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Short Running Head: Arsenic and global methylation of PBMC DNA

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ABSTRACT

BACKGROUND: Several studies employing cell culture and animal models suggest that arsenic (As) exposure induces global DNA hypomethylation. However, As has been associated with global DNA hypermethylation in human study populations. It has been hypothesized that this discrepancy may reflect a non-linear relationship between As dose and DNA methylation.

OBJECTIVE: The objective of this study was to examine the dose-response relationship between As and global methylation of peripheral blood mononuclear cell (PBMC) DNA in apparently healthy Bangladeshi adults chronically exposed to a wide range of As concentrations in drinking water.

METHODS: Global PBMC DNA methylation, plasma folate, blood *S*-adenosylmethionine (SAM), and concentrations of As in drinking water, blood, and urine were measured in 320 adults. DNA methylation was measured using the [³H]-methyl incorporation assay, which provides disintegration per minute (DPM) values that are negatively associated with global DNA methylation.

RESULTS: Water, blood, and urinary As were positively correlated with global PBMC DNA methylation ($P < 0.05$). In multivariable adjusted models, 1- $\mu\text{g/L}$ increases in water and urinary As were associated with 27.6 unit (95% CI, 6.3 to 49.0) and 22.1 unit (95% CI, 0.5 to 43.8) decreases in DPM per μg DNA, respectively. Categorical models indicated that estimated mean levels of PBMC DNA methylation were highest in participants with the highest As exposures.

CONCLUSIONS: These results suggest that As is positively associated with global methylation of PBMC DNA over a wide range of drinking water As concentrations. Further research is necessary to elucidate underlying mechanisms and physiologic implications.

INTRODUCTION

An estimated 70 million people in Bangladesh are chronically exposed to arsenic (As)-contaminated drinking water at levels over the World Health Organization (WHO) limit of 10 $\mu\text{g/L}$ (Loewenberg 2007). Exposure to As has been associated with cancers of the skin, lung, bladder, liver, and kidney (Navarro Silvera and Rohan 2007), hypertension and cardiovascular disease (Chen et al. 1995; Chen et al. 2011; Tseng et al. 2003), respiratory outcomes (Chattopadhyay et al. 2010; Dauphine et al. 2011; Parvez et al. 2010), and reduced cognitive function in children (Hamadani et al. 2011; Wasserman et al. 2004). The mechanism(s) responsible for the pleiotropic health effects of As are not completely understood.

DNA methylation involves the covalent addition of a methyl group from *S*-adenosylmethionine (SAM) to the 5' position of cytosine bases in CpG dinucleotides (Zhao et al. 1997). SAM biosynthesis is regulated by one-carbon metabolism, a biochemical pathway for the methylation of numerous substrates that is dependent on folate for the recruitment of methyl groups from serine (Chiang et al. 1996). Methylation of CpG dinucleotides is associated with gene silencing: CpGs in intergenic regions and repetitive elements are usually methylated (Robertson 2001), while CpGs in gene promoter regions of transcriptionally active genes are generally less methylated than in other regions (Razin and Kantor 2005). Global DNA hypomethylation is associated with genomic instability, including loss of heterozygosity (Matsuzaki et al. 2005), aneuploidy (Dodge et al. 2005), and chromosomal alterations (Schulz et al. 2002), and is commonly observed in tumors and transformed cells (Gaudet et al. 2003). In addition, recent work by the ENCODE project (Consortium 2004) has highlighted important regulatory roles of methylation of transposable elements and other non-coding regions of the genome (previously

coined “junk DNA”) in cell-type dependent transcriptional regulation (Bernstein et al. 2012; Thurman et al. 2012).

Evidence based on *in vitro* and *in vivo* models suggests that chronic As exposure induces global DNA hypomethylation, in conjunction with hypermethylation of promoter regions of tumor suppressor genes (Benbrahim-Tallaa et al. 2005; Chen et al. 2004; Reichard et al. 2007; Ren et al. 2011). Based on this literature, we initially hypothesized that As exposure would be associated with a reduction in global methylation of DNA in a population chronically exposed to As-contaminated drinking water, providing a mechanistic link between As exposure and increased cancer risk. We further hypothesized that this would be exacerbated by folate deficiency. However, in our first population-based study on this subject, contrary to our *a priori* hypotheses, chronic As exposure was positively associated with global methylation of peripheral blood leukocyte (PBL) DNA among subjects with sufficient folate levels (Pilsner et al. 2007). While these findings were unanticipated, the positive association between As exposure and global DNA methylation in humans has been replicated by our group (Pilsner et al. 2012; Pilsner et al. 2009) and by others (Kile et al. 2012; Lambrou et al. 2012; Majumdar et al. 2010). We hypothesized that the discrepancy between the experimental and human studies is related to the dose and duration of As exposure: High acute As exposures might result in DNA hypomethylation, whereas a non-linear pattern of association might be observed within the range of As exposures in human populations, many of whom have been exposed to As for decades.

The primary objective of this analysis was to evaluate the relationship between As exposure and global methylation of peripheral blood mononuclear cell (PBMC) DNA. We used data from the Folate and Oxidative Stress (FOX) study (Hall et al. 2013), which selected study participants having a wide range of As exposure with the primary objective to assess the relationship between

As exposure and oxidative stress. This study provided a unique opportunity to examine the dose-response relationship between As exposure and global DNA methylation in a population with higher levels of As exposure than previous study populations.

SUBJECTS AND METHODS

Eligibility criteria and study design

For the FOX study, we recruited 379 men and women between the ages of 35 and 65 y between April 2007 and April 2008 in Araihasar, Bangladesh. Participants were selected based on well water As (wAs) exposure such that the final study sample represented the full range of wAs concentrations in the region. We aimed to recruit 75 participants from each of 5 exposure categories (Group A, 0-10 $\mu\text{g/L}$; Group B, 10-100 $\mu\text{g/L}$; Group C, 100-200 $\mu\text{g/L}$; Group D, 200-300 $\mu\text{g/L}$; and Group E, >300 $\mu\text{g/L}$) but had difficulty recruiting participants in the highest exposure categories because households using wells that tested positive for As (i.e., > 50 $\mu\text{g/L}$, the Bangladesh standard) were encouraged to switch to lower-As wells (Chen et al. 2007). Thus, the final participant numbers for the 5 categories were Group A, N=76; Group B, N=104; Group C, N=86; Group D, N=67, and Group E, N=45. Note that a random sample in this region would have likely yielded 40-50% of participants consuming wAs \leq 50 $\mu\text{g/L}$. Participants must have been drinking from the same well for a minimum of 3 months prior to recruitment. Participants were excluded if they were pregnant; were currently taking nutritional supplements (or had done so within 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 assent form to the study participants. This study was approved by the institutional

review boards of the Bangladesh Medical Research Council and of Columbia University Medical Center.

Analytic techniques

Sample collection and handling

Blood samples were drawn and processed immediately at our field clinic laboratory in Araihasar. Blood samples were centrifuged at $3,000 \times g$ for 10 min at 4°C , and buffy coat and plasma were separated from red cells. Aliquots of blood and plasma were stored at -80°C . Urine samples were collected in 50 mL acid-washed polypropylene tubes and frozen at -20°C . Blood, plasma, and urine samples were transported to Dhaka on dry ice and stored at -80°C . Samples were then shipped, frozen on dry ice, to Columbia University for analysis.

Water As

Field sample collection and laboratory procedures have been described in detail (Cheng et al. 2004; Van Geen et al. 2005). Briefly, at the recruitment visit of the FOX study, new water samples were collected in 20 mL polyethylene scintillation vials and acidified to 1% with high-purity Optima HCl (Fisher Scientific, Pittsburg, PA, USA) at least 48 hr before analysis (van Geen et al. 2007). Water samples were analyzed by high-resolution inductively coupled plasma mass spectrometry after 1:10 dilution and addition of a Ge spike to correct fluctuations in instrument sensitivity. A standard with an As concentration of $51 \mu\text{g/L}$ was run multiple times in each batch; the intra- and inter-assay coefficients of variation (CVs) for this standard were 6.0% and 3.8%, respectively.

Total urinary As and urinary creatinine

Urinary As (uAs) metabolites were speciated using HPLC separation of arsenobetaine (AsB), arsenocholine (AsC), As^V, As^{III}, MMA (MMA^{III} + MMA^V), and DMA (DMA^V), followed by detection using ICP-MS (Vela et al. 2001). Total uAs was calculated by summing the concentrations of As^V, As^{III}, MMA, and DMA; AsC and AsB were not included in the sum. The limit of detection for each uAs metabolite was 0.1 µg/L. Arsenic levels in urine were determined with and without adjustment for urinary creatinine (uCr), which was analyzed by a colorimetric assay based on the Jaffe reaction (Slot 1965). The intra-assay CVs were 4.5% for As^V, 3.8% for As^{III}, 1.5% for MMA, and 0.6% for DMA; the inter-assay CVs were 10.6% for As^V, 9.6% for As^{III}, 3.5% for MMA, and 2.8% for DMA.

Total blood As

Total blood As (bAs) was analyzed using Perkin-Elmer Elan DRC II ICP-MS equipped with an AS 93+ autosampler, with a limit of detection of 0.1 µg/L, as previously described (Hall et al. 2006). The intra- and inter-assay CVs were 3.2% and 5.7%, respectively.

PBMC DNA isolation

PBMCs were isolated from fresh blood samples in the field clinic laboratory in Araihaazar. To isolate PBMCs, 4 mL Ficoll solution was added to a tube containing 4 mL blood (with serum removed) and 11 mL PBS. Tubes were centrifuged at 400 x g for 30 min, and the mononuclear cell layer was extracted and washed with PBS. Cells were sedimented by centrifuging at 200 x g for 10 min. The cell pellet was resuspended with 4 mL lysis solution and 50 µl Proteinase K solution. PBMC lysates were stored at 4°C until shipment to Columbia University. PBMC DNA was isolated from 4 mL PBMC lysate using 1 mL Protein Precipitation Solution (5-Prime,

New York, NY) and standard isopropanol extraction following the manufacturer's protocol. DNA was stored at -20°C until further analysis.

Global DNA methylation

Global DNA methylation was measured using the [³H]-methyl incorporation assay developed by Balaghi and Wagner (Balaghi and Wagner 1993), as previously described (Pilsner et al. 2007). The assay employs ³H-labeled SAM and SssI methylase to add ³H-labeled methyl groups to unmethylated CpG sequences. Thus, disintegration per minute (DPM) values are negatively associated with global DNA methylation. Samples were run in duplicate, and each run included a blank (mixture including all reaction components except SssI enzyme), hypomethylated control (HeLa cell DNA), and positive control (DNA extracted from whole blood sample). PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR) was used to quantify the exact amount of double-stranded DNA (dsDNA) used in each reaction. The mean DPM values from the duplicate samples were expressed per µg DNA, as determined by PicoGreen. The intra- and inter-assay CVs were 3.4% and 10.4%, respectively.

Plasma folate

Plasma folate was analyzed by radioproteinbinding assay (SimulTRAC-S, MP Biomedicals, Orangeburg, NY). This method requires heating plasma to 100°C to denature endogenous binding substances. For folate concentration determination, folic acid as pteroylglutamic acid was used for calibration, and its ¹²⁵I-labeled analog was used as the tracer. The intra- and inter-assay CVs were 6% and 14%, respectively.

Blood S-adenosylmethionine

SAM was measured in whole blood as described previously (Poirier et al. 2001). SAM was detected at 254 nm using a 996 Photodiode Array ultraviolet absorbance detector (Waters Inc, Milford MA) and quantified relative to standard curves (Sigma, St. Louis, MO). The inter-assay CV was 9.6%.

Statistical methods

Descriptive statistics (means and standard deviations) were calculated for the overall sample. Spearman correlations (for continuous variables) and the Wilcoxon rank-sum test (for dichotomous variables) were used to examine bivariate associations between As variables and other covariates with [³H]-methyl incorporation. Certain confounders (sex, age, and ever versus never cigarette smoking) were selected based on biologic plausibility and our previous studies (Pilsner et al. 2007). Other potential confounders (BMI, years of education, plasma folate, plasma vitamin B₁₂, ever betelnut use, and television ownership) were selected based on bivariate associations with markers of As exposure and [³H]-methyl incorporation in this dataset, but none met our criteria for inclusion in the final models ($P > 0.20$ for associations of the potential confounder with both the exposure and the outcome).

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 urinary As values. The uCr-adjusted urinary As variable was used for all analyses involving urinary As.

We used locally estimated scatterplot smoothing (LOESS) curves of unadjusted associations between As exposure variables and [³H]-methyl incorporation to visually identify potential nonlinear or nonmonotonic relationships. A SAS macro for restricted cubic splines (RCS) was used to estimate associations between markers of As exposure and [³H]-methyl incorporation adjusted for sex, age, and smoking (Desquilbet and Mariotti 2010). An RCS function represents the sum of piecewise cubic polynomial splines with continuity and constraints at 3 to 5 specified knots on the continuous exposure variables (Desquilbet and Mariotti 2010). A Wald chi-square test with K-2 degrees of freedom, where K = number of knots and K-2 = number of spline variables, was used to test the null hypothesis of linear relationship between the exposure and outcome variables. Failure to reject the null hypothesis of linearity would support the use of linear regression to estimate associations between markers of As exposure (modeled as untransformed continuous variables) and [³H]-methyl incorporation.

Associations were also estimated by modeling As exposure using categorical variables. Water As categories were created with the reference category reflecting the Bangladeshi drinking water standard for As of 50 µg/L: 0-50 µg/L, 50-100 µg/L, 100-200 µg/L, 200-300 µg/L, and 300-700 µg/L. Urinary As was first adjusted for urinary Cr using the residual method; quintiles of uAs exposures were then constructed based on these adjusted values. Blood As was categorized based on quintiles. Separate general linear models were constructed with each categorized As exposure variable as the predictor and [³H]-methyl incorporation as the outcome, with gender, age, and ever cigarette smoking included in the model as covariates. Least squares means for [³H]-methyl incorporation were calculated for each level of each As exposure variable, assuming the mean value for age (43.175 years) and a value of 0.5 for the dichotomous covariates sex and ever cigarette smoking.

[³H]-methyl incorporation values were excluded from the analysis if duplicate assays had coefficients of variation > 15% (N=48), if less than 10 µg/mL of DNA was used in the assay (N=8), or if DPM values were extreme outliers, defined as values that exceeded the 75th percentile of the DPM values by more than three interquartile ranges (N=2). Additionally, we were unable to extract DNA from 1 lysate, leaving a final dataset of N=320 for the analyses. All statistical analyses were conducted using SAS (version 9.2; SAS Institute Inc., Cary, NC); statistical tests were two sided with a significance level of 0.05.

RESULTS

The characteristics of the study population are shown in Table 1. The mean age was 43.2 years, and there were roughly equal numbers of males and females. Using a cutoff of < 9.0 nmol/L for folate deficiency and > 13 µmol/L for hyperhomocysteinemia, 31.3% and 17.5% of the participants were classified as having folate deficiency and hyperhomocysteinemia, respectively. Using more stringent cut-offs (10.4 µmol/L for females and 11.4 µmol/L for males), 29% of the participants were classified as having hyperhomocysteinemia. Water As concentrations ranged from 0.4 to 700 µg/L, with a mean of 145 µg/L. By design, wells that exceeded the Bangladeshi standard of 50 µg/L were the primary drinking water source for 70.9% of participants.

All As variables (wAs, bAs, and uAs adjusted for uCr) were negatively correlated with [³H]-methyl incorporation (Table 2), indicating that As exposure was positively correlated with PBMC DNA methylation. Spearman correlation coefficients were similar between groups when stratified by folate deficiency (plasma folate < 9.0 nmol/L) (data not shown), suggesting that folate status did not modify associations between As exposure markers and [³H]-methyl incorporation in this sample. Correlations also were similar between groups defined using more

stringent definitions of folate deficiency (plasma levels < 8.0 nmol/L, N=69; or < 7.0 nmol/L, N=38) (data not shown). We also examined correlations of As and [³H]-methyl incorporation with blood SAM, which has been hypothesized to mediate the relationship between As exposure and DNA methylation (Mass and Wang 1997). While blood SAM was negatively correlated with [³H]-methyl incorporation, As was not correlated with blood SAM (Table 2). In addition, methylated metabolites of As were not correlated with [³H]-methyl incorporation (data not shown). We did not observe significant correlations between plasma folate and [³H]-methyl incorporation or blood SAM, or between age and [³H]-methyl incorporation (Table 2).

The shapes of the LOESS curves suggested that [³H]-methyl incorporation decreased linearly with increasing levels of wAs, bAs, and uAs (data not shown). Using RCS functions with 5 knots, we similarly observed a decrease in [³H]-methyl incorporation as As levels increased (data not shown). Tests of departures from the null hypothesis of linear dose-response relations were not statistically significant for any of the As predictors (all $P > 0.30$).

Unadjusted linear regression models of wAs, uAs, or bAs as predictors of [³H]-methyl incorporation indicated negative associations between As exposure and [³H]-methyl incorporation that were statistically significant for wAs ($\beta = -28.2$; 95% CI: -49.5, -6.8, $P = 0.01$) and borderline significant for uAs ($\beta = -20.2$; 95% CI: -41.9, 1.4, $P = 0.07$) and bAs ($\beta = -254.5$; 95% CI: -523.5, 14.6, $P = 0.06$) (Table 3). Estimates for the difference in mean DPM estimates associated with a 1-unit increase in As exposure adjusted for sex, age, ever smoking, and uCr (for uAs), were similar to unadjusted estimates for wAs ($\beta = -27.6$; 95% CI: -49.0, -6.3, $P = 0.01$) and uAs ($\beta = -22.1$; 95% CI: -43.8, -0.5, $P = 0.045$), but the association for bAs was

attenuated ($\beta = -211.7$; 95% CI: -483.9, 60.5, $P = 0.13$ compared with $\beta = -254.5$; 95% CI: -523.5, 14.6 before adjustment).

Linear models were constructed to estimate least squares mean [^3H]-methyl incorporation levels by As category, with gender, age, and ever smoking included in the models as covariates (Figure 1). Mean [^3H]-methyl incorporation levels were similar between the lowest two wAs categories (Figure 1A) and among the lowest three uAs quintiles (Figure 1B). Higher wAs and uAs exposure categories were associated with lower estimated least squares mean values for [^3H]-methyl incorporation: estimated mean [^3H]-methyl incorporation was 7.0% lower (95% CI: 2.2, 11.8%) among participants in the highest wAs category (300-700 $\mu\text{g/L}$) compared with the 0-50 $\mu\text{g/L}$ wAs referent group ($P = 0.02$, Figure 1A) and was 6.4% lower (95% CI: 2.5, 10.3%) among those in the highest uAs quintile compared with the lowest uAs quintile ($P = 0.02$, Figure 1B). While the pattern of association for [^3H]-methyl incorporation by quintile of bAs was similar to the pattern for wAs and uAs (Figure 1C), the least squares means were not significantly different from one another.

DISCUSSION

The primary objective of this study was to assess the dose-response relationship between As exposure and global methylation of PBMC DNA in adults chronically exposed to a wide range of As in drinking water in Bangladesh. In agreement with previous findings (Pilsner et al. 2007, 2009), As exposure was positively associated with global DNA methylation of PBMC DNA. Furthermore, within the range of As exposures in our study, we did not detect a statistically significant departure from linearity in the association between As and DNA methylation. Adjusted mean DNA methylation levels were similar among the lower As exposure categories

but increased in the higher As exposure categories, with the highest estimated mean DNA methylation levels in the highest As exposure categories (water As > 300 µg/L). However, we cannot rule out the possibility of non-linearity at extremely high doses often used in animal studies, e.g., As exposures ranging from 1 to 85 ppm (1,000 to 85,000 µg/L) (Reichard and Puga 2010).

In the present study, we did not find a significant correlation between age and [³H]-methyl incorporation. It is possible that this is attributable to the demographics of the study population. In our first study of As exposure and global DNA methylation (Pilsner et al. 2007), global DNA methylation decreased with age across the 18-29, 30-36, and 37-45 year age categories. However, global DNA methylation levels did not differ significantly between the 37-45 and 46-66 year age groups (Pilsner et al. 2007). The narrower age range of the FOX study (30-65 years) may therefore have contributed to the lack of a significant association between global DNA methylation and age in our study population.

We also did not find a correlation between plasma folate and [³H]-methyl incorporation. The mean plasma folate level was higher in the FOX study population than in our first study of As exposure and global DNA methylation (12.6 vs. 8.6 nmol/L, respectively), and a smaller proportion was folate deficient (31.3 vs. 64.6%). Folate might only influence DNA methylation under conditions in which folate is limiting. In support of this hypothesis, folic acid supplementation was not associated with higher global DNA methylation in study populations whose participants were folate sufficient at baseline (Basten et al. 2006; Jung et al. 2011). In the present study population, associations between As and DNA methylation were similar between those with and without folate deficiency, even when more stringent definitions of folate

deficiency were used. However, our ability to evaluate an interaction may have been limited by the small numbers of participants with severe folate deficiency.

Other epidemiologic studies in adults have reported that associations between As exposure and global DNA methylation were modified by folate status. In a study of elderly men in Massachusetts, toenail As concentrations were positively associated with Alu methylation only among men with plasma folate levels below the study median (Lambrou et al. 2012). Although this seemingly contradicts our previous finding of an association between As exposure and DNA methylation that was limited to participants with sufficient plasma folate levels (Pilsner et al. 2007), as noted by Lambrou et al. (2012), plasma folate levels in our folate-sufficient group (> 9.0 nmol/L) overlapped with levels in their low folate group (< 32 nmol/L). In the present study, all but 6 participants had plasma folate levels < 32 nmol/L.

Two studies of prenatal As exposure have reported positive associations between As exposure and global DNA methylation. A study of 113 mother/newborn pairs in Bangladesh reported that As exposure was associated with higher methylation of LINE-1 in both umbilical cord blood and maternal leukocytes (Kile et al. 2012). In a similar study of 101 mother/newborn pairs in Bangladesh, maternal As exposure also was associated with increased global DNA methylation based on [³H]-methyl-incorporation in DNA extracted from umbilical cord blood leukocytes (Pilsner et al. 2012). However, the direction of the associations between maternal As exposure and other markers of global DNA methylation (Alu, LINE-1, and LUMA) differed by sex: associations were positive in male newborns, but negative in female newborns (Pilsner et al. 2012).

Several mechanisms of As-induced epigenetic dysregulation have been proposed. For example, because SAM is required for As metabolism, it has been hypothesized that As methylation may influence DNA methylation through competition for methyl groups (Mass and Wang 1997). In an *in vitro* model, low-dose As exposure decreased SAM concentrations in keratinocytes cultured in folic acid-deficient medium (Reichard et al. 2007). While blood SAM was positively correlated with DNA methylation in our study population, it was not correlated with markers of As exposure. In addition, in contrast with what one might expect if there were competition between DNA methyltransferases and arsenic methyltransferase for SAM, As metabolites also were not correlated with DNA methylation in our study population. Quantitatively, methylation of As and DNA consumes only a very small proportion of total SAM (Gamble and Hall 2012).

There is growing evidence that As exposure may alter post-translational modifications of lysine residues in histone tails. For example, exposure to NaAsO_2 led to increased global histone acetylation and more open chromatin formation in human HepG2 hepatocarcinoma cells (Ramirez et al. 2008), and exposure to InAs^{III} led to increased H3K9 dimethylation (H3K9me₂) and decreased H3K27 trimethylation (H3K27me₃) in human lung carcinoma A549 cells (Zhou et al. 2008). Occupational exposure to inhalable As was significantly associated with H3K4me₂ and H3K9 acetylation (H3K9ac) in histones purified from WBCs in a study of steel workers in Italy (Cantone et al. 2011). In ongoing studies, we are evaluating associations of As exposure on global histone modifications in As-exposed Bangladeshis (Chervona et al. 2012) and whether these histone marks are additionally associated with global DNA methylation.

The physiologic implications of increased global DNA methylation in apparently healthy adults are unclear, given that global DNA *hypomethylation* has been associated with multiple diseases, including As-induced skin lesions. An increase in global DNA methylation might be associated

with an increased spontaneous mutation rate: methylated cytosines are more prone to deamination than unmethylated cytosines, which increases the likelihood of C→T transitions (Ehrlich et al. 1986). Additionally, DNA methylation is associated with heterochromatic regions (Beisel and Paro 2011), and somatic mutation density in human cancer genomes were found to be highest in heterochromatin-like domains (Schuster-Bockler and Lehner 2012).

Additionally, previous findings suggest that As-induced global DNA hypermethylation might be associated with concomitant increases in As-induced gene-specific promoter methylation of tumor suppressors or other disease-related genes. Kile et al. reported that urinary As was associated with both increased LINE-1 methylation and increased *P16* promoter methylation of leukocyte DNA from umbilical cord blood and maternal blood (Kile et al. 2012). Promoter methylation of *P16* and *P53* tumor suppressors was elevated in both As-exposed individuals and As-associated skin cancer cases in West Bengal, India (Chanda et al. 2006), and methylation of the tumor suppressors *RASSF1A* and *PRSS3* was associated with toenail As concentrations and invasive tumor stage in bladder cancer cases in New Hampshire (Marsit et al. 2006). In women from the Argentinean Andes, urinary As concentrations were positively associated with promoter methylation of *P16* and the DNA repair gene *MLH1* (Hossain et al. 2012).

We cannot dismiss the possibility that our findings may be explained by As-induced shifts in blood cell type distributions. In our previous studies (Pilsner et al. 2007, 2009), DNA was isolated from PBLs, while our current study used PBMCs. Because PBMCs are a subset of the cells present in PBLs, this would reduce cell type variability to some extent. Arsenic exposure was not significantly associated with proportions of total T, Tc, B, or NK cells in PBMCs isolated from As-exposed children in Mexico (Soto-Pena et al. 2006). Furthermore, average

[³H]-methyl incorporation levels were not significantly different among DNA samples isolated from granulocytes, mononuclear cells, and white blood cells (Wu et al. 2011).

In the present study population, the p-value for the association of [³H]-methyl-incorporation with blood As (0.13) was larger than p-values for corresponding associations with water or urine As (0.01 and 0.045, respectively). Blood As is the most proximal marker of PBMC exposure, so one might expect that blood As would have the strongest and/or most significant association with PBMC DNA methylation. While blood As was strongly correlated with water As (Spearman $r = 0.75$) and urinary As adjusted for urinary Cr (Spearman $r = 0.93$), the range of concentrations of As in blood (1.2 – 57.0 ug/L) was much smaller than in urine (10 – 548 ug/L) or water (0.4 – 700 ug/L), resulting in reduced statistical power to predict [³H]-methyl incorporation.

This study has several limitations. First, PBMCs are not known to be direct targets of As-induced carcinogenesis. However, As trioxide is a highly effective therapeutic drug for the treatment of acute promyelocytic leukemia (Powell et al. 2010), demonstrating that As distributes to bone marrow progenitor cells and influences their cellular function (Petrie et al. 2009; Soignet et al. 1998). We do not know the extent to which methylation of PBMC DNA reflects the methylation of other target tissues. Finally, it is possible that our results are explained by unmeasured confounding. Notably, we do not have measures of all possible contaminants in well water that may co-occur with As and potentially confound our observed associations. However, mass spectrometry data for a panel of 33 elements from well water samples from our study area indicated that only As and Mn were elevated (Cheng et al. 2004), and Mn was not associated with DNA methylation (data not shown).

In conclusion, As exposure was positively associated with global methylation of PBMC DNA in Bangladeshi adults with a wide range of As exposures, consistent with previous studies. Furthermore, we did not observe statistical evidence of non-linear relationships between As and global DNA methylation within the range of As exposures in our population. Future studies, in addition to investigating the influence of As on histone modifications, should evaluate the potential physiological implications of increased global DNA methylation.

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Table 1. Demographic and clinical data for current study (N=320) -

Baseline variables	Mean \pm SD (range) or N(%)
Age (yrs)	43.2 \pm 8.3 (30 - 63)
Male	159 (49.7)
BMI (kg/m ²)	20.3 \pm 3.5 (13.8 - 35.3)
Underweight (BMI < 18.5 kg/m ²)	112 (35.1)
Ever cigarette smoking	123 (38.4)
Ever betel nut use	142 (44.4)
Television ownership	185 (57.8)
Water As (μ g/L) ^a	145 \pm 123 (0.4 - 700)
Water As > 50 μ g/L ^a	226 (70.9)
Urinary As (μ g/L)	213 \pm 242 (2 - 1800)
Urinary creatinine (mg/dL)	54.5 \pm 43.7 (4.3 - 223.5)
Urinary As adj. for urinary creatinine (μ g/L)	167 \pm 122 (10 - 548)
Blood As (μ g/L)	13.9 \pm 9.8 (1.2 - 57.0)
Plasma folate (nmol/L) ^a	12.6 \pm 7.0 (2.4 - 60.6)
Folate deficient ^a (<9 nmol/L)	100 (31.3)
Plasma Hcys (μ mol/L)	11.2 \pm 12.0 (3.0 - 165.4)
Hyperhomocysteinemia (>13 μ mol/L)	56 (17.5)
Blood SAM (μ M) ^b	1.29 \pm 0.50 (0.44 - 3.38)
DPM per μ g DNA	149,123 \pm 23,932 (61,398 - 215,666)

^a N=319^b N=312

Table 2. Spearman correlation coefficients for arsenic variables, blood s-adenosylmethionine, plasma folate, and [³H]-methyl incorporation in PBMC DNA (N=320)

	Blood As	Urinary As	Urinary As, adj. for urinary Cr	DPM per µg DNA	Blood SAM^b	Plasma folate^a	Age
Water As^a	0.75***	0.63***	0.75***	-0.14*	-0.03	-0.04	-0.01
Blood As		0.67***	0.93***	-0.13*	-0.03	-0.06	0.00
Urinary As			0.71***	-0.08	0.00	0.03	-0.02
Urinary As, adj. for urinary Cr				-0.12*	-0.04	0.00	0.01
DPM per µg DNA					-0.12*	0.03	0.07
Blood SAM^b						-0.07	0.13*
Plasma folate^a							-0.12*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$

^a N=319

^b N=312

Table 3. Estimated regression coefficients from separate adjusted linear regression models of associations between arsenic exposure variables and [³H]-methyl incorporation in PBMC DNA (N=320)

Predictor	Model	B (95% CI)	P
Water As^a	Unadjusted	-28.2 (-49.5, -6.8)	0.01
	Adjusted ^b	-27.6 (-49.0, -6.3)	0.01
Urinary As, adjusted for urinary Cr	Unadjusted	-20.2 (-41.9, 1.4)	0.07
	Adjusted ^b	-22.1 (-43.8, -0.5)	0.045
Blood As	Unadjusted	-254.5 (-523.5, 14.6)	0.06
	Adjusted ^b	-211.7 (-483.9, 60.5)	0.13

^a N=319

^b Adjusted for sex, age, and ever cigarette smoking

Figure Legend

Figure 1. Least Squares Mean Values of [³H]-Methyl Incorporation by Category Means of Water (A), Urinary (B), and Blood (C) Arsenic. Data points represent estimated mean DPM values (with 95% CI) according to the mean As value for each As exposure category assuming the mean value for age (43.2 years) and a value of 0.5 for the dichotomous covariates sex and ever cigarette smoking. Categories for blood and urinary As are quintiles, while categories for water As reflect study sampling categories and the Bangladeshi As standard of 50 µg/L.

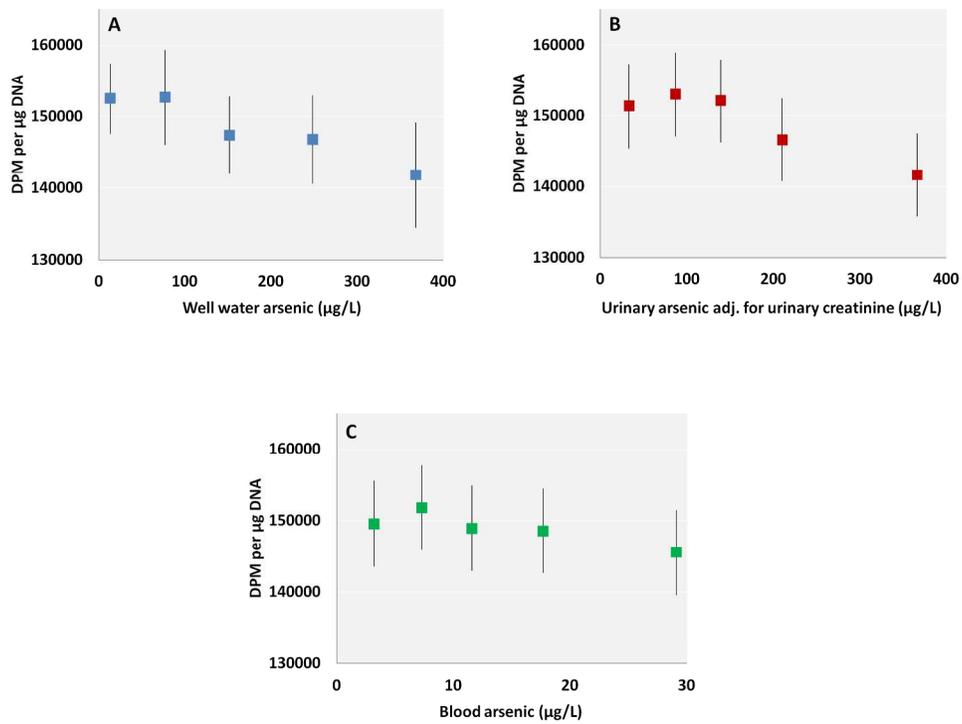


Figure 1

328x247mm (205 x 205 DPI)