns in urine (higher %iAs and %MMA and lower %DMA) have been associated with higher risk of skin lesions, cancer and cardiovascular disease in populations exposed to arsenic in drinking water (Chen et al. 2003; Del Razo et al. 1997; Hsueh et al. 1997 ; Kile et al. 2011; Steinmaus et al. 2006; Wu et al. 2006; Yu et al. 2000). In a small study population from Mexico

(N = 16) arsenic species have been associated with gene-specific promoter methylation in 812 genes (Bailey et al. 2013). In this study, increasing absolute levels of iAs and MMA were mostly associated with decreasing levels of promoters' DNA methylation, whereas increasing levels of DMA were inconsistently associated both with increasing and decreasing methylation of specific promoters (Bailey et al. 2013). The interpretation of these findings is unclear because absolute levels of arsenic metabolites depend not only on arsenic metabolism capability, but also on arsenic exposure levels. The association of arsenic metabolism and global DNA methylation, measured as %iAs, %MMA and %DMA, has been seldom explored. In our study the relationships of global DNA methylation and global DNA hydroxymethylation with %iAs, %MMA and %DMA were consistent in the cross-sectional and prospective analyses. Individuals with higher %DMA (faster methylators of inorganic arsenic) also had higher global DNA methylation and global DNA hydroxymethylation while those with higher %iAs and %MMA (slower methylators) tended to have lower global DNA methylation and global DNA hydroxymethylation.

Both DNA methylation and arsenic metabolism require S-adenosylmethionine (SAM) as the methyl donor. Competitive demand between arsenic metabolism and DNA methylation for SAM could affect the DNA methylation status throughout the genome (Lee et al. 2009). It is also possible that the arsenic methylation profile reflects general methylation capability in the body or that common enzymes are involved in both arsenic and DNA methylation processes. In a recent genome-wide linkage scan on arsenic metabolism in our study population (Tellez-Plaza et al. 2013), we found suggestive peaks near genes encoding several methyltransferases including PRDM9 (PR domain zinc finger protein 9 gene, a protein with histone methyltransferase activity) and EHMT1 (histone methyltransferase gene). Other minor, borderline suggestive,

peaks were near AS3MT (Arsenic (III) methyltransferase), METTL20 (methyltransferase-like 20 gene) and RNMT (RNA methyltransferase gene). In 103 Argentinean women and 127 women from Bangladesh delivering singleton infants, *AS3MT* haplotypes associated with efficient arsenic metabolism were associated with DNA methylation and gene expression of *AS3MT* and other genes in chromosome 10 (Engstrom et al. 2013). In this study, which assayed DNA methylation in a genome-wide basis by using a microarray technology, the association of *AS3MT* efficiency and arsenic metabolism and DNA methylation status in other chromosomes and genomic regions was not reported.

Evidence for plausible mechanisms for metals' effects other than the connection between arsenic and DNA-methylation metabolism on DNA-methylation is relatively scarce. Experimental *ex-vivo* evidence using M.SssI DNMT (a bacterial DNMT that recognizes the same sequence as mammalian's DNMTs) showed that cadmium exposure is an effective, non-competitive, inhibitor of DNMT (Takiguchi et al. 2003). In rat liver cells, short-term cadmium exposure induced DNA global hypomethylation. Prolonged exposure, however, resulted in global DNA hypermethylation (Takiguchi et al. 2003). Other studies are consistent with these findings (Benbrahim-Tallaa et al. 2007; Jiang et al. 2008; Poirier and Vlasova 2002). Metals could indirectly impair both one-carbon (Lee et al. 2009) and citric acid metabolic (Chia et al. 2011) pathways. It is proposed TETs are regulated by redox reaction (Dao et al. 2014). TET proteins were found to be activated under high oxygen condition and alpha-ketoglutarate (a-KG) which is generated in citric acid cycle (Chowdhury et al. 2011; Xu et al. 2011). It has been suggested that imbalance of redox reaction in cells in response to environmental stress/toxin may affect the ratio of oxidants and eventually alter production of a-KG and activation of TETs (Chia et al. 2011; Dao et al. 2014). Whether metals exposure-induced oxidative stress (Prozialeck et al. 2008;

Valko et al. 2005) affects TET-mediated hydroxymethylation is still unknown. It is also possible that, in addition to cadmium, other divalent metals can inhibit DNMT and other enzymes involved not only in one-carbon metabolism and citric acid metabolism pathways, but also in histone acetylation, deacetylation and methylation pathways (Chervona and Costa 2012; Dai and Wang 2014). More mechanistic research evaluating the role of metals in inducing DNA-methylation changes is needed.

The major limitation of this study is the small sample size. Another limitation is the tissue-dependent nature of DNA methylation and DNA hydroxymethylation changes. In our study we measured global methylation and global hydroxymethylation in blood cell DNA, which is composed of different cell types, each with a different DNA methylation profile. Information on blood cell counts was only available for visit 3. For visit 3 we could thus incorporate information on cell heterogeneity in the analysis, showing largely consistent and somewhat stronger associations (Table 3, model 2). DNA methylation markers measured in blood samples, moreover, have been related to immunologic disease, mental disease, cardiovascular disease and cancer, suggesting that blood cells can be an adequate tissue for conducting epigenetic studies in epidemiologic samples (Terry et al. 2011). Finally, creatinine may be a surrogate for several key mediators of the DNA methylation and arsenic metabolism processes as DNA methylation, arsenic methylation (Lee et al. 2009; Loenen 2006) and creatine synthesis (Brosnan et al. 2011) use S-adenosyl methionine (SAM) as the methyl-donor. Creatinine, a break-down product of creatine phosphate in muscle is generally produced at a constant rate depending on muscle mass (Heymsfield et al. 1983). Experimental models are needed to evaluate if the connection between DNA methylation and arsenic metabolism and measures of body composition are related to muscle mass.

The strengths of the study include the availability of information in several potential determinants of global DNA methylation and hydroxymethylation, including metals and arsenic metabolism, the high quality of standardized protocols for the recruitment of participants, conduction of interviews, physical examinations, collection of biological samples and laboratory analysis. Importantly, American Indian populations are an important ethnic group that has often been understudied. Findings from American Indian populations have proven to be applicable to other groups with high rates of diabetes mellitus and obesity. Our findings can thus be relevant to many populations in the US and around the world increasingly affected by the obesity and diabetes epidemics. Finally, in addition to evaluating determinants of traditional global DNA methylation, we also evaluated determinants of global DNA hydroxymethylation, which is a novel and relatively unknown epigenetic marker.

In conclusion, we found a positive correlation between global DNA methylation and global DNA hydroxymethylation in human blood samples collected in the same individuals at 2 time points, with confirmation of findings in an independent population with low burden of disease, supporting that both epigenetic measures are related at the population level. The consistency in the trend of the associations between these epigenetic modifications and categories of available determinants, specially arsenic exposure and metabolism, suggests the need for understanding which of the two measures is a better biomarker for environmental epigenetic effects in future large-scale epidemiologic studies.

References

Andrew AS, Jewell DA, Mason RA, Whitfield ML, Moore JH, Karagas MR. 2008. Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. Population. Environmental health perspectives 116:524-531.

Arita A, Costa M. 2009. Epigenetics in metal carcinogenesis: Nickel, arsenic, chromium and cadmium. Metallomics : integrated biometal science 1:222-228.

Bailey KA, Wu MC, Ward WO, Smeester L, Rager JE, Garcia-Vargas G, et al. 2013. Arsenic and the epigenome: Interindividual differences in arsenic metabolism related to distinct patterns of DNA methylation. Journal of biochemical and molecular toxicology 27:106-115.

Bailey KA, Fry RC. 2014. Arsenic-associated changes to the epigenome: What are the functional consequences?. Current environmental health reports 1:22–34.

Baubec T, Ivanek R, Lienert F, Schubeler D. 2013. Methylation-dependent and -independent genomic targeting principles of the MBD protein family. Cell 153:480-492.

Benbrahim-Tallaa L, Waterland RA, Dill AL, Webber MM, Waalkes MP. 2007. Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of de novo DNA methyltransferase. Environmental health perspectives 115:1454-1459.

Bornhorst JA, Hunt JW, Urry FM, McMillin GA. 2005. Comparison of sample preservation methods for clinical trace element analysis by inductively coupled plasma mass spectrometry. American journal of clinical pathology 123:578-583.

Bourdonnay E, Morzadec C, Sparfel L, Galibert MD, Jouneau S, Martin-Chouly C, et al. 2009. Global effects of inorganic arsenic on gene expression profile in human macrophages. Molecular immunology 46:649-656.

Branco MR, Ficz G, Reik W. 2012. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. Nature reviews Genetics 13:7-13.

Breton CV, Marutani AN. 2014. Air pollution and epigenetics: Recent findings. Current environmental health reports 1:35-45.

Brosnan JT, da Silva RP, Brosnan ME. 2011. The metabolic burden of creatine synthesis. Amino acids 40:1325-1331.

Casasnovas JA, Alcaide V, Civeira F, Guallar E, Ibanez B, Borreguero JJ, et al. 2012. Aragon workers' health study--design and cohort description. BMC cardiovascular disorders 12:45.

Castillo P, Ibanez F, Guajardo A, Llanos MN, Ronco AM. 2012. Impact of cadmium exposure during pregnancy on hepatic glucocorticoid receptor methylation and expression in rat fetus. PloS one 7:e44139.

Chen YC, Su HJ, Guo YL, Hsueh YM, Smith TJ, Ryan LM, et al. 2003. Arsenic methylation and bladder cancer risk in taiwan. Cancer causes & control : CCC 14:303-310.

Cheng TF, Choudhuri S, Muldoon-Jacobs K. 2012. Epigenetic targets of some toxicologically relevant metals: A review of the literature. Journal of applied toxicology : JAT 32:643-653.

Chervona Y, Costa M. 2012. The control of histone methylation and gene expression by oxidative stress, hypoxia, and metals. Free radical biology & medicine 53:1041-1047.

Chia N, Wang L, Lu X, Senut MC, Brenner C, Ruden DM. 2011. Hypothesis: Environmental regulation of 5-hydroxymethylcytosine by oxidative stress. Epigenetics : official journal of the DNA Methylation Society 6:853-856.

Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR, et al. 2011. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO reports 12:463-469.

Cimmino L, Abdel-Wahab O, Levine RL, Aifantis I. 2011. Tet family proteins and their role in stem cell differentiation and transformation. Cell stem cell 9:193-204.

Dai H, Wang Z. 2014. Histone modification patterns and their responses to environment. Current environmental health reports 1:11–21.

Dao T, Cheng RYS, Revelo MP, Mitzner W, Tang WT. 2014. Hydroxymethylation as a novel environmental biosensor. Current environmental health reports 1:1–10.

Del Razo LM, Garcia-Vargas GG, Vargas H, Albores A, Gonsebatt ME, Montero R, et al. 1997. Altered profile of urinary arsenic metabolites in adults with chronic arsenicism. A pilot study. Archives of toxicology 71:211-217.

Engstrom KS, Hossain MB, Lauss M, Ahmed S, Raqib R, Vahter M, et al. 2013. Efficient arsenic metabolism--the AS3MT haplotype is associated with DNA methylation and expression of multiple genes around AS3MT. PloS one 8:e53732.

Epigentek. 2014. DNA methylation quantification. Available: http://www.epigentek.com/catalog/dna-methylation-quantification-c-75_21_48.html [Accessed March 2014].

Feinberg AP. 2010. Genome-scale approaches to the epigenetics of common human disease. Virchows Archiv : an international journal of pathology 456:13-21.

Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, et al. 2011. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473:398-402.

Figueroa-Romero C, Hur J, Bender DE, Delaney CE, Cataldo MD, Smith AL, et al. 2012. Identification of epigenetically altered genes in sporadic amyotrophic lateral sclerosis. PloS one 7:e52672.

Gribble MO, Tang W, Shang T, Pollak J, Umans JG, Francesconi KA, et al. 2014. Differential methylation of the arsenic (III) methyltransferase promoter according to arsenic exposure. Archives of toxicology 88(2):275-82.

Heymsfield SB, Arteaga C, McManus C, Smith J, Moffitt S. 1983. Measurement of muscle mass in humans: Validity of the 24-hour urinary creatinine method. The American journal of clinical nutrition 37:478-494.

Hossain MB, Vahter M, Concha G, Broberg K. 2012. Low-level environmental cadmium exposure is associated with DNA hypomethylation in argentinean women. Environmental health perspectives 120:879-884.

Hou L, Zhang X, Wang D, Baccarelli A. 2012. Environmental chemical exposures and human epigenetics. International journal of epidemiology 41:79-105.

Hsueh YM, Chiou HY, Huang YL, Wu WL, Huang CC, Yang MH, et al. 1997. Serum beta-carotene level, arsenic methylation capability, and incidence of skin cancer. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 6:589-596.

Huang D, Zhang Y, Qi Y, Chen C, Ji W. 2008. Global DNA hypomethylation, rather than reactive oxygen species (ROS), a potential facilitator of cadmium-stimulated K562 cell proliferation. Toxicology letters 179:43-47.

Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A. 2010. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PloS one 5:e8888.

Jiang G, Xu L, Song S, Zhu C, Wu Q, Zhang L, et al. 2008. Effects of long-term low-dose cadmium exposure on genomic DNA methylation in human embryo lung fibroblast cells. Toxicology 244:49-55.

Jones PA. 2012. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nature reviews Genetics 13:484-492.

Karagas MR, Le CX, Morris S, Blum J, Lu X, Spate V, et al. 2001. Markers of low level arsenic exposure for evaluating human cancer risks in a US population. International journal of occupational medicine and environmental health 14:171-175.

Kile ML, Hoffman E, Rodrigues EG, Breton CV, Quamruzzaman Q, Rahman M, et al. 2011. A pathway-based analysis of urinary arsenic metabolites and skin lesions. American journal of epidemiology 173:778-786.

Kile ML, Baccarelli A, Hoffman E, Tarantini L, Quamruzzaman Q, Rahman M, et al. 2012. Prenatal arsenic exposure and DNA methylation in maternal and umbilical cord blood leukocytes. Environmental health perspectives 120:1061-1066.

Koestler DC, Avissar-Whiting M, Houseman EA, Karagas MR, Marsit CJ. 2013. Differential DNA methylation in umbilical cord blood of infants exposed to low levels of arsenic in utero. Environmental health perspectives 121:971-977.

Lam LL, Emberly E, Fraser HB, Neumann SM, Chen E, Miller GE, et al. 2012. Factors underlying variable DNA methylation in a human community cohort. Proceedings of the National Academy of Sciences of the United States of America 109 Suppl 2:17253-17260.

Lambrou A, Baccarelli A, Wright RO, Weisskopf M, Bollati V, Amarasiriwardena C, et al. 2012. Arsenic exposure and DNA methylation among elderly men. Epidemiology 23:668-676.

Lee DH, Jacobs DR, Jr., Porta M. 2009. Hypothesis: A unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environmental health perspectives 117:1799-1802.

Lee ET, Welty TK, Fabsitz R, Cowan LD, Le NA, Oopik AJ, et al. 1990. The Strong Heart Study. A study of cardiovascular disease in American Indians: Design and methods. American journal of epidemiology 132:1141-1155.

Loenen WA. 2006. S-adenosylmethionine: Jack of all trades and master of everything? Biochemical Society transactions 34:330-333.

Majumdar S, Chanda S, Ganguli B, Mazumder DN, Lahiri S, Dasgupta UB. 2010. Arsenic exposure induces genomic hypermethylation. Environmental toxicology 25:315-318.

Navas-Acien A, Umans JG, Howard BV, Goessler W, Francesconi KA, Crainiceanu CM, et al. 2009. Urine arsenic concentrations and species excretion patterns in American Indian communities over a 10-year period: The Strong Heart Study. Environmental health perspectives 117:1428-1433.

North KE, Howard BV, Welty TK, Best LG, Lee ET, Yeh JL, et al. 2003. Genetic and environmental contributions to cardiovascular disease risk in American Indians: The Strong Heart Family study. American journal of epidemiology 157:303-314.

Ordovas JM, Smith CE. 2010. Epigenetics and cardiovascular disease. Nature reviews Cardiology 7:510-519.

Pilsner JR, Liu X, Ahsan H, Ilievski V, Slavkovich V, Levy D, et al. 2007. Genomic methylation of peripheral blood leukocyte DNA: Influences of arsenic and folate in bangladeshi adults. The American journal of clinical nutrition 86:1179-1186.

Pilsner JR, Hall MN, Liu X, Ilievski V, Slavkovich V, Levy D, et al. 2012. Influence of prenatal arsenic exposure and newborn sex on global methylation of cord blood DNA. PloS one 7:e37147.

Poirier LA, Vlasova TI. 2002. The prospective role of abnormal methyl metabolism in cadmium toxicity. Environmental health perspectives 110 Suppl 5:793-795.

Prozialeck WC, Edwards JR, Nebert DW, Woods JM, Barchowsky A, Atchison WD. 2008. The vascular system as a target of metal toxicity. Toxicological sciences : an official journal of the Society of Toxicology 102:207-218.

Reichard JF, Puga A. 2010. Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2:87-104.

Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. 2011. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environmental health perspectives 119:11-19.

Ryan PB, Huet N, MacIntosh DL. 2000. Longitudinal investigation of exposure to arsenic, cadmium, and lead in drinking water. Environmental health perspectives 108:731-735.

Scheer J, Findenig S, Goessler W, Francesconi KA, Howard B, Umans JG, et al. 2012. Arsenic species and selected metals in human urine: Validation of HPLC/ICPMS and ICPMS procedures for a long-term population-based epidemiological study. Analytical methods : advancing methods and applications 4:406-413.

Shen L, Wu H, Diep D, Yamaguchi S, D'Alessio AC, Fung HL, et al. 2013. Genome-wide analysis reveals TET- and TDG-dependent 5-methylcytosine oxidation dynamics. Cell 153:692-706.

Smeester L, Rager JE, Bailey KA, Guan X, Smith N, Garcia-Vargas G, et al. 2011. Epigenetic changes in individuals with arsenicosis. Chemical research in toxicology 24:165-167.

Song CX, Szulwach KE, Dai Q, Fu Y, Mao SQ, Lin L, et al. 2013. Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. Cell 153:678-691.

Steinmaus C, Bates MN, Yuan Y, Kalman D, Atallah R, Rey OA, et al. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine 48:478-488.

Steinmaus CM, Yuan Y, Smith AH. 2005. The temporal stability of arsenic concentrations in well water in western nevada. Environmental research 99:164-168.

Stuart EA, Hanna DB. 2013. Should epidemiologists be more sensitive to design sensitivity? Epidemiology 24:88-89.

Su PF, Hu YJ, Ho IC, Cheng YM, Lee TC. 2006. Distinct gene expression profiles in immortalized human urothelial cells exposed to inorganic arsenite and its methylated trivalent metabolites. Environmental health perspectives 114:394-403.

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324:930-935.

Tajuddin SM, Amaral AF, Fernandez AF, Rodriguez-Rodero S, Rodriguez RM, Moore LE, et al. 2013. Genetic and non-genetic predictors of LINE-1 methylation in leukocyte DNA. Environmental health perspectives 121:650-656.

Takiguchi M, Achanzar WE, Qu W, Li G, Waalkes MP. 2003. Effects of cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. Experimental cell research 286:355-365.

Tellez-Plaza M, Gribble MO, Voruganti VS, Francesconi KA, Goessler W, Umans JG, et al. 2013. Heritability and preliminary genome-wide linkage analysis of arsenic metabolites in urine. Environmental health perspectives 121(3):345-51.

Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. 2011. DNA methylation in white blood cells: Association with risk factors in epidemiologic studies. Epigenetics : official journal of the DNA Methylation Society 6:828-837.

Vahter M. 2000. Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity. Toxicology letters 112-113:209-217.

Valko M, Morris H, Cronin MT. 2005. Metals, toxicity and oxidative stress. Current medicinal chemistry 12:1161-1208.

Wu MM, Chiou HY, Hsueh YM, Hong CT, Su CL, Chang SF, et al. 2006. Effect of plasma homocysteine level and urinary monomethylarsonic acid on the risk of arsenic-associated carotid atherosclerosis. Toxicology and applied pharmacology 216:168-175.

Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. 2011. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer cell 19:17-30.

Yu RC, Hsu KH, Chen CJ, Froines JR. 2000. Arsenic methylation capacity and skin cancer. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 9:1259-1262.

Zubizarreta JR, Cerda M, Rosenbaum PR. 2013. Effect of the 2010 chilean earthquake on posttraumatic stress: Reducing sensitivity to unmeasured bias through study design. Epidemiology 24:79-87.

Table 1. Baseline participant characteristics in the Strong Heart Study.

Characteristics	**SHS sample-Visit 1 (N= 48)**	**SHS and SHFS cohort[a] (N=517)**
Age, years	54.9 ± 7.2	55.0 ± 7.3
Women, number (%)	33 (68.7)	343 (66.3)
Education <high school, number (%)	19 (39.6)	214 (41.4)
Body mass index, kg/m^2	31.0 ± 5.8	31.6 ± 6.0
Waist circumference, cm	105.0 ± 14.0	106.7 ±14.3
% Body fat	36.8 ± 8.8	37.9 ± 8.9
Ever smokers, number (%)	28 (58.3)	326 (63.1)
Cumulative smoking, pack-years	8.5 ±15.5	9.3 ± 17.40
Ever alcohol drinkers, number (%)	40 (83.3)	421 (81.4)
Urine arsenic, µg/g[b]	10.02 (6.32, 16.20)	8.10 (5.10, 14.6)
%iAs	8.53 (5.53, 10.71)	7.63 (5.44, 10.30)
%MMA	14.35 (11.02, 17.82)	13.69 (10.60, 16.93)
%DMA	78.32 (71.27, 81.39)	78.69 (72.15, 83.14)
Cadmium, µg/g	0.88 (0.52, 1.45)	0.92 (0.61, 1.45)
Antimony, µg/g	0.27 (0.17, 0.46)	0.22 (0.15, 0.36)
Tungsten, µg/g	0.13 (0.08, 0.27)	0.14 (0.07, 0.25)

Data in the table are mean ± SD, number (%) or median (interquartile range).

Abbreviations: NA, not available; %iAs, Percentage of inorganic arsenic over the sum of inorganic and methylated arsenic species; %MMA, percentage of monomethylarsonate over the sum of inorganic and methylated arsenic species; %DMA, percentage of dimethylarsinate over the sum of inorganic and methylated arsenic species.

The sample size for body mass index was only 47 and for waist circumference and cigarette pack-years it was 46.

[a]Strong Heart Study participants with urine metal measures who also participated in the Strong Heart Family Study. [b]Sum of inorganic and methylated arsenic species in urine.

Table 2. Cross-sectional associations (Odds ratio [95% CI]) of global DNA methylation (% 5-mC) and global DNA hydroxymethylation (% 5-hmC) in 1989-1991 (visit 1) by participant characteristics in the Strong Heart Study.

Characteristics	N	Methylation, Model 1	Methylation, Model 2	Hydroxymethylation, Model 1	Hydroxymethylation, Model 2
Age, years					
<54	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥54	24	0.79 (0.48, 1.30)	0.78 (0.46, 1.32)	0.76 (0.46, 1.26)	0.90 (0.54, 1.50)
Sex					
Male	15	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Female	33	1.37 (0.80, 2.33)	1.36 (0.77, 2.38)	1.20 (0.69, 2.07)	1.23 (0.72, 2.10)
Education, years					
≥12	29	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
<12	19	1.23 (0.74, 2.05)	1.18 (0.67, 2.08)	0.64 (0.39, 1.07)	0.74 (0.43, 1.26)
BMI, kg/m^2					
<30	20	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥30	27	1.35 (0.81, 2.25)	1.35 (0.80, 2.28)	1.20 (0.73, 1.98)	1.17 (0.71, 1.95)
Waist circumference, cm					
<Sex-specific median[a]	20	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥Sex-specific median	26	0.90 (0.53, 1.53)	0.88 (0.52, 1.50)	1.05 (0.63, 1.75)	0.99 (0.59, 1.65)
% Body fat					
<Sex-specific median	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥Sex-specific median	24	0.94 (0.56, 1.56)	0.90 (0.53, 1.51)	1.47 (0.91, 2.38)	1.58 (0.98, 2.56)
Smoking					
Never	20	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Ever	28	0.81 (0.49, 1.35)	0.81 (0.47, 1.38)	1.02 (0.61, 1.72)	1.09 (0.65, 1.82)
Alcohol status					
Never	8	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Ever	40	0.97 (0.49, 1.90)	1.06 (0.46, 2.43)	0.85 (0.43, 1.68)	0.71 (0.32, 1.58)
Urine arsenic, µg/g[b]					
<7.2	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥14.0	24	1.05 (0.64, 1.74)	1.05 (0.61, 1.80)	0.74 (0.44, 1.22)	0.73 (0.44, 1.22)
%iAs					
<8.5	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥8.5	23	0.85 (0.51, 1.43)	0.78 (0.44, 1.39)	0.56 (0.34, 0.92)	0.61 (0.36, 1.01)
%MMA					
< 14.4	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥ 14.4	23	0.73 (0.44, 1.21)	0.78 (0.44, 1.40)	0.59 (0.36, 0.97)	0.71 (0.42, 1.20)
%DMA					
<78.3	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥78.3	23	1.56 (0.95, 2.57)	1.64 (0.93, 2.88)	1.76 (1.07, 2.88)	1.59 (0.95, 2.66)
Cadmium, µg/g					
<0.87	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.87	24	1.64 (1.02, 2.65)	1.75 (0.96, 3.20)	1.16 (0.70, 1.94)	1.08 (0.59, 1.97)
Antimony, µg/g					
<0.27	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.27	24	1.37 (0.84, 2.24)	1.24 (0.71, 2.15)	1.07 (0.64, 1.78)	1.08 (0.64, 1.85)
Tungsten, µg/g					
<0.13	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.13	24	1.50 (0.92, 2.45)	1.46 (0.85, 2.52)	1.25 (0.76, 2.08)	1.13 (0.66, 1.92)
Global DNA methylation, % 5-mC (visit 1)					
<0.32	24	NA	NA	1.00 (Ref)	1.00 (Ref)
≥0.32	24	NA	NA	1.36 (0.82, 2.25)	1.56 (0.95, 2.56)

Characteristics	N	Methylation, Model 1	Methylation, Model 2	Hydroxymethylation, Model 1	Hydroxymethylation, Model 2
Global DNA hydroxy-methylation, % 5-hmC (visit 1)					
<0.12	23	1.00 (Ref)	1.00 (Ref)	NA	NA
≥0.12	25	1.63 (1.01, 2.64)	1.93 (1.15, 3.26)	NA	NA

Abbreviations: NA, Not available; %iAs, Percentage of inorganic arsenic over the sum of inorganic and methylated arsenic species; %MMA, percentage of monomethylarsonate over the sum of inorganic and methylated arsenic species; %DMA, percentage of dimethylarsinate over the sum of inorganic and methylated arsenic species.
Model 1 was unadjusted. Model 2 was adjusted for age (years), sex (men, women), smoking status (never, former, current) and body mass index (kg/m^2).

[a]Sex-specific median for waist circumference and percent body fat were 130 cm and 29.8% in men and 130 cm and 41.40% in women.

[b]Sum of inorganic and methylated arsenic species in urine.

Table 3. Prospective associations (odds ratio [95% CI]) of global DNA methylation (% 5-mC) and global DNA hydroxymethylation (% 5-hmC) in 1993-1995 (visit 3) by participant characteristics in the Strong Heart Study.

Characteristics	N	Methylation, Model 1	Methylation, Model 2	Methylation, Model 3	Hydroxymethylation, Model 1	Hydroxymethylation, Model 2	Hydroxymethylation, Model 3
Age, years							
<54	21	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥54	23	0.62 (0.34, 1.10)	0.62 (0.35, 1.12)	0.56 (0.31, 1.03)	0.63 (0.38, 1.10)	0.63 (0.38, 1.05)	0.64 (0.37, 1.10)
Sex							
Male	15	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Female	29	0.76 (0.41, 1.42)	0.80 (0.42, 1.53)	0.69 (0.36, 1.33)	1.20 (0.70, 2.08)	1.24 (0.70, 2.18)	1.16 (0.66, 2.04)
Education, years							
≥12	27	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
<12	17	1.08 (0.58, 1.99)	1.06 (0.56, 2.01)	1.07 (0.54, 2.09)	1.20 (0.71, 2.05)	1.17 (0.67, 2.04)	1.23 (0.69, 2.19)
BMI, kg/m^2							
<30	18	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥30	25	1.67 (0.92, 3.05)	1.69 (0.91, 3.14)	1.69 (0.92, 3.10)	1.05 (0.61, 1.80)	1.08 (0.62, 1.89)	1.01 (0.59, 1.73)
Waist circumference, cm							
<Sex-specific median[a]	19	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥Sex-specific median	24	1.19 (0.64, 2.20)	1.16 (0.63, 2.17)	1.16 (0.63, 2.14)	0.86 (0.51, 1.47)	0.85 (0.50, 1.47)	0.82 (0.49, 1.38)
% Body fat							
<Sex-specific median	21	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥Sex-specific median	22	1.61 (0.89, 2.92)	1.60 (0.87, 2.93)	1.45 (0.79, 2.65)	1.48 (0.88, 2.49)	1.50 (0.89, 2.55)	1.41 (0.84, 2.36)
Smoking							
Never	19	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Ever	25	0.58 (0.32, 1.03)	0.61 (0.33, 1.13)	0.51 (0.27, 0.98)	0.69 (0.41, 1.16)	0.71 (0.41, 1.24)	0.68 (0.39, 1.18)
Alcohol status							
Never	8	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
Ever	36	0.86 (0.40, 1.87)	0.84 (0.38, 1.85)	0.93 (0.35, 2.44)	0.89 (0.45, 1.75)	0.89 (0.45, 1.78)	0.90 (0.39, 2.07)
Urine arsenic, µg/g[b]							
<7.2	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥14.0	21	0.58 (0.33, 1.03)	0.54 (0.30, 0.97)	0.59 (0.32, 1.10)	0.78 (0.46, 1.30)	0.73 (0.43, 1.25)	0.80 (0.46, 1.39)
%iAs							
<8.5	21	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥8.5	22	0.91 (0.50, 1.68)	0.90 (0.48, 1.67)	0.78 (0.40, 1.50)	0.83 (0.49, 1.40)	0.79 (0.46, 1.37)	0.71 (0.40, 1.25)
%MMA							
<14.4	21	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥14.4	22	0.73 (0.40, 1.34)	0.73 (0.40, 1.34)	0.70 (0.36, 1.37)	0.58 (0.35, 0.96)	0.58 (0.35, 0.98)	0.62 (0.35, 1.10)
%DMA							
<78.3	22	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥78.3	21	1.57 (0.87, 2.85)	1.61 (0.88, 2.94)	1.75 (0.92, 3.32)	1.34 (0.79, 2.26)	1.38 (0.81, 2.36)	1.32 (0.75, 2.35)
Cadmium, µg/g							
<0.87	24	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.87	20	0.86 (0.47, 1.56)	0.90 (0.49, 1.66)	1.03 (0.50, 2.11)	1.04 (0.62, 1.76)	1.07 (0.62, 1.83)	0.97 (0.52, 1.80)

Characteristics	N	Methylation, Model 1	Methylation, Model 2	Methylation, Model 3	Hydroxymethylation, Model 1	Hydroxymethylation, Model 2	Hydroxymethylation, Model 3
Antimony, µg/g							
<0.27	22	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.27	22	1.71 (0.96, 3.04)	1.93 (1.07, 3.47)	2.15 (1.15, 4.01)	1.15 (0.69, 1.94)	1.22 (0.71, 2.10)	1.16 (0.65, 2.07)
Tungsten, µg/g							
<0.13	23	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.13	21	0.84 (0.46, 1.52)	0.90 (0.47, 1.71)	0.93 (0.46, 1.86)	1.25 (0.75, 2.10)	1.40 (0.81, 2.44)	1.32 (0.73, 2.37)
Global DNA methylation, % of 5-mC (visit 1)							
<0.32	22	NA	NA	NA	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
≥0.32	22	NA	NA	NA	1.24 (0.74, 2.08)	1.21 (0.71, 2.06)	1.15 (0.67, 1.96)
Global DNA hydroxy-methylation, % of 5-hmC (visit 1)							
<0.12	20	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)	NA	NA	NA
≥0.12	24	1.23 (0.68, 2.24)	1.16 (0.63, 2.16)	1.28 (0.67, 2.45)	NA	NA	NA

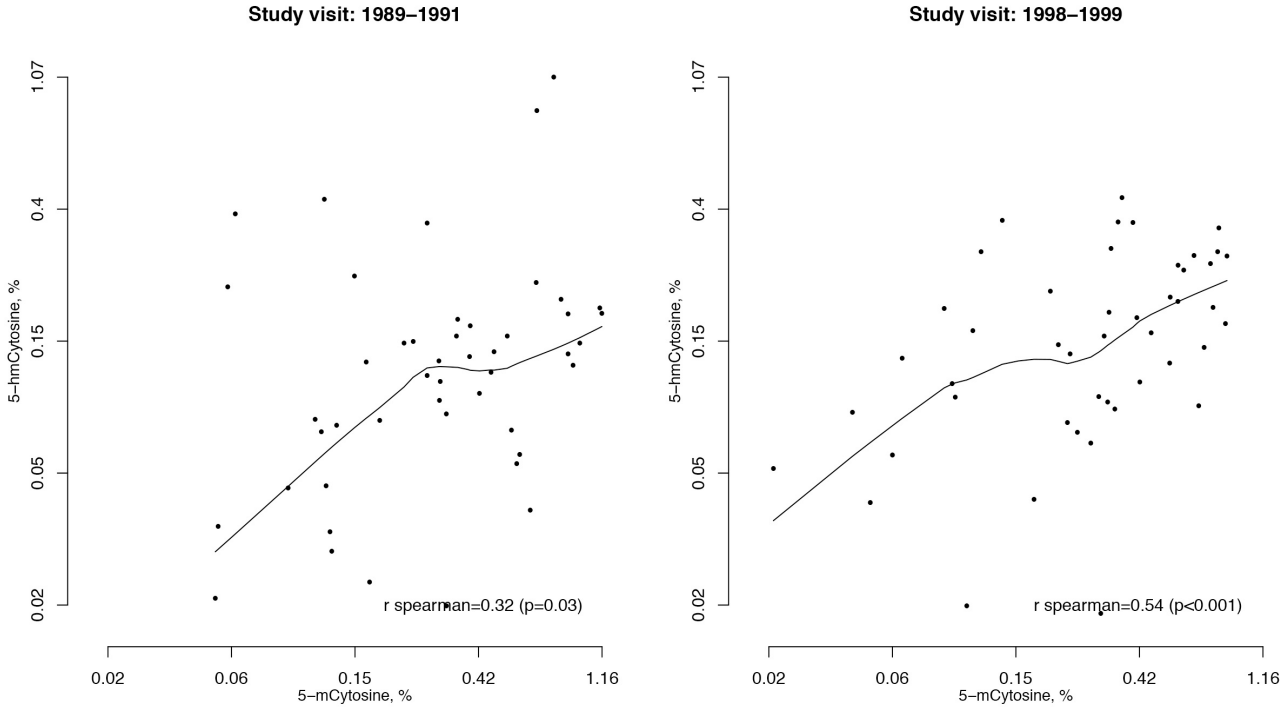
Abbreviations: NA, Not available; %iAs, Percentage of inorganic arsenic over the sum of inorganic and methylated arsenic species; %MMA, percentage of monomethylarsonate over the sum of inorganic and methylated arsenic species; %DMA, percentage of dimethylarsinate over the sum of inorganic and methylated arsenic species.

Model 1 was unadjusted. Model 2 was adjusted for log-transformed total count of white blood cells and percent of neutrophils. Model 3 was model 2 further adjusted for age (years), sex (men, women), smoking status (never, former, current) and body mass index (kg/m^2). [a]Sex-specific median for waist circumference and percent body fat were 130 cm and 29.8% in men and 130 cm and 41.40% in women. [b]Sum of inorganic and methylated arsenic species in urine.

Figure legend

Figure 1. Relationship of global DNA methylation (% 5-mC) and global DNA hydroxymethylation (% 5-hmC) in blood collected in two study visits 10 years apart, Strong Heart Study.

Figure 1


Study visit: 1989–1991
5–hmCytosine, %
1.07
0.4
0.15
0.05
0.02
r spearman=0.32 (p=0.03)
0.02
0.06
0.15
0.42
1.16
5–mCytosine, %
Study visit: 1998–1999
5–hmCytosine, %
1.07
0.4
0.15
0.05
0.02
r spearman=0.54 (p<0.001)
0.02
0.06
0.15
0.42
1.16
5–mCytosine, %