Genomic methylation of peripheral blood leukocyte DNA: influences of arsenic and folate in Bangladeshi adults

J Richard Pilsner, Xinhua Liu, Habibul Ahsan, Vesna Ilievski, Vesna Slavkovich, Diane Levy, Pam Factor-Litvak, Joseph H Graziano, and Mary V Gamble

ABSTRACT

Background: Studies in cell culture and animal models indicate that arsenic exposure induces modifications in DNA methylation, including genome-wide DNA hypomethylation. It is not known whether arsenic exposure influences genomic DNA methylation in human populations chronically exposed to arsenic-contaminated drinking water.

Objective: The objective of this study was to determine whether arsenic is associated with genomic hypomethylation of peripheral blood leukocyte (PBL) DNA in Bangladeshi adults who are chronically exposed to arsenic. We also investigated whether arsenic-induced alterations in DNA methylation may be influenced by folate nutritional status.

Design: PBL DNA methylation and concentrations of plasma folate, plasma arsenic, and urinary arsenic were assessed in 294 adults in Araihazar, Bangladesh. Genomic PBL DNA methylation was measured by using a [3H]-methyl incorporation assay.

Results: Urinary arsenic, plasma arsenic, and plasma folate were positively associated with the methylation of PBL DNA (P = 0.009, 0.03, and 0.03, respectively). Stratification of participants by folate nutritional status [<9 nmol/L (n = 190) or ≥9 nmol/L (n = 104)] showed that the associations between arsenic exposure and methylation of PBL DNA were restricted to persons with folate concentrations ≥ 9 nmol/L.

Conclusions: Contrary to our a priori hypothesis, arsenic exposure was positively associated with genomic PBL DNA methylation in a dose-dependent manner. This effect is modified by folate, which suggests that arsenic-induced increases in DNA methylation cannot occur in the absence of adequate folate. The underlying mechanisms and physiologic implications of increased genomic DNA methylation are unclear, and they warrant further study.


KEY WORDS DNA methylation, epigenetics, folate, folic acid, folate deficiency, one-carbon metabolism, S-adenosylmethionine, SAM, arsenic, arsenicosis, Bangladesh, well water

INTRODUCTION

The installation of tube wells has been promoted in Bangladesh since the 1960s in an effort to reduce the high infant mortality due to waterborne microbial diseases contracted by drinking contaminated surface water (1). It is estimated that nearly 35 million Bangladeshis are being exposed to arsenic concentrations that exceed the Bangladeshi standard of 50 μg/L, and 57 million are exposed to concentrations >10 μg/L (2), the World Health Organization standard, which is based on the extrapolation of cancer risk from studies in Taiwan (3, 4).

Arsenic exposure is associated with greater risk of cancers of the skin, liver, lung, and bladder (5). Noncancer health effects include ischemic heart disease (6), peripheral vascular disease, and neurologic deficits in adults and in children (7, 8). The mechanisms responsible for these multiple adverse outcomes have not been clearly elucidated and likely are multifactorial.

DNA methylation, or the covalent addition of a methyl group from S-adenosylmethionine (SAM) to cytosine by DNA methyltransferases (DNMTs), is an essential epigenetic modification of the genome in mammalian cells (9, 10). DNA methylation occurs almost exclusively at CpG dinucleotides in repetitive sequences as well as in coding and intronic regions (the latter are termed genomic DNA methylation). In contrast, most cytosine residues in CpG islands or in the G+C-rich regions found in the 5’ regions of many genes are unmethylated. Appropriate DNA methylation plays important roles in transcriptional regulation, X chromosome inactivation and imprinting, suppression of parasitic DNA sequences, DNA mismatch repair, and maintenance of genomic stability (10). Genomic hypomethylation of DNA is thought to constitute an early event in some cancers, and it is commonly seen in tumors and transformed cells, where hypomethylation may induce chromosomal instability (11, 12).

Environmental exposures such as smoking, pesticides, radiation, and cadmium (13, 14) and other factors such as folate deficiency (15, 16) and age (17, 18) are associated with altered DNA methylation. Cell culture and animal studies suggest that arsenic exposure induces genomic hypomethylation of DNA (19, 20).
Arsenic-induced gene-specific hypermethylation of promoter regions in p53 and p16 have been reported in both cell culture and in peripheral blood leukocyte (PBL) DNA from persons with arsenic-induced skin lesions (21, 22).

Folate is an important regulator of one-carbon metabolism, the biochemical pathway that mediates the transfer of methyl groups from SAM to numerous substrates, including DNA and arsenic. Methyl-deficient diets (ie, those deficient in folate, methionine, choline, and vitamin B-12) (23, 24) or folate deficiency alone (25) can induce genomic hypomethylation of DNA. Methyl-deficient diets are thought to induce methylation errors in DNA by reducing the methyl pool during replication, which can lead to heritable changes in DNA methylation and subsequent chromosomal instability, loss of imprinting, reactivation of transposable elements, and aberrant gene expression patterns. We hypothesized that arsenic exposure is associated with genomic hypomethylation of PBL DNA in Bangladeshi adults who are chronically exposed to arsenic in drinking water and that this association may be exacerbated by inadequate folate nutritional status.

SUBJECTS AND METHODS

The Nutritional Influences on Arsenic Toxicity (NIAT) study was designed to assess the prevalences of folate deficiency and hyperhomocysteinemia in Bangladesh (26), to determine whether or not folate nutritional status is associated with methylation of arsenic (27), and to determine whether folate deficiency, arsenic exposure, or both are associated with genomic methylation of PBL DNA. The NIAT study has worked in collaboration with the Health Effects of Arsenic Longitudinal Study, a large prospective cohort study of adults exposed to a wide range of water arsenic concentrations (28), from which the adult subjects evaluated in the present study were selected.

The study region

The study site is a 25-km² region within the thana of Araihazar (a thana is an administrative unit, or subdivision, of 1 of the 64 divisions of Bangladesh), ~30 km east of Dhaka, Bangladesh. The variability of well-water arsenic concentrations (0.1–860 ng/L) in this region offers a unique opportunity for the study of dose-response relations between arsenic exposure and arsenic-related health outcomes. Our data on socioeconomic status and the data of Columbia University’s Center for International Earth Science Information Network (CIESIN; 29) indicate that this region is not particularly poor by Bangladeshi standards.

Eligibility criteria and study design

The Health Effects of Arsenic Longitudinal Study includes 11 746 men and women between the ages of 18 and 65 y who were recruited between October 2000 and May 2002 and who continue to be followed at 2-y intervals. As part of the NIAT study, a cross-sectional study of 1650 of these participants, reported elsewhere (26), was first conducted to determine the prevalences of folate and cobalamin deficiencies and of hyperhomocysteinemia and to identify a pool of participants with low plasma folate for recruitment into a folic acid (FA) intervention study. The 200 participants enrolled in the FA supplementation trial were a random sample of the 550 participants who were in the lowest tertile of plasma folate in the cross-sectional study (27). Participants were excluded if they were pregnant or cobalamin deficient (vitamin B-12 ≤ 185 pmol/L) or if they were taking vitamin supplements.

For these analyses, we include baseline data from 195 participants who were enrolled in the FA intervention study in which high-quality DNA was obtained (30). To capture wider ranges of arsenic exposure and folate nutritional status, an additional 100 participants were recruited from the cohort study. These participants were known to have continued to drink from the same well for at least the past 4 y (so that exposure was not altered), and we oversampled participants who were drinking from a high-arsenic well so that 70% were drinking water with >50 µg As/L. Aside from these criteria, these 100 participants were randomly selected. In total, the current study consists of 294 Bangladeshi adults. To determine whether folate nutritional status modifies the relation between arsenic exposure and genomic PBL DNA methylation, we stratified participants by folate nutritional status. The low-folate (LF) group included all participants with plasma folate concentrations < 9 nmol/L (n = 190), and the high-folate (HF) group included all participants with plasma folate concentrations ≥ 9 nmol/L (n = 104). Normal reference values (95% CIs) for cobalamin and plasma folate were reported by Christenson et al (31) from ostensibly healthy blood donors with the use of the Quantaphase II radioimmunoassay (Bio-Rad, Richmond, CA), and those reference values were used to stratify participants in the present study into HF or LF groups.

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 Columbia Presbyterian Medical Center and the Bangladesh Medical Research Council.

Analytic techniques

Sample collection and handling

Blood samples for buffy coats, plasma total homocysteine (tHcy), folate, and total cobalamin were obtained by venipuncture at the time of recruitment. Blood was collected into heparin-containing evacuated tubes that were placed in IsoRack cool packs (Brinkmann Instruments, Westbury, NY), which were designed to maintain samples at 0 °C for 6 h. Within 4 h, samples were transported in hand-carried coolers to our local laboratory, which is situated at our field clinic in Araihazar. Samples were centrifuged at 3000 × g for 10 min at 4 °C, and the buffy coat and plasma were separated from red cells. Aliquots of plasma were stored at −80 °C and shipped, frozen on dry ice, to Columbia University for analysis. Urine samples were collected in 50-mL acid-washed polypropylene tubes, which were kept in portable coolers, frozen at −20 °C within 4 h, and similarly shipped on dry ice.

Water arsenic

A survey of all wells in the study region measured the water arsenic concentrations of the tube well at each participant’s home between January and May 2000 (32). Samples were analyzed at Columbia University’s Lamont Doherty Earth Observatory by using graphite furnace atomic absorption (GFAA) spectrometry, which has a detection limit of 5 µg/L. Those samples found by GFAA to have nondetectable arsenic were subsequently analyzed with the use of inductively coupled mass spectrometry (ICP-MS), which has a detection limit of 0.1 µg/L (33).
Total urinary arsenic

Total urinary arsenic concentrations were measured by using GFAA spectrometry in a graphite furnace system (AAnalyst 600; Perkin-Elmer, Shelton, CT) in the Columbia University Trace Metals Core Laboratory, as described previously (34). This laboratory participates in a quality-control program for total urinary arsenic, which is coordinated by Philippe Weber at the Québec Toxicology Center (Québec, Canada). During the course of this study, intraclass correlation coefficients between our laboratory’s values and the samples calibrated at the Québec laboratory were 0.99. Urinary creatinine was analyzed by using a method based on the Jaffe reaction (35) and was used to correct for differences in urine concentration.

Plasma arsenic

Plasma samples were analyzed for arsenic by using dynamic reaction cell (DRC) technology (Elan DRC II ICP-MS; Perkin-Elmer) equipped with an AS 93+ autosampler. The ICP-MS-DRC method for measuring metals in plasma was developed according to published procedure (36) with modifications for plasma sample preparation developed in our laboratory. Plasma samples were thawed, thoroughly mixed, and analyzed in duplicate after dilution 100× with 2% HNO₃ and 0.2% Triton (Fischer Scientific, Fairlawn, NJ). One multiple-element standard solution was used for instrument calibration, where concentrations were chosen to cover the expected ranges of arsenic concentrations in plasma samples. Special attention was given to correction of matrix-induced and spectral interferences. Matrix suppression is very well compensated by the selection of suitable internal standards, which are matched to masses and, if possible, to ionization properties of the analytes. For arsenic, we used iridium. A stock iridium-spiking solution was prepared, which ultimately delivered 5 ng Ir to each tube. Spectral interferences for arsenic were solved with DRC technology by introducing oxygen as a second gas. The detection limit of the method for plasma arsenic was 0.01 μg/L. After calibrating the instrument and after every 10–12 samples, we ran quality-control serum samples with known arsenic concentrations obtained from the Laboratoire de Santé Publique du Québec. Quality-control serum samples covered the entire range of observed arsenic concentrations. During the month in which these study samples were analyzed, the intraassay CV for arsenic concentration in control serum arsenic was 10.4.

Total blood arsenic and blood arsenic metabolites

Whole-blood specimens were digested according to method of Csanaky and Gregus (37). Frozen samples were thawed and mixed with 0.1 volume of 5.5% Triton X-100 (Fischer Scientific). After the addition of 0.1 volume of 150 mmol aqueous nitrate–ammonium phosphate/L, pH 9.1. Arsenocholine (AsC), arsenobetaine (AsB), monomethylarsenic acid (MMA), dimethylarsinic acid (DMA), arsenite (As⁺³), and arsenate (As⁻⁵) are detectable with great precision, even in blood samples with total arsenic concentrations as low as 3 μg/L. We report inorganic arsenic (InAs) as total InAs because As⁺³ can oxidize to As⁻⁵ during sample transport and preparation. However, we note that most of the InAs in blood appeared as As⁺³. We use 2 types of quality-control samples. We have blood samples (purchased from the Institut de Santé Publique du Québec) with known concentrations of 23 different elements, including arsenic. We also have our own set of blood samples, which are spiked with AsC, As⁺³, DMA, MMA, and As⁻⁵ at 3 different concentrations to cover the expected range of arsenic in unspiked samples. We ran both sets of quality-control samples at the beginning of every working day and throughout the day, after every 10 samples.

Isolation of peripheral blood leukocyte DNA

Buffy coats were transferred to 1 mL red blood cell solution (GenomicPrep Blood DNA Isolation Kit; Amersham Biosciences, Piscataway, NJ) and centrifuged at 16 000 × g for 5 min at 4 °C to separate PBLs from contaminating red blood cells. PBLs were subsequently lysed in the presence of a DNA preservative and stored at 4 °C. Samples were subsequently shipped at 4 °C to Columbia University, where the isolation of PBL DNA was completed according to the manufacturer’s protocol.

Genomic DNA methylation

Genomic DNA methylation was measured by using the methyl acceptance assay according to the method of Balaghi and Wagner (25). DNA was incubated with [³²P]-SAM in the presence of the SssI bacterial methylase enzyme, which indiscriminately methylates all unmethylated CpG sequences. Therefore, the ability of DNA to incorporate [³²P]methyl groups in vitro is inversely related to endogenous DNA methylation. Briefly, 0.5 μg DNA was incubated with 3 U SssI methylase (New England Biolabs, Beverly, MA), 3.8 μM (1.1 μCi) [³²P]-labeled SAM (Perkin-Elmer), EDTA, dithiothreitol, and Tris-HCl (pH 8.2) in a 30-μL mixture for 1 h at 37 °C. The reaction was terminated on ice, and 15 μL of the reaction mixture was applied onto filter paper (DE81; Whatman Inc, Clifton, NJ). The filter was washed 3 times on a vacuum filtration apparatus with 5 mL of 0.5 mol sodium phosphate buffer/L (pH 8.0) and then with 2 mL each of 70% and 100% ethanol. Dried filters were each placed in a vial with 5 mL scintillation fluid (Scintisafe; Fisher Scientific, Fair Lawn, NJ) and were analyzed by using a liquid scintillation counter (Tri-Carb 2100TR; Packard Instruments, Downers Grove, IL). Each DNA sample was processed in duplicate, and each processing run included samples for background (the reaction mixture included all components except the SssI enzyme), a hypomethylation control (HeLa cell DNA), and a quality-control sample (DNA extracted from a whole-blood sample) to determine the intraassay and interassay CVs (1.8% and 5.3%, respectively). To quantify the amount of double-stranded DNA (dsDNA) in each reaction, an aliquot of the assayed DNA was used to determine DNA concentrations by using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR). All disintegration per minute (DPM) values were expressed per μg DNA as quantified with the use of PicoGreen.
Plasma folate (nmol/L) 6.5 ± 1.42
Age (y) 39.5 ± 10
Education (y) 3.5 ± 3.4
BMI (kg/m²) 19.6 ± 3.2
Children (n) 3.2 ± 1.7
Water arsenic (µg/L) 117 ± 109
Urinary arsenic (µg/L) 161 ± 185
Urinary arsenic/creatinine (µg/g Cr) 313 ± 300
Plasma arsenic (µg/L) 7.2 ± 3.8
Plasma creatinine (mg/dL) 61 ± 48
Blood arsenic (µg/L)3
Plasma vitamin B-12 (pmol/L) 272 ± 120
Plasma homocysteine (µmol/L) 12.2 ± 5.9
Pre-DNA methylation (DPM/µg DNA) 42 639 ± 3881

Table 1
Demographic and clinical data of subjects in the current study

<table>
<thead>
<tr>
<th>Baseline variables</th>
<th>Low-folate group (n = 190)</th>
<th>High-folate group (n = 104)</th>
<th>Group difference</th>
<th>Total (n = 294)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>6.5 ± 1.42</td>
<td>12.3 ± 3.5</td>
<td>&lt; 0.00014</td>
<td>8.6 ± 3.7</td>
</tr>
<tr>
<td>Age (y)</td>
<td>39.5 ± 10</td>
<td>34.9 ± 10</td>
<td>&lt; 0.0014</td>
<td>37.9 ± 10.4</td>
</tr>
<tr>
<td>Education (y)</td>
<td>3.5 ± 3.4</td>
<td>3. ± 3.6</td>
<td>0.264</td>
<td>3.3 ± 3.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>19.6 ± 3.2</td>
<td>20 ± 3.3</td>
<td>0.083</td>
<td>19.8 ± 3.2</td>
</tr>
<tr>
<td>Children (n)</td>
<td>3.2 ± 1.7</td>
<td>3.0 ± 1.7</td>
<td>0.254</td>
<td>3.1 ± 1.7</td>
</tr>
<tr>
<td>Water arsenic (µg/L)</td>
<td>117 ± 109</td>
<td>105 ± 104</td>
<td>0.673</td>
<td>112 ± 107</td>
</tr>
<tr>
<td>Urinary arsenic (µg/L)</td>
<td>161 ± 185</td>
<td>185 ± 144</td>
<td>0.023</td>
<td>170 ± 171</td>
</tr>
<tr>
<td>Urinary arsenic/creatinine (µg/g Cr)</td>
<td>313 ± 300</td>
<td>370 ± 300</td>
<td>0.013</td>
<td>333 ± 301</td>
</tr>
<tr>
<td>Plasma arsenic (µg/L)</td>
<td>7.2 ± 3.8</td>
<td>7.8 ± 3.8</td>
<td>0.133</td>
<td>7.5 ± 3.8</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dL)</td>
<td>61 ± 48</td>
<td>60 ± 37</td>
<td>0.864</td>
<td>61 ± 44</td>
</tr>
<tr>
<td>Blood arsenic (µg/L)</td>
<td>9.9 ± 6.3</td>
<td>9.9 ± 6.5</td>
<td>0.834</td>
<td>9.9 ± 6.3</td>
</tr>
<tr>
<td>Plasma vitamin B-12 (pmol/L)</td>
<td>272 ± 120</td>
<td>265 ± 119</td>
<td>0.524</td>
<td>270 ± 120</td>
</tr>
<tr>
<td>Plasma homocysteine (µmol/L)</td>
<td>12.2 ± 5.9</td>
<td>8.6 ± 2.3</td>
<td>&lt; 0.00014</td>
<td>10.9 ± 5.2</td>
</tr>
<tr>
<td>Pre-DNA methylation (DPM/µg DNA)</td>
<td>42 639 ± 3881</td>
<td>41 517 ± 4017</td>
<td>0.034</td>
<td>42 242 ± 3959</td>
</tr>
<tr>
<td>Male [n (%)]</td>
<td>112 (59)</td>
<td>31 ± 22</td>
<td>&lt; 0.0001</td>
<td>144 (49)</td>
</tr>
<tr>
<td>Cigarette smoker [n (%)]</td>
<td>87 (46)</td>
<td>20 ± 21</td>
<td>&lt; 0.0001</td>
<td>109 (37)</td>
</tr>
<tr>
<td>Use of betel nut [n (%)]</td>
<td>75 (39)</td>
<td>23 ± 24</td>
<td>0.01</td>
<td>99 (34)</td>
</tr>
<tr>
<td>House type [n (%)]</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Thatched or other</td>
<td>11 (6)</td>
<td>4 ± 4</td>
<td>0.65</td>
<td>15 (5)</td>
</tr>
<tr>
<td>Corrugated tin</td>
<td>165 (87)</td>
<td>90 ± 94</td>
<td>259 (88)</td>
<td></td>
</tr>
<tr>
<td>Semi-pakka</td>
<td>14 (7)</td>
<td>6 ± 6</td>
<td>20 (7)</td>
<td></td>
</tr>
<tr>
<td>Children [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4 (2.0)</td>
<td>0 ± 0</td>
<td>0.21</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>1–2</td>
<td>73 (40)</td>
<td>47 ± 47</td>
<td>120 (43)</td>
<td></td>
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<tr>
<td>≥3</td>
<td>104 (58)</td>
<td>53 ± 53</td>
<td>157 (56)</td>
<td></td>
</tr>
<tr>
<td>Education [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 y</td>
<td>73 (49)</td>
<td>52 ± 46</td>
<td>0.65</td>
<td>119 (50)</td>
</tr>
<tr>
<td>1–5 y</td>
<td>34 (23)</td>
<td>25 ± 22</td>
<td>113 (24)</td>
<td></td>
</tr>
<tr>
<td>&gt;5 y</td>
<td>42 (28)</td>
<td>23 ± 20</td>
<td>62 (26)</td>
<td></td>
</tr>
</tbody>
</table>

1 n = 294. Semi-pakka, a construction with half in concrete and half in another material (eg, corrugated tin).
2 ± SD (all such values).
3 P values were determined with Wilcoxon’s rank-sum test.
4 n = 137 and 87 in the low-folate and high-folate groups, respectively.
5 P values were determined with a chi-square test.

Plasma folate and vitamin B-12
Plasma folate and total cobalamin were analyzed by using the Quantaphase II radioimmunoassay as previously reported (26). The within- and between-day CVs for folate were 3% and 11%, respectively, and those for cobalamin were 4% and 8%, respectively.

Plasma total homocysteine concentrations
Plasma thcy concentrations were measured by using HPLC with fluorescence detection according to the method described by Pfeiffer et al (39) and as previously reported (26, 27). The within- and between-day CVs for thcy were 5 and 8%, respectively.

Statistical analysis
Descriptive statistics for characteristics of the study sample were calculated for the LF and the HF groups separately and combined. Group differences in quantitative variables were tested by using Wilcoxon’s rank-sum test. Chi-square tests were used to test for group differences in the categorical variables.

We used Spearman correlations to assess bivariate associations for quantitative variables. Linear regression analyses examined associations between the continuous outcome—ie, [3H]-methyl incorporation—and predictors of interest, such as urinary and plasma arsenic and plasma folate, after control for age, smoking, and sex, as well as urinary creatinine when urinary arsenic was included in a model. All analyses were conducted with the use of SAS software (version 9.1: SAS Institute, Cary, NC). Variables with skewed distributions were log transformed to reduce the effect of extreme values in the regression analysis. The log-transformed variables included plasma concentrations of thcy, folate, arsenic, and cobalamin and urinary concentrations of arsenic and creatinine.

RESULTS
The characteristics of the study population are given in Table 1. There were no significant differences between the LF and HF groups in BMI, number of children, and sociodemographic variables such as the type of house and years of education. Water arsenic, plasma arsenic, and total blood arsenic also did not differ significantly between groups. However, the LF group had significantly lower concentrations of urinary arsenic, whether adjusted for urinary creatinine or not adjusted (P = 0.01 and 0.02, respectively). Participants in the LF group were more likely to be male, to smoke cigarettes, and to chew betel nut.
which is consistent with our previous work indicating that these variables are associated with low plasma folate (26). The LF group had significantly ($P < 0.0001$) higher plasma tHcy concentrations than the HF group. Age ($P < 0.0001$) and $[^3H]$-methyl incorporation of PBL DNA ($P = 0.03$) also were significantly higher in the LF group than in the HF group.

The associations (Spearman correlation coefficients) between $[^3H]$-methyl incorporation in PBL DNA and biological variables are shown in Table 2 for the LF and HF groups separately and combined. In the combined sample, plasma arsenic, urinary arsenic, and the amount of urinary arsenic in relation to creatinine ($\mu g$ As/g Cr) were negatively correlated with the extent of $[^3H]$-methyl incorporation ($P = 0.03$, $0.009$, and $0.04$, respectively), which suggests that arsenic exposure is positively associated with genomic methylation of PBL DNA. In agreement with other investigators (40), we found that age was positively ($P < 0.0001$) associated and plasma folate was negatively ($P = 0.03$) associated with $[^3H]$-methyl incorporation. When the LF and HF groups were analyzed separately, the magnitude of effect of arsenic exposure on $[^3H]$-methyl incorporation differed by group (Table 2), so that the significant negative relation between arsenic exposure and $[^3H]$-methyl incorporation observed in the combined sample was apparent only in the HF group.

In the HF group, urinary arsenic was associated with a dose-dependent decrease in $[^3H]$-methyl incorporation after control for age, sex, urinary creatinine, and smoking (Figure 1; $P$ for trend $= 0.009$); no significant dose response was observed in the LF group. Estimated regression coefficients of urinary and plasma arsenic and age as predictors of $[^3H]$-methyl incorporation of PBL DNA for the LF and HF groups separately and combined are shown in Table 3. In the combined sample, linear regression analysis found a negative association between urinary arsenic and $[^3H]$-methyl incorporation that persisted after control for age, urinary creatinine, smoking, and sex ($P = 0.04$). Plasma arsenic also was associated with $[^3H]$-methyl incorporation ($P = 0.07$). Age, unadjusted and adjusted, was the strongest predictor of $[^3H]$-methyl incorporation ($P < 0.0001$ and $P = 0.002$, respectively).

Plasma folate was inversely correlated with $[^3H]$-methyl incorporation ($P = 0.01$). However, it lost significance and the effect was reduced after control for age, smoking, and sex ($P = 0.10$). In stratified regression (Table 3), we found that, within the HF group, urinary and plasma arsenic concentrations were negatively associated with $[^3H]$-methyl incorporation after control for covariates ($P = 0.002$ and 0.04, respectively). No significant associations between arsenic exposure and $[^3H]$-methyl incorporation were found in the LF group. Linear regression analysis substantiated that the significant associations between arsenic biomarkers and $[^3H]$-methyl incorporation in the combined sample of the LF and HF groups were largely attributable to the strong associations found in the HF group and that those associations persisted after control for additional covariates. Total blood arsenic concentrations were analyzed in a subset of 224 of these participants (a subset having greater exposure), and the results were consistent with those for plasma arsenic: in the HF group after adjustment for sex, smoking, and age, total blood arsenic was significantly associated with $[^3H]$-methyl incorporation ($P = 0.01$), whereas no significant association was found in the LF group. Finally, with the combined LF and HF groups sample, we found significant interactions between the arsenic exposure variables (plasma arsenic, total blood arsenic, and urinary arsenic adjusted for creatinine) and folate ($<9$ compared with $\geq 9$ nmol/L) in predicting PBL DNA methylation after control for covariates ($P = 0.06$, $0.006$, and $0.005$, respectively).

The increase in genomic $[^3H]$-methyl incorporation in PBL DNA associated with increasing age is shown in Figure 2. The mean ± SE DPM, after adjustment for sex, urinary arsenic, creatinine, and smoking, is plotted for each age quartile. A significant age-dependent increase in $[^3H]$-methyl incorporation was observed ($P = 0.001$).

**DISCUSSION**

We investigated the association between arsenic exposure and genomic methylation of PBL DNA and examined the influence

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**TABLE 2**

<table>
<thead>
<tr>
<th>Low-folate group</th>
<th>High-folate group</th>
<th>Group</th>
<th>Total difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>0.24±</td>
<td>0.08±</td>
<td>-0.15±</td>
</tr>
<tr>
<td>Water arsenic ($\mu g$/L)</td>
<td>-0.31±</td>
<td>0.08±</td>
<td>0.008±</td>
</tr>
<tr>
<td>Urinary arsenic ($\mu g$/L)</td>
<td>-0.29±</td>
<td>0.02±</td>
<td>0.13±</td>
</tr>
<tr>
<td>Urinary arsenic/creatinine ($\mu g$/g Cr)</td>
<td>0.00±</td>
<td>0.02±</td>
<td>0.12±</td>
</tr>
<tr>
<td>Plasma arsenic ($\mu g$/L)</td>
<td>0.03±</td>
<td>0.03±</td>
<td>0.03±</td>
</tr>
</tbody>
</table>

1 PBL, peripheral blood leukocyte.
2 $P < 0.0001$.
3 $P < 0.01$.
4 $P \leq 0.05$.
5 $n = 137$ and 87 in the low-folate and high-folate groups, respectively.

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**FIGURE 1.** Mean (±SE) $[^3H]$-methyl incorporation, measured as disintegration per minute (DPM) per $\mu g$ DNA, by quartile of urinary arsenic for the low-folate ($n = 190$) and high-folate ($n = 104$) groups. Values were adjusted for age, urinary creatinine, sex, and smoking. $P$ for trend $= 0.98$ and 0.009 for low-folate and high-folate groups, respectively. Folate × urinary arsenic interaction, $P = 0.005$. 

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**FIGURE 2.** Mean (±SE) $[^3H]$-methyl incorporation, measured as disintegration per minute (DPM) per $\mu g$ DNA, by quartile of age for the low-folate ($n = 190$) and high-folate ($n = 104$) groups. Values were adjusted for urinary creatinine, sex, and smoking. $P$ for trend $= 0.009$. 

of folate nutritional status on this association. To our knowledge, this publication is the first report of this relation in a human population study.

As anticipated, folate was positively associated with DNA methylation, although the association was not strong and became nonsignificant after adjustment for covariates. Age was the most robust predictor of genomic methylation of PBL DNA, which supports the notion that there is a gradual and cumulative age-dependent loss of genomic DNA methylation (18). In the present study, humans were chronically exposed to sodium arsenite (45 mg/L for 48 wk) induced hepatic genomic DNA hypomethylation and gene-specific hypomethylation of the estrogen receptor α gene (19). Some have proposed that methylation of arsenic may deplete the methyl donor pool and deprive the DNA methylation process of SAM, thereby causing DNA hypomethylation (20). However, it is not known whether PBLs or their progenitor cells methylate arsenic. In the present study, none of the speciated arsenic metabolites (ie, inorganic arsenic or monomethylarsenic or dimethylarsenic acids) were differentially associated with genomic methylation of PBL DNA (data not shown).

Arsenic exposure has also been shown to produce gene-specific promoter hypermethylation of p53 in human lung adenocarcinoma cells (22, 46) and of p16^{INK4a} and RASSF1A in mice (47). Transcriptional suppression of numerous genes by arsenic exposure in human urothelial cells was restored by 5-aza-deoxyctidine, a DNMT inhibitor, which suggests that promoter DNA hypermethylation is involved in arsenic-induced gene silencing (18). Moreover, a recent study in humans exposed to arsenic in West Bengal, India, observed significant promoter hypermethylation of p53 and p16 in PBL DNA (21).

The results of the current study are contrary to our a priori hypothesis that arsenic exposure would be associated with genomic hypomethylation of PBL DNA, and they contradict the findings of the studies described above (19, 20, 45). There are many plausible explanations that can account for these disparities. First, the doses of arsenic used in the animal model studies are nearly 3 orders of magnitude greater than the exposure of our participants [45 mg/L in animal models compared with median water arsenic of 80 μg/L (range: 0.1–716 μg/L)]. Second, the duration of exposure is difficult to mimic when experimental models are used. In the present study, humans were chronically

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Low-folate group (n = 190)</th>
<th>High-folate group (n = 104)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B ± SE P</td>
<td>B ± SE P</td>
<td>B ± SE P</td>
</tr>
<tr>
<td>Urinary arsenic&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted for creatinine</td>
<td>−48.3 ± 351 0.89</td>
<td>−1818 ± 516 0.0006</td>
<td>−684 ± 291 0.02</td>
</tr>
<tr>
<td>Adjusted for creatinine, sex, smoking, and age</td>
<td>−87.7 ± 358 0.81</td>
<td>−1674 ± 528 0.002</td>
<td>−614 ± 298 0.04</td>
</tr>
<tr>
<td>Plasma arsenic&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>−195 ± 590 0.74</td>
<td>−1844 ± 809 0.02</td>
<td>−863 ± 479 0.07</td>
</tr>
<tr>
<td>Adjusted for sex, smoking, and age</td>
<td>−145 ± 596 0.80</td>
<td>−1729 ± 814 0.04</td>
<td>−741 ± 482 0.13</td>
</tr>
<tr>
<td>Total blood arsenic&lt;sup&gt;4,5&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>606 ± 536 0.26</td>
<td>−1912 ± 664 0.005</td>
<td>−342 ± 432 0.43</td>
</tr>
<tr>
<td>Adjusted for sex, smoking, and age</td>
<td>601 ± 546 0.27</td>
<td>−1857 ± 736 0.01</td>
<td>−328 ± 441 0.46</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>59.9 ± 27.1 0.03</td>
<td>110 ± 36.6 0.003</td>
<td>85.5 ± 21.6 &lt;0.0001</td>
</tr>
<tr>
<td>Adjusted for urinary arsenic, creatinine, sex, and smoking</td>
<td>66.1 ± 30.2 0.03</td>
<td>97.0 ± 40.0 0.02</td>
<td>82.7 ± 24.3 0.0008</td>
</tr>
</tbody>
</table>

<sup>1</sup> DPM, disintegration per minute; PBL, peripheral blood leukocyte.
<sup>2</sup> P for interaction (folate × plasma arsenic) = 0.005.
<sup>3</sup> P for interaction (folate × plasma arsenic) = 0.06.
<sup>4</sup> n = 137 and 87 in low-folate and high-folate groups, respectively.
<sup>5</sup> P for interaction (folate × total blood arsenic) = 0.006.

![FIGURE 2](image-url) Mean (±SE) [3H]-methyl incorporation, measured as disintegration per minute (DPM), by quartile of age (n = 294). Values were adjusted for urinary arsenic, urinary creatinine, sex, and smoking. P for trend = 0.001.
exposed to arsenic throughout most of their lives, whereas, in experimental studies, the exposures span <1 y. Third, although cell culture studies have applied arsenic concentrations that are more comparable to environmental human exposures, they cannot account for the toxicokinetic aspects of tissue distribution and metabolism of arsenic. Fourth, animal models permit assessment of DNA methylation in the liver and other tissues. Because the extent to which factors that influence hepatic DNA methylation may similarly influence DNA methylation in PBLs is not currently known, these differences in the source of DNA further compromise our ability to make meaningful comparisons. Further studies will be needed to determine the utility of PBL DNA methylation as a biomarker of DNA methylation in other tissues, to ascertain its predictive value for adverse health outcomes, or both.

There are several additional limitations of using PBLs as our sole source of DNA. First, circulating PBLs are not targets of arsenic-induced carcinogenesis. However, arsenate distributes to bone because of its structural similarity to phosphate, and it presumably could influence the methylation of PBL DNA during hematopoiesis. Second, ≈62% of PBLs are neutrophils, which are relatively short-lived—ie, the life span of neutrophils generally is <1 wk. Because PBL progenitor cells are rapidly dividing, they may respond more quickly to factors that influence DNA methylation than do cells that turn over more slowly. For this reason, PBL DNA methylation may not identify DNA methylation errors that are directly relevant to cancer, which is a disease process that occurs over decades. Third, because PBLs are not a single cell type but rather a mix of cell types, we cannot rule out the possibility that differences in the relative distribution of cell types may account for our observed findings; the similarity of genomic DNA methylation between these cell types is not known.

We can only speculate about a mechanism by which arsenic exposure may increase genomic DNA methylation. InAsIII is a well-known inhibitor of many enzymes, and thus it is plausible that arsenic could directly or indirectly influence the activity of DNMTs. Reports of the effects of arsenic exposure on overall DNMT activity are inconsistent; some reports indicate arsenic exposure results in reduced DNMT activity without any significant changes in the expression of specific DNMT isoforms (45), whereas other reports indicate that arsenic exposure increased DNMT activity (48). There are other examples, eg, S-adenosylhomocysteine (SAH) hydrolase deficiency, in which genomic DNA methylation is paradoxical, increased (49–51). Clearly, the regulation of genomic DNA methylation is complex and incompletely understood. It is possible that the observed increase in DNA methylation may reflect a compensatory or protective response to alterations in an upstream regulatory function, such as histone modifications, that may subsequently influence DNA methylation. Although little is known about the effect of inorganic arsenic exposure on histone modifications, arsenic trioxide, which is an effective treatment of acute promyelocytic leukemia, has been shown to promote the acetylation of histone tails (52). In this manner, arsenic exposure may influence chromatin remodeling, which could lead to a more open conformation that could result in increased DNA methylation. Our data imply that this outcome would be contingent on an adequate availability of methyl groups, either for the synthesis of SAM or the removal of the product inhibitor, SAH. Plasma SAH concentrations have been shown to be negatively correlated with genomic methylation of lymphocyte DNA (53). SAH is a potent inhibitor of most methylation reactions, including the methylation of arsenic and DNA (54–56). Concentrations of SAH can be lowered by downstream remethylation of homocysteine using the methyl group donated by 5-methyltetrahydrofolate.

The consequences of genomic DNA hypermethylation also remain unclear; however, an increase in genomic DNA methylation could increase the risk of subsequent mutations because methylcytosines are deaminated more frequently than are non-methylated cytosines, and they yield C→T transitions. Thus, aberrant DNA methylation could be one pathway by which arsenic acts as a cocarcinogen, either as a cancer initiator or a cancer promoter.

In conclusion, the results of this study indicate that arsenic exposure is positively associated with genomic methylation of PBL DNA among Bangladeshi adults who are chronically exposed to arsenic in drinking water and that folate nutritional status modifies this effect. The causal mechanisms and physiologic implications of greater genomic DNA methylation are unclear. Future lines of investigation should aim to identify the mechanism by which arsenic influences DNA methylation. Further studies also are warranted to determine whether alterations in genomic DNA methylation are associated with arsenic-induced disease outcomes.

We are grateful to Mominul Islam for directing the field staff and Shafiu Alam for overseeing laboratory operations in Araihazar and to our staff, the fieldworkers, and the study participants in Bangladesh, without whom this work would not have been possible. We also extend our appreciation to Jennie Kline for her contributions to the interpretations of these findings.

The authors’ responsibilities were as follows—MVG and JRP: study design, interpretation of results, and manuscript preparation; XL: statistical analyses; HA, PF-L, and JHG: assisted in manuscript preparation and interpretation of results; DL: data management; and JRP, VI, and VS: laboratory analyses.

None of the authors had a personal or financial conflict of interest.

REFERENCES

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