Research Article

Sex-Specific Associations of Arsenic Exposure with Global DNA Methylation and Hydroxymethylation in Leukocytes: Results from Two Studies in Bangladesh

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Abstract

Background: Depletion of global 5-hydroxymethylcytosine (5-hmC) is observed in human cancers and is strongly implicated in skin cancer development. Although arsenic (As)—a class I human carcinogen linked to skin lesion and cancer risk—is known to be associated with changes in global %5-methylcytosine (%5-mC), its influence on 5-hmC has not been widely studied.

Methods: We evaluated associations of As in drinking water, urine, and blood with global %5-mC and %5-hmC in two studies of Bangladeshi adults: (i) leukocyte DNA in the Nutritional Influences on Arsenic Toxicity study ($n = 196$; 49% male, 19–66 years); and (ii) peripheral blood mononuclear cell DNA in the Folate and Oxidative Stress study ($n = 375$; 49% male, 30–63 years).

Results: Overall, As was not associated with global %5-mC or %5-hmC. Sex-specific analyses showed that associations of As

exposure with global %5-hmC were positive in males and negative in females (P for interaction < 0.01). Analyses examining interactions by elevated plasma total homocysteine (tHcys), an indicator of B-vitamin deficiency, found that tHcys also modified the association between As and global %5-hmC (P for interaction < 0.10).

Conclusion: In two samples, we observed associations between As exposure and global %5-hmC in blood DNA that were modified by sex and tHcys.

Impact: Our findings suggest that As induces sex-specific changes in 5-hmC, an epigenetic mark that has been associated with cancer. Future research should explore whether altered %5-hmC is a mechanism underlying the sex-specific influences of As on skin lesion and cancer outcomes. *Cancer Epidemiol Biomarkers Prev; 24(11); 1748–57. ©2015 AACR.*

Introduction

Roughly 70 million people in Bangladesh are exposed to inorganic arsenic (As) in drinking water at concentrations above the World Health Organization (WHO) guideline of 10 µg/L (1). As a class I human carcinogen (2), chronic As exposure is associated with the development of precancerous skin lesions (i.e., melanosis and keratosis) and increased risks for cancers of

the skin, liver, lung, bladder, and kidney (3–6). Susceptibility to As-induced health outcomes varies dramatically across individuals and is often sex-dependent (7). For example, men have a higher incidence for skin lesions (8), whereas women appear to be at greater risk for several cancers (9). However, mechanisms of As toxicity in humans, particularly the mechanisms underlying the sex differences in As-related health outcomes, are not well understood.

Accumulating evidence suggests that As-induced changes in DNA methylation might be an important pathway of As toxicity. Because methylation of As and CpGs both require methyl groups from *S*-adenosylmethione (SAM), a product of B-vitamin–dependent one-carbon metabolism, it was initially hypothesized that As exposure would lead to decreased global DNA methylation through competition for methyl groups, and that this would be exacerbated by B-vitamin deficiency (10). However, in the first human study on the subject, our group found that chronic As exposure was positively associated with global methylation of leukocyte DNA, contingent upon adequate folate status (11). Several subsequent human studies have observed associations between As exposure and changes in global and gene-specific DNA methylation in leukocytes (12–24) and have also identified changes in DNA methylation that are associated with As-induced health conditions (25–33).

In 2009, Tahiliani and colleagues reported that ten-eleven translocation (TET) enzymes catalyze the oxidation of

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5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC; ref. 34). The 5-hmC mark is an intermediate in active and passive DNA demethylation pathways (35), and as opposed to 5-mC, increased 5-hmC abundance is found in gene bodies (36) and is generally associated with gene activation and cellular pluripotency (37). Although the biologic functions of 5-hmC are incompletely understood, global 5-hmC depletion has been implicated as a biomarker of malignant transformation (38, 39) and is an epigenetic "hallmark" of melanoma (40). However, little information is available regarding the influence of As exposure on global 5-hmC in humans, primarily because common methods used to assess DNA methylation in epidemiologic studies do not distinguish between 5-mC and 5-hmC (41).

Thus, our primary objective was to examine the association between As exposure and global %5-mC and %5-hmC in leukocyte DNA in two sets of Bangladeshi adults who were chronically exposed to As in drinking water. Based on our previous observations of sex-specific effects of As on epigenetic disruption (23, 42), we wished to examine whether these associations differed by sex. The first sample examined leukocyte DNA from 196 subjects from the Nutritional Influences of Arsenic Toxicity (NIAT) folic acid clinical trial at baseline (43). The second sample examined peripheral blood mononuclear cell (PBMC) DNA from 375 subjects from the Folate and Oxidative Stress (FOX) study, a cross-sectional study originally designed to assess the relationship between As exposure and oxidative stress (44). In addition, we explored whether plasma total homocysteine (tHcys), a sensitive biomarker of B-vitamin status, modified these associations.

Materials and Methods

Eligibility criteria and study design

The NIAT trial has been described previously (43). Participants were drawn from a cross-sectional study of 1,650 participants designed to assess the prevalence of folate and vitamin B_{12} deficiencies in Araihazar, Bangladesh (45); participants ($n = 200$) were randomly selected from subjects in the lowest tertile of plasma folate ($n = 550$; ref. 43). Participants were excluded if they were pregnant or B_{12} deficient (plasma vitamin $B_{12} \leq 185$ pmol/L) or were taking nutritional supplements. The current study includes all NIAT subjects with DNA samples at baseline ($n = 196$).

The FOX study design has also been described previously (44). Briefly, 378 subjects were recruited in Araihazar based on well water As (wAs) exposure categories such that the final study sample represented the full range of wAs concentrations in the region. Participants were excluded if they were pregnant, had taken nutritional supplements in the past 3 months, or had known diabetes, cardiovascular, or renal disease.

Oral informed consent was obtained by our Bangladeshi field staff physicians, who read an approved consent form to the study participants. The studies were approved by the Institutional Review Boards of the Bangladesh Medical Research Council and of Columbia University Medical Center.

Analytic techniques

Sample collection and handling. For the NIAT study, blood samples were drawn in the field (11). For the FOX study, blood samples were collected at the field clinic laboratory in Araihazar and immediately processed (12). Aliquots of blood and plasma were stored at $-80°C$, urine samples were stored at $-20°C$ in acid-washed polypropylene tubes, and PBMC lysates were stored in preservative buffer at $4°C$. All samples were shipped on dry ice to Columbia University.

Water As. In NIAT, water samples were analyzed using graphite furnace atomic absorption (GFAA) spectrometry, with a detection limit of 5 µg/L; water samples with nondetectable As using GFAA were analyzed with inductively coupled mass spectrometry (ICP-MS), with a detection limit of 0.1 µg/L. In FOX, all water samples were analyzed using ICP-MS. The intra- and interassay coefficients of variation (CV) in FOX were 6% and 4%, respectively.

Urinary As metabolites and urinary creatinine. Urinary As (uAs) metabolites—arsenobetaine (AsB), arsenocholine (AsC), As^V, As^{III}, MMA (MMA^{III} + MMA^V), and DMA (DMA^V)—were separated using high-performance liquid chromatography (HPLC), and metabolite concentrations were measured using ICP-MS (46). Total uAs was calculated by summing the concentrations of As^V, As^{III}, MMA, and DMA, excluding AsC and AsB. Urinary creatinine (uCr) was analyzed by a colorimetric assay (47) and used to adjust for urine concentration. In FOX, the intraassay CVs were 5% for As^V, 4% for As^{III}, 2% for MMA, and 1% for DMA, and the interassay CVs were 11% for As^V, 10% for As^{III}, 4% for MMA, and 3% for DMA.

Blood As. In FOX, total blood As (bAs) was analyzed using ICP-MS, as previously described (48). The intra- and interassay CVs were 3% and 6%, respectively.

Plasma homocysteine. Plasma tHcys was measured using the method of Pfeiffer and colleagues (49) as previously described (44, 45). In NIAT, the within- and between-day CVs for tHcys were 5% and 8%, respectively, and in FOX, the within- and between-day CVs were 2% and 9%, respectively.

DNA isolation. In NIAT, DNA was isolated from whole blood using silica membrane spin columns (GenomicPrep Blood DNA Isolation Kit; Amersham Biosciences; ref. 11). In FOX, DNA was isolated from PBMC lysates with Protein Precipitation Solution (5-Prime) and standard isopropanol extraction (12).

Global %5-mC and %5-hmC. Global %5-mC and %5-hmC were analyzed using LC-MS/MS with biosynthetic [U-^{15}N]deoxycytidine and [U-^{15}N]methyldeoxycytidine internal standards (50). Briefly, DNA samples (1 µg DNA in 50 µL water) were hydrolyzed into nucleosides, transferred to vials with 4 µL [U-^{15}N]DNA internal standard, and measured using LC-MS/MS (Agilent Technologies). Detailed instrumentation settings can be found in Supplementary Materials and Methods.

In FOX, one aliquot (10 µg) of a DNA control sample was hydrolyzed, and aliquots were run in duplicate with each sample batch which were run over 5 days; the intra- and interday CVs for %5-mC were 1% and 2%, respectively, and for %5-hmC, the intra- and interday CVs were 7% and 14%, respectively. In NIAT, a subset of 48 subjects were selected for which aliquots of their original DNA samples were hydrolyzed and analyzed in a separate batch, using a different batch of [U-^{15}N]DNA internal standard; for these samples, the interassay CVs for %5-mC and %5-hmC were 3% and 12%, respectively.

Statistical methods

Descriptive statistics (mean and SDs for continuous variables, counts, and proportions for dichotomous variables) were calculated for each sample overall and separately by sex. χ^2 tests and Wilcoxon rank sum tests were used to detect sex differences in categorical and continuous variables for sample characteristics. To examine bivariate associations between demographic characteristics and global %5-mC and %5-hmC, Spearman correlations were used for continuous variables, and the Kruskal–Wallis test was used to detect difference in each continuous variable among the categories of a categorical variable. Linear regression models were used to examine the associations between total As exposure variables and global %5-mC and %5-hmC, adjusting for covariates. Model covariates were selected based on biologic plausibility (sex and age for both global %5-mC and %5-hmC) and/or their associations with any As exposure variables and with either global %5-mC and/or %5-hmC [ever cigarette smoking for global %5-mC and %5-hmC, ever betel nut chewing and body mass index (BMI) for global %5-hmC]. The Wald test was used to detect differences in estimated coefficients of As variables between sex-specific and HHcys-specific models.

Urinary As was adjusted for urinary Cr using the residual method. To estimate these adjusted values, linear regression models were constructed with log-transformed uCr as the predictor of log-transformed uAs. The residuals from this model were added back to the mean log-transformed uAs and exponentiated to get the final uCr-adjusted uAs values. The uCr-adjusted urinary As variable was used for all analyses involving uAs.

From the total FOX sample of 378 subjects, there were 2 subjects with no DNA and 1 subject with a global %5-hmC value (0.0042%) identified as an extreme outlier (defined as > 3 interquartile ranges below the 25th percentile for the study sample) who was excluded, resulting in a final sample size of 375 subjects. All statistical analyses were conducted using SAS (version 9.3; SAS Institute Inc.).

Results

Demographic and clinical characteristics are presented in Table 1. In both samples, males were older, had a lower BMI, and had a higher proportion of individuals who reported a history of smoking cigarettes. Mean wAs and uAs concentrations were similar between males and females, whereas the prevalence of HHcys was higher among males (NIAT, 50% in males, 23% in females; FOX, 36% in males, 19% in females).

Global %5-mC and %5-hmC were normally distributed overall and by sex. Global %5-mC and %5-hmC were not correlated in NIAT (males, Spearman $r = -0.04$, $P = 0.67$; females, Spearman $r = 0.01$, $P = 0.91$) and positively correlated in FOX (males, Spearman $r = 0.44$, $P < 0.0001$; females, Spearman $r = 0.51$, $P < 0.0001$). In unadjusted analyses, males had 0.06% higher mean global %5-mC (NIAT: males, 4.64% ± 0.09%, females, 4.58% ± 0.09%, $P < 0.0001$; FOX: males, 4.60% ± 0.11%, females, 4.54% ± 0.12%, $P < 0.0001$), whereas mean global %5-hmC was two orders of magnitude lower than global %5-mC (NIAT, 0.032% ± 0.004%; FOX, 0.031% ± 0.006%) and did not differ by sex (Table 1). Bivariate analyses showed that age was negatively correlated with global %5-mC in FOX (NIAT, Spearman $r = 0.05$, $P = 0.47$; FOX, Spearman $r =$

-0.12, $P = 0.02$) and with global %5-hmC in both studies (NIAT, Spearman $r = -0.24$, $P = 0.0006$; FOX, Spearman $r = -0.17$, $P = 0.0012$).

Associations between As exposure (as measured by wAs, uAs, and bAs) with global %5-mC and %5-hmC are shown in Table 2. (Due to the small effect estimate values, all effect estimates are reported with a multiplier of 10^4, i.e., an effect estimate of 1.0 in the table corresponds to an actual effect estimate of 0.0001.) In the overall samples, As exposure was not associated with global %5-mC in models adjusting for sex, age, and smoking. Upon stratification by sex, the As variables were positively associated with global %5-mC in males, whereas no consistent relationship was observed in females. Formal tests of interaction indicated that associations for wAs and bAs differed by sex in both samples (P for interaction < 0.10).

Similar to the overall results for global %5-mC, As exposure was not associated with global %5-hmC in overall adjusted models (Table 2). However, sex-specific models showed that As exposure was positively associated with global %5-hmC in males and negatively associated in females (P for interaction < 0.01 for all As variables). In order to illustrate visually the interaction by sex, we generated forest plots separately by sex and across the two studies (Fig. 1). Although the effect estimates appeared small in magnitude, a small effect estimate corresponded to an appreciable estimated change in %5-hmC. For example, the effect estimate [95% confidence interval (CI)] for a 10 μg/L increase in wAs in NIAT males was 0.00007 (0.00001–0.00014; $P = 0.03$). To put this into context, for a hypothetical subject with a global %5-hmC value of 0.03255% (the mean value in NIAT males), the model estimates that an increase in wAs exposure from 50 μg/L to 150 μg/L is associated with an increase in global %5-hmC to 0.03325%, an estimated 2.2% increase in the global 5-hmC level.

Next, we explored whether HHcys modified the associations of wAs with global %5-mC and %5-hmC (Table 3). For global %5-mC, we did not find evidence that the association of wAs was modified by HHcys overall or among males in either study. Results were suggestive of a possible interaction among females, where wAs was negatively associated with global %5-mC in females with HHcys (i.e., with evidence of B-vitamin deficiency; P for interaction = 0.15 in NIAT and FOX). For global %5-hmC, overall effect estimates of the associations of wAs with global %5-hmC were more negative in subjects with HHcys. In stratified analyses, the positive association of wAs with global %5-hmC was restricted to males without HHcys (i.e., without evidence of B-vitamin deficiency), and in females, the negative association of wAs with global %5-hmC was observed in both strata, but was stronger among females with HHcys (i.e., females with evidence of B-vitamin deficiency).

To further explore the associations of As exposure and other study characteristics with global %5-hmC, we constructed regression models adjusted for sex, age, cigarette smoking, betel nut chewing, BMI, HHcys, As, and As*sex and As*HHcys cross-product terms. As shown in Table 4, interactions of As exposure with sex and HHcys were found for all As variables in both samples (P for interaction < 0.10, with the exception of the uAs*HHcys cross-product term in NIAT, with P for interaction = 0.27). In addition, we found a strong negative association of age with global %5-hmC ($P < 0.0001$). Effect estimates for age and other study characteristics were not found to differ by sex (data not shown). To examine whether any differences between NIAT and FOX were related to the different age ranges between the studies, we

Table 1. Demographic and clinical characteristics of NIAT and FOX study samples

Variables	NIAT Overall $n=196$	NIAT Males $n=96$	NIAT Females $n=100$	NIAT Sex diff. (P)	FOX Overall $n=375$	FOX Males $n=183$	FOX Females $n=192$	FOX Sex diff. (P)
Demographic								
Age (y)	38.7 ± 10.4 (19–66)	42.3 ± 10.2 (25–66)	35.1 ± 9.3 (19–57)	<0.0001	43.1 ± 8.3 (30–63)	44.5 ± 8.6 (31–63)	41.7 ± 7.7 (30–62)	0.002
BMI (kg/m²)[a]	19.9 ± 3.1 (14.4–32.5)	19.5 ± 2.7 (15.1–25.9)	20.2 ± 3.5 (14.4–32.5)	0.14	20.4 ± 3.5 (13.8–35.3)	19.7 ± 3.2 (14.2–32.3)	21.0 ± 3.7 (13.8–35.3)	0.0004
Cigarette smoking (ever)	81 (41.3)	75 (78.1)	6 (6.0)	<0.0001	137 (36.5)	126 (68.9)	11 (5.7)	<0.0001
Betel nut chewing (ever)	76 (38.8)	42 (43.8)	34 (34.0)	0.16	160 (42.7)	81 (44.3)	79 (41.2)	0.54
Arsenic exposure								
Water As (µg/L)	105 ± 103 (0.1–435)	107 ± 107 (0.1–399)	102 ± 97 (0.4–435)	0.94	139 ± 125 (0.4–700)	145 ± 135 (0.4–700)	134 ± 114 (0.4–493)	0.75
Blood As (µg/L)	—	—	—	—	13.4 ± 9.8 (1.2–57.0)	15.0 ± 10.9 (1.3–57.0)	11.9 ± 8.4 (1.2–39.4)	0.008
Urinary As adjusted for uCr (µg/L)[b]	121 ± 99 (12–544)	108 ± 87 (12–544)	133 ± 107 (19–544)	0.07	161 ± 120 (10–548)	154 ± 117 (10–548)	167 ± 124 (10–503)	0.39
Urinary %InAs	15.2 ± 6.8 (6.0–60.1)	15.1 ± 6.0 (7.1–43.2)	15.3 ± 7.5 (6.0–60.1)	0.72	17.7 ± 5.5 (6.7–51.8)	17.4 ± 4.9 (8.3–42.9)	18.0 ± 6.1 (6.7–51.8)	0.56
Urinary %MMA	12.9 ± 4.3 (3.8–26.9)	15.0 ± 4.0 (5.4–24.1)	11.3 ± 4.0 (3.8–26.9)	<0.0001	14.0 ± 5.0 (3.6–30.0)	16.0 ± 5.0 (5.6–30.0)	12.0 ± 4.3 (3.6–26.7)	<0.0001
Urinary %DMA	79.1 ± 8.4 (36.2–87.9)	70.4 ± 7.7 (39.3–87.5)	73.3 ± 8.7 (36.2–87.9)	0.003	68.3 ± 7.9 (38.3–88.0)	66.6 ± 7.5 (39.5–82.1)	70.0 ± 7.9 (38.3–88.0)	<0.0001
DNA methylation								
Global %5-mC (% of total C)[c]	4.61 ± 0.10 (4.36–4.99)	4.58 ± 0.09 (4.36–4.88)	4.64 ± 0.09 (4.45–4.99)	<0.0001	4.57 ± 0.12 (4.24–4.85)	4.60 ± 0.11 (4.26–4.85)	4.54 ± 0.12 (4.24–4.83)	<0.0001
Global %5-hmC (% of total C)	0.032 ± 0.004 (0.025–0.044)	0.033 ± 0.004 (0.025–0.044)	0.032 ± 0.004 (0.015–0.049)	0.70	0.031 ± 0.006 (0.015–0.051)	0.031 ± 0.006 (0.015–0.049)	0.031 ± 0.006 (0.018–0.051)	0.61
Homocysteine								
Plasma tHcys (µmol/L)[c]	10.9 ± 5.1 (0.2–39.8)	12.6 ± 5.2 (6.0–39.8)	9.2 ± 4.4 (0.2–36.5)	<0.0001	11.2 ± 13.0 (3.0–165.6)	14.0 ± 17.6 (5.0–165.6)	8.6 ± 5.0 (3.0–57.6)	<0.0001
HHcys (males > 11.4 µmol/L, females > 10.4 µmol/L)	71 (36.2)	48 (50.0)	23 (23.0)	0.0001	104 (27.7)	67 (36.6)	37 (19.3)	0.0002

[a]NIAT overall, $n = 194$; NIAT females, $n = 98$; FOX overall, $n = 374$, FOX females, $n = 191$.
[b]NIAT overall, $n = 190$; NIAT males, $n = 92$; NIAT females, $n = 98$.
[c]NIAT overall, $n = 194$; NIAT males, $n = 95$; NIAT females, $n = 99$.

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Table 2. Associations of As exposure with global %5-mC and %5-hmC

Outcome	Sample	Predictor, unit	Overall Effect estimate (95% CI)[a]	Overall P	Males Effect estimate (95% CI)[a]	Males P	Females Effect estimate (95% CI)[a]	Females P	P for interaction by sex[b]
Global %5-mC[c]	NIAT[d]	wAs, 10 μg/L	−0.17 (−13.47, 13.13)	0.98	14.39 (−4.12, 32.88)	0.13	−11.05 (−30.96, 8.86)	0.27	0.07
		uAs, 10 μg/L	6.89 (−7.68, 21.46)	0.35	18.25 (−5.36, 41.86)	0.13	5.13 (−14.09, 24.35)	0.60	0.39
	FOX[e]	wAs, 10 μg/L	−0.82 (−9.91, 8.27)	0.86	10.51 (−1.45, 22.47)	0.08	−14.11 (−28.26, 0.03)	0.05	0.01
		uAs, 10 μg/L	3.17 (−6.24, 12.58)	0.51	11.61 (−2.00, 25.23)	0.09	−3.79 (−16.96, 9.39)	0.57	0.11
		bAs, 1 μg/L	5.66 (−6.04, 17.37)	0.34	15.75 (1.07, 30.44)	0.04	−8.66 (−28.03, 10.71)	0.38	0.05
Global %5-hmC[f]	NIAT[d]	wAs, 10 μg/L	0.14 (−0.39, 0.66)	0.61	0.75 (0.05–1.44)	0.03	−0.81 (−1.59, −0.04)	0.04	0.003
		uAs, 10 μg/L	0.25 (−0.32, 0.82)	0.38	1.02 (0.16–1.89)	0.02	−0.61 (−1.38, 0.15)	0.11	0.005
	FOX[e]	wAs, 10 μg/L	−0.23 (−0.68, 0.23)	0.33	0.55 (−0.08, 1.18)	0.09	−1.01 (−1.70, −0.32)	0.01	0.001
		uAs, 10 μg/L	−0.01 (−0.49, 0.47)	0.96	0.82 (0.10–1.55)	0.03	−0.61 (−1.25, 0.04)	0.07	0.004
		bAs, 1 μg/L	0.16 (−0.43, 0.75)	0.59	0.91 (0.14–1.67)	0.02	−0.76 (−1.71, 0.18)	0.11	0.007

[a]Effect estimates expressed with multiplier of 10^4 (i.e., effect estimate of 1.0 in table corresponds to actual effect estimate of 0.0001).
[b]Interaction P value from Wald test for group difference in the regression coefficient.
[c]Adjusted for sex, age (categorical), and cigarette smoking.
[d]Overall, $n = 196$; males, $n = 100$; females, $n = 96$; $n = 4$ males and $n = 2$ females with missing uAs; $n = 2$ females with missing BMI.
[e]Overall, $n = 375$; males, $n = 183$; females, $n = 192$; $n = 1$ female with missing BMI.
[f]Adjusted for sex, age (categorical), cigarette smoking, betel nut chewing, and BMI.

conducted a sensitivity analysis in the subset of NIAT participants ≥ 30 years (the minimum age in FOX), but estimates were consistent with the overall sample.

Discussion

The primary objective of this study was to examine the associations of As exposure with global methylation and hydroxymethylation of blood DNA in two samples of As-exposed Bangladeshi adults. The most striking findings relate to the sex-specific associations of As exposure with global DNA methylation and hydroxymethylation: in males, As exposure was positively associated with global %5-mC and %5-hmC, whereas in females, As exposure was negatively associated with global %5-hmC. Many As-induced health outcomes have sex-specific risk profiles, although the mechanisms remain unclear (51). Thus, our findings may have implications for understanding the mechanisms underlying these sex-specific health effects.

In previous work, we found that the positive associations between As exposure and global DNA methylation, as measured by the methyl-incorporation assay (which measures predomi-nantly %5-mC), were apparent only among folate-sufficient individuals (11, 25). Similarly, here we found that positive associations between As exposure and global %5-hmC were restricted to males without HHcys, i.e., less metabolic evidence of B-vitamin deficiency, and while negative associations with %5-hmC were found in all females, they were more negative in females with HHcys. Sex-specific interactions of As exposure and dietary methyl donor status have been identified in experimental models: in a 5-month study in C57BL/6 mice, Nohara and colleagues found that exposure to 50 ppm As in drinking water, in combination with a methyl-deficient diet, significantly reduced liver %5-mC and DNMT1 expression in males, whereas in females, this same treatment led to increased liver %5-mC (52). Although little is known about the influence of methyl donor status on 5-hmC, a recent study in mice by Takumi and colleagues found that a methionine-choline–deficient diet upregulated several enzymes involved in 5-hmC dynamics, including Tet2 and Tet3 (53). In support of this finding, we observed a positive association between HHcys and global %5-hmC in fully-adjusted models. Future studies are necessary to investigate the nutritional influences on 5-hmC regulation and to explore mechanisms through
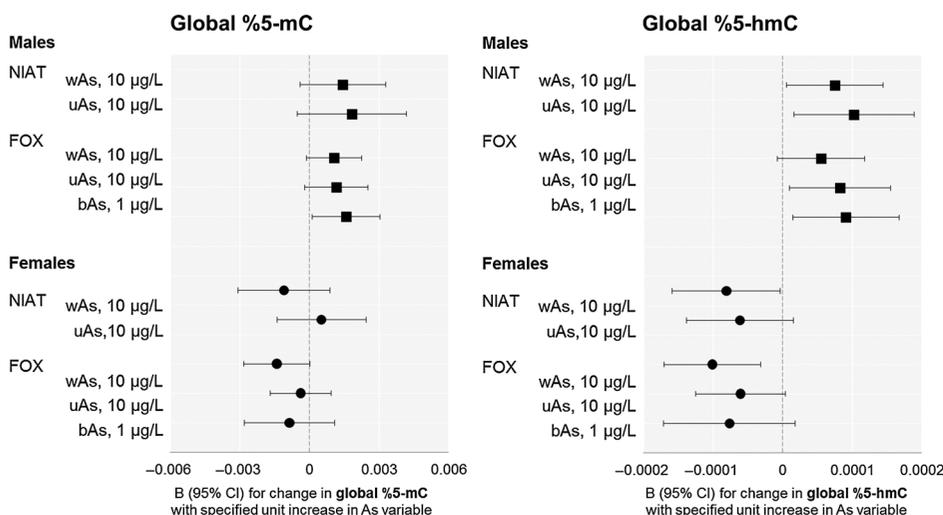

Figure 1.
Covariate-adjusted regression coefficients of As variables predicting global %5-mC and %5-hmC, stratified by sex. Plots represent B ± 95% CIs for the change in global %5-mC (left panel) and %5-hmC (right panel) with the specified unit change in each As variable (wAs and uAs, 10 μg/L; bAs, 1 μg/L) from sex-specific linear regression models adjusting for age (categorical) and cigarette smoking (global %5-mC), and age (categorical), cigarette smoking, betel nut chewing, and BMI (global %5-hmC), as shown in Table 2.

Table 3. Associations of wAs (10 μg/L unit increase) with global %5-mC and %5-hmC by HHcys

Outcome	Sample	Stratum	n	Overall Effect estimate (95% CI)[a]	P	n	Males Effect estimate (95% CI)[a]	P	n	Females Effect estimate (95% CI)[a]	P
Global %5-mC[b]	NIAT (n = 194)	Without HHcys	123	2.03 (−16.22, 20.28)	0.83	47	11.52 (−16.27, 39.31)	0.41	76	1.52 (−24.69, 27.72)	0.91
		With HHcys	71	−2.62 (−23.29, 18.05)	0.80	48	17.10 (−9.44, 43.64)	0.20	23	−28.23 (−61.35, 4.89)	0.09
		P interaction[c]		0.74			0.77			0.15	
	FOX (n = 375)	Without HHcys	271	−1.39 (−12.80, 10.01)	0.81	116	11.97 (−5.03, 28.96)	0.17	155	−12.14 (−27.85, 3.58)	0.13
		With HHcys	104	−1.44 (−17.32, 14.44)	0.86	67	8.04 (−9.17, 25.25)	0.35	37	−41.04 (−78.42, −3.66)	0.03
		P interaction[c]		1.00			0.75			0.15	
Global %5-hmC[d]	NIAT (n = 192)	Without HHcys	121	0.52 (−0.18, 1.22)	0.15	47	1.20 (0.30–2.11)	0.01	74	−0.64 (−1.68, 0.40)	0.22
		With HHcys	71	−0.47 (−1.28, 0.33)	0.25	48	0.01 (−1.12, 1.13)	0.99	23	−0.82 (−2.03, 0.38)	0.17
		P interaction[c]		0.07			0.09			0.81	
	FOX (n = 374)	Without HHcys	270	−0.06 (−0.64, 0.52)	0.83	116	0.90 (−0.06, 1.85)	0.06	154	−0.85 (−1.59, −0.12)	0.02
		With HHcys	104	−0.57 (−1.37, 0.23)	0.16	67	−0.14 (−0.98, 0.70)	0.75	37	−2.60 (−4.84, −0.36)	0.02
		P interaction[c]		0.31			0.11			0.13	

[a]Effect estimates expressed with multiplier of 10^4 (i.e., effect estimate of 1.0 in table corresponds to actual effect estimate of 0.0001).
[b]Adjusted for sex, age, and cigarette smoking.
[c]Interaction P value from Wald test for group difference in the regression coefficient.
[d]Adjusted for sex, age, cigarette smoking, betel nut chewing, and BMI.

which As exposure might interact with nutritional status to influence 5-hmC.

Previous studies of adults (reviewed in ref. 54) have generally found that As exposure is positively associated with global DNA methylation. In a cohort of elderly men in Massachusetts, Lambrou and colleagues (16) observed a positive association between toenail As and leukocyte Alu methylation that was restricted to men with folate and B$_{12}$ levels below the study median, although it is important to note that their cutoffs for "low" folate and B$_{12}$ levels (plasma folate < 32 nmol/L, plasma B$_{12}$ < 351 pmol/L) are at the upper end of the plasma folate and B$_{12}$ ranges in our Bangladeshi populations (43, 55). Although no studies in adults have presented results stratified by sex, in a study of 101 newborn infants in Bangladesh, our group previously found that the association between prenatal As exposure and cord blood global DNA methylation (Alu, LINE-1, and LUMA) was positive in males and negative in females (23), consistent with our current findings.

To our knowledge, only one experimental study and one epidemiologic study have examined the relationship between As exposure and global DNA hydroxymethylation. Consistent with our observations in males, male Sprague-Dawley rats exposed to As in drinking water (0.5, 2, or 10 ppm) for 8 weeks had increased global 5-hmC levels in several tissues, with dose-dependent increases in global %5-hmC found in heart and spleen (56). In leukocyte DNA from 15 males and 33 females of Native American descent, Navas-Acien's group observed that having a higher proportion of the dimethyl arsenic (DMA) metabolite in urine (urinary %DMA ≥ 78.3%) was associated with higher global %5-hmC in unadjusted models, a finding not observed in our current study (data not shown). Also in contrast with our study, no association was observed between uAs and global %5-hmC; sex-specific associations were not reported (57). Additional studies are needed to examine the relationships of As exposure and other environmental exposures with global 5-mC and 5-hmC.

There are several mechanisms whereby As exposure could influence 5-mC and/or 5-hmC. For example, a recent study by Liu and colleagues found that arsenite can bind to the zinc finger domains of Tet family of proteins, leading to a dose-dependent decrease in global 5-hmC levels in HEK293T and mouse embryonic cells (58). Alternatively, As inhibits the base excision repair (BER) pathway (59), which would interfere with the active

removal of 5-hmC (60). Another possibility is through As-induced perturbations of the tricarboxylic acid (TCA) cycle, because alterations in energy metabolism have been shown to dynamically alter 5-hmC levels through alpha-ketoglutarate (α-KG), the substrate required for TET-catalyzed oxidation of 5-mC to 5-hmC (61). Namely, As is an inhibitor of pyruvate dehydrogenase, an enzyme complex that synthesizes acetyl coenzyme A (acetyl-CoA), an important TCA cycle precursor (62). A potential sex-specific mechanism is through As-induced inhibition of poly(ADP-ribose) polymerase 1 (PARP1), which can occur at physiologically-relevant concentrations as low as 1 nmol/L *in vitro* (63). PARP1 is involved in the BER pathway (64), and it also modulates chromatin structure through PARylation of histone-related proteins (65) and regulation of DNMT1 (66). Inhibition of PARP1 prevents the active removal of 5-hmC through BER, leading to increased 5-hmC (60), and also results in increased DNMT1 expression and global DNA hypermethylation (67). This would be consistent with our observation in males that As exposure was positively associated with both %5-mC and %5-hmC—and interestingly, the biologic effects of altered PARP1 activity are often stronger in males than females (68).

Sex hormones can influence the expression of DNA methylation-related enzymes, and evidence indicates that As acts as an endocrine disruptor. For example, estradiol treatment increased DNMT3a and DNMT3b expression in the hippocampus in mice (69), and reduced DNMT expression was observed in human endometrial samples during the mid-secretory phase of the menstrual cycle (70). Hamilton and colleagues have related noncytotoxic levels of As exposure to As-induced disruption of steroid hormone receptor:response element binding (71–75). In addition, work from the Waalkes lab demonstrated that As exposure (500 nmol/L) induced expression of aromatase, a key enzyme involved in estrogen synthesis, and that treatment with an aromatase inhibitor reversed the observed cancer cell phenotype (76). Although the mechanism(s) underlying sex-specific effects of As on DNA methylation remain unclear, it is tempting to speculate that this may be related to As-induced endocrine disruption and effects of this disruption on components of the epigenetic machinery.

There is a great deal of evidence that As exposure influences disease outcomes in a sex-specific manner, and we speculate that

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Table 4. Adjusted effect estimates of As variables and other covariates predicting global %5-hmC

		NIAT (n = 192) Effect estimate (95% CI)[a]	P	NIAT, age ≥ 30 (n = 151) Effect estimate (95% CI)[a]	P	FOX (n = 374) Effect estimate (95% CI)[a]	P
wAs model[b]							
Sex	Male	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	Female	12.97 (−7.43, 33.36)	0.21	14.88 (−6.86, 36.61)	0.18	36.27 (16.56–55.98)	0.0003
Age (y)	19–29	Ref.	Ref.	—	—	—	—
	30–35	−17.08 (−32.94, −1.22)	0.04	Ref.	Ref.	Ref.	Ref.
	36–41	−24.54 (−42.27, −6.81)	0.01	−9.52 (−26.69, 7.64)	0.27	−15.31 (31.34, 0.73)	0.06
	42–49	−30.36 (−48.35, −12.38)	0.001	−16.30 (−33.66, 1.07)	0.07	−32.07 (−48.58, −15.56)	0.0002
	≥50	−40.66 (−60.88, −20.45)	0.0001	−27.31 (−46.89, −7.73)	0.01	−31.16 (−49.75, −12.56)	0.001
Smoking	Never	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	Ever	3.28 (−12.93, 19.50)	0.69	7.33 (−9.57, 24.23)	0.39	18.08 (2.21–33.96)	0.03
Betel nut	Never	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	Ever	9.60 (−2.46, 21.67)	0.12	11.97 (−0.99, 24.92)	0.07	−6.72 (−19.41, 5.98)	0.30
BMI (kg/m^2)	1 kg/m^2	−1.09 (−2.91, 0.73)	0.24	−1.17 (−3.15, 0.82)	0.25	−1.13 (−2.78, 0.51)	0.18
HHcys	Without	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
	With	15.67 (−0.70, 32.04)	0.06	17.73 (0.04–35.50)	0.05	14.96 (−3.07, 33.00)	0.10
wAs (μg/L)	10 μg/L	1.03 (0.18–1.89)	0.02	1.22 (0.30–2.14)	0.01	1.05 (0.26–1.84)	0.01
wAs*female		−1.11 (−2.14, −0.08)	0.04	−1.30 (−2.44, −0.17)	0.02	−1.87 (−2.85, −0.89)	0.0002
wAs*HHcys		−0.94 (−2.00, 0.11)	0.08	−1.06 (−2.18, 0.07)	0.07	−1.25 (−2.23, −0.27)	0.01
uAs model[b,c]							
uAs (μg/L)	10 μg/L	1.08 (0.03–2.12)	0.04	1.14 (0.07–2.20)	0.04	0.98 (0.21–1.74)	0.01
uAs*female		−1.04 (−2.21, 0.14)	0.08	−1.03 (−2.30, 0.23)	0.11	−1.36 (−2.30, −0.42)	0.01
uAs*HHcys		−0.85 (−2.37, 0.67)	0.27	−0.90 (−2.47, 0.66)	0.26	−1.15 (−2.32, 0.02)	0.05
bAs model[b]							
bAs (μg/L)	1 μg/L	—	—	—	—	1.09 (0.25–1.92)	0.01
bAs*female		—	—	—	—	−1.64 (−2.85, −0.44)	0.01
bAs*HHcys		—	—	—	—	−1.23 (−2.58, 0.13)	0.08

[a]Effect estimates expressed with multiplier of 10^4 (i.e., effect estimate of 1.0 in table corresponds to actual effect estimate of 0.0001).
[b]Adjusted for sex, age (categorical), cigarette smoking, betel nut chewing, BMI, HHcys, specified As variable, and cross-product terms for As*sex and As*HHcys.
[c]NIAT: overall, n = 187; age ≥ 30 years, n = 148.

our findings might represent one of several mechanisms underlying these sex differences. For example, several studies show that men have a higher incidence of As-induced skin lesions—likely due to coexposures such as ultraviolet radiation—whereas women develop skin lesions at lower levels of As exposure (8, 77–79). Previously, our group found that global hypomethylation of leukocyte DNA and HHcys were risk factors for As-induced skin lesions (25). Our current observation in women that As exposure is associated with decreased global %5-mC and %5-hmC in the presence of HHcys might explain why women are more susceptible to skin lesions at lower levels of As exposure. The As-associated decrease in global %5-hmC in women might also contribute to sex differences in As-related cancers: loss of global 5-hmC is a hallmark of numerous cancers (39), and studies in Taiwan and Chile indicate that As-associated risk for several cancers is greater among women (80–82).

While the health consequences of alterations in genomic 5-mC levels have been widely studied, particularly with regard to cancer, the functional implications of changes in global 5-hmC are only recently emerging. Recent evidence suggests that global depletion of 5-hmC has an independent role in tumor progression. In 2012, Lian and colleagues found that loss of 5-hmC is a molecular marker of melanoma, with marked decreases in global 5-hmC found in human melanomas compared with benign nevi (40). Furthermore, the re-establishment of global 5-hmC levels resulted in decreased melanoma growth and increased tumor-free survival in a mouse model (40). Increased 5-hmC levels may also be detrimental: Supek and colleagues found that hydroxymethylated cytosines are prone to C→G transversion mutations (83).

Increasing evidence suggests that aberrant global 5-hmC may be an important diagnostic and/or prognostic disease marker. A

recent study by Kroeze and colleagues found that global %5-hmC levels in bone marrow and leukocyte DNA from healthy subjects were distributed within a tight range (1.5-fold difference), whereas the ranges in patients with acute myeloid leukemia (AML) were markedly wider (15-fold difference; ref. 84). In addition, when AML patients were divided into groups based on global 5-hmC levels, these groups were found to have different disease characteristics: the group of AML patients with low global %5-hmC was primarily comprised of patients with TET2 and isocitrate dehydrogenase gene mutations, and high global %5-hmC was found to correlate negatively with overall survival (84). This suggests that the measurement of global %5-hmC as a biomarker in AML and possibly other cancers might improve the understanding and treatment of disease subtypes with different etiologies.

Age was negatively associated with global %5-hmC in both of our studies. Aging is associated with hematopoietic decline and increased risk of leukemias, which might result, in part, from a decline in hematopoietic stem cell (HSC) integrity (85). A study examining epigenetic signatures associated with aging HSCs found that global 5-hmC levels in purified HSCs were lower in older C57BL/6 mice, which was associated with decreased HSC differentiation potential and increased HSC self-renewal (86). In healthy elderly people, Busque and colleagues found a decrease in 5-hmC levels in myeloid cell DNA compared with younger subjects; the authors speculated that 5-hmC depletion may play a role in the aging hematopoietic system (87).

Our study had several limitations. First, our study was limited to measuring alterations in DNA methylation in blood cells. It is possible that our findings are explained by As-induced shifts in blood cell subtypes or counts, and we were unable to adjust for

cell types in our models. While we cannot dismiss this possibility, we note that the effect estimates were consistent between our two study samples, which examined DNA from different populations of cells (total leukocytes and PBMCs). In addition, although As is known to target bone marrow progenitor cells (88), and epigenetic modifications of these progenitor cells are likely to be propagated through subsequent cell divisions, the effects of As exposure on blood DNA may or may not reflect alterations in other tissue targets, such as skin, liver, or bladder. However, evidence suggesting that blood cell DNA may reflect systemic effects of As exposure on 5-hmC is provided in a study employing male rats by Zhang and colleagues, in which As exposure increased 5-hmC in several organs, with the strongest effects in the spleen, an organ with important roles in immune function and hematopoiesis (56). Second, due to the NIAT selection criteria on the basis of folate deficiency and B_{12} sufficiency, we did not have adequate sample sizes to assess effect modification by folate and B_{12} status. We plan to investigate folate and B_{12}, along with other nutrients that are important for the regulation of one-carbon metabolism and the TCA cycle, such as choline and betaine, in future studies. Finally, we cannot determine the mechanisms through which As exposure might induce sex-specific changes in 5-mC and 5-hmC, but our findings can inform the generation of hypotheses for future studies. Further study will be required to determine whether our findings are generalizable to non-Bangladeshi populations, and to explore the relationship between As and global %5-mC and %5-hmC in populations with different dietary intakes and coexposures.

Collectively, our findings suggest that As exposure is significantly associated with global %5-mC and %5-hmC in leukocyte and PBMC DNA; these associations are modified by sex, as they are positive among males and negative among females. Furthermore, we found some evidence of sex-specific effect modification by tHcys such that the positive associations between As and %5hmC are stronger among males with normal tHcys, whereas the negative associations among females are stronger in those with HHcys. The mechanisms underlying these observations warrant further investigation. We previously found that decreases in %5-mC are associated with As-induced skin lesion risk. In future studies, we plan to evaluate whether changes in global %5-hmC contribute to the sex-specific risk profiles observed for As-induced melanosis and keratosis outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences, the NCI, the National Center for Advancing Translational Sciences, or the NIH.

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