Influence of Prenatal Arsenic Exposure and Newborn Sex on Global Methylation of Cord Blood DNA

J. Richard Pilsner¹, Megan N. Hall², Xinhua Liu³, Vesna Ilievski⁴, Vesna Slavkovich⁵, Diane Levy³, Pam Factor-Litvak², Mahammad Yunus⁶, Mahfuzar Rahman⁶, Joseph H. Graziano⁴, Mary V. Gamble⁴*

¹Division of Environmental Health Sciences, School of Public Health and Health Sciences, University of Massachusetts, Amherst, Massachusetts, United States of America, ²Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, New York, United States of America, ³Department of Biostatistics, Mailman School of Public Health, Columbia University, New York, New York, United States of America, ⁴Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York, United States of America, ⁵International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh, ⁶Columbia University Arsenic Project in Bangladesh, Dhaka, Bangladesh

Abstract

**Background:** An emerging body of evidence indicates that early-life arsenic (As) exposure may influence the trajectory of health outcomes later in life. However, the mechanisms underlying these observations are unknown.

**Objective:** The objective of this study was to investigate the influence of prenatal As exposure on global methylation of cord blood DNA in a study of mother/newborn pairs in Matlab, Bangladesh.

**Design:** Maternal and cord blood DNA were available from a convenience sample of 101 mother/newborn pairs. Measures of As exposure included maternal urinary As (uAs), maternal blood As (mbAs) and cord blood As (cbAs). Several measures of global DNA methylation were assessed, including the [3H]-methyl-incorporation assay and three Pyrosequencing assays: Alu, LINE-1 and LUMA.

**Results:** In the total sample, increasing quartiles of maternal uAs were associated with an increase in covariate-adjusted means of newborn global DNA methylation as measured by the [3H]-methyl-incorporation assay (quartile 1 (Q1) and Q2 vs. Q4; p = 0.06 and 0.04, respectively). Sex-specific linear regression analyses, while not reaching significance level of 0.05, indicated that the associations between As exposures and Alu, LINE-1 and LUMA were positive among male newborns (N = 58) but negative among female newborns (N = 43); tests for sex differences were borderline significant for the association of cbAs and mbAs with Alu (p = 0.05 and 0.09, respectively) and for the association between maternal uAs and LINE-1 (p = 0.07). Sex-specific correlations between maternal urinary creatinine and newborn methyl-incorporation, Alu and LINE-1 were also evident (p < 0.05).

**Conclusions:** These results suggest that prenatal As exposure is associated with global DNA methylation in cord blood DNA, possibly in a sex-specific manner. Arsenic-induced epigenetic modifications in utero may potentially influence disease outcomes later in life. Additional studies are needed to confirm these findings and to examine the persistence of DNA methylation marks over time.

Introduction

Naturally occurring arsenic (As) is a ubiquitous element in the environment. Through processes that are incompletely understood, As in the solid phase can become solubilized and released into groundwater. Numerous aquifers around the world are enriched with this metalloid, posing a tremendous public health challenge in populations whose drinking water is obtained from contaminated aquifers [1]. Bangladesh is one of the most severely affected regions; approximately 35% of all tube wells contain concentrations of As exceeding the Bangladesh drinking water standard of 50 μg/L, and an estimated 35–77 million people are at risk for As-induced health outcomes [2]. Arsenic is classified as a human carcinogen by the International Agency for Research on Cancer and the U.S. Environmental Protection Agency [3]. However, it is often referred to as a ‘paradoxical’ human carcinogen because animal models have generally shown that As does not act as a complete carcinogen [4]. Lack of an ideal animal model due to variability in the response to As exposure across mammalian species [5] has slowed progression toward obtaining a complete mechanistic understanding of As-induced health outcomes. In 2003, Waalkes et al developed a
mouse model in which brief in utero exposure to As induced sex-specific tumor formation in adulthood. Male offspring developed liver and adrenal tumors and female offspring developed ovarian specific tumor formation in adulthood. Male offspring developed.

Ethics Statement

Methods

In our study of mother/newborn pairs from 101 pregnancies in Bangladesh, we previously demonstrated strong positive correlations between maternal blood As (mbAs) and cord blood As (cbAs), indicating that As readily crosses the placenta [27]. The objective of the present analysis was to test the hypothesis that maternal As exposure influences global methylation of cord blood DNA.

Total Urinary and Blood As Measurements

Urinary As (uAs) concentrations were assayed by graphite furnace atomic absorption spectrophotometry (GFAA) using a Perkin-Elmer AAnalyst 600 system (Shelton, CT) as described [30]. Our laboratory participates in a quality control program coordinated by the Quebec Toxicology Center in Quebec, Canada. During the course of this study, intraclass correlation coefficients between our laboratory’s values and samples calibrated at the Quebec Toxicology Center were 0.99. The detection limit of the method is 2 μg/L. In order to correct for urine dilution, we also measured urinary creatinine (uCrn) concentrations using a standard colorimetric method based on Jaffe’s reaction [31] with reagents from Sigma Diagnostics (Sigma, St. Louis, MO).

Maternal and cord blood samples were analyzed for total blood As concentration using a Perkin-Elmer Elan DRC II ICP-MS equipped with an AS 93+ autosampler as described [27]. Briefly, the As concentration of the standard solution used for instrument calibration was chosen to cover the expected range of As concentrations in the blood samples: 5, 25 and 50 μg/L. Matrix suppression was compensated for by the use of iridium (Ir) as an internal standard, selected to match to the ionization potential of As. Spectral interferences for As were resolved with Dynamic Reaction Cell (DRC) technology by introducing oxygen as a second gas. The intraclass correlation coefficients between our laboratory’s values and samples calibrated at the Quebec Toxicology Center were 0.99. The detection limit of the method is 0.06 μg/L.
DNA extraction

Genomic DNA was isolated at Columbia University from 300 μl of frozen whole blood samples using the FlexiGene DNA kit (Qiagen) following the manufacturer’s protocol, except for an additional centrifugation step at 10,000 × g for 5 min immediately after protease digestion to pellet any remaining proteins or lipids. The supernatant was then transferred into a new microcentrifuge tube containing 150 μl of isopropanol. The remainder of the procedures were conducted according to the manufacturer’s protocol. All extractions yielded ≥2 μg of DNA with a 260/280 ratio ≥1.7.

Global DNA methylation

Methyl Incorporation Assay. The methyl-incorporation assay was performed by the method of Balaghi and Wagner [32] as we have previously described in detail [21,33]. DNA was incubated with [3H]-SAM in the presence of the SssI prokaryotic methylase enzyme, which indiscriminately methylates all unmethylated cytosines in CpG sequences. The ability of DNA to incorporate [3H] methyl groups in vitro is inversely related to endogenous DNA methylation. Briefly, 250 ng of DNA was incubated for 1 hr at 37°C with 3 U of SssI methylase (New England Biolabs, Beverly, MA), 3.8 mM (1.1 μg) [3H]-labeled SAM (GE Healthcare), and EDTA, DTT and Tris-HCL (pH 8.2). The reaction mixtures were applied onto Whatman DE81 filter papers and washed on a vacuum filtration apparatus and air dried. Dried filters were analyzed by a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer. Each DNA sample was processed in duplicate and each processing run included samples for background (reaction mixture with all components except SssI enzyme), a hypomethylation control (HeLa cell DNA) and a quality control sample (DNA extracted from a whole-blood sample). The intra- and inter-day coefficients of variation (CVs) were 1.8% and 5.3%, respectively. To quantify the amount of double-stranded input DNA in each reaction, an aliquot of the assayed DNA was used to determine DNA concentrations using PicoGreen dsDNA Quantitation Reagent (Molecular Probes). All disintegrations per minute (DPM) values were expressed per μg DNA.

Alu and LINE-1 methylation. DNA samples (500 ng at 20 ng/μl) were bisulfite-treated using the EZ-96 DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA). Bisulfite conversion of DNA changes unmethylated cytosine to uracil and subsequently to thymidine after PCR whereas methylated cytosines are protected from bisulfite conversion, resulting in methylation-dependent differences in DNA sequences. Bisulfite-converted DNA was stored at -20°C until further use. LINE-1 and Alu methylation was measured by quantitative Pyrosequencing (Qiagen) using primers and conditions as described previously [34,35]. Pyrosequencing employs a primer extension reaction, using a biotin-labeled single stranded PCR amplicon as template, in which pyrophosphatase (PPi) is released during the incorporation of each nucleotide in equimolar proportion to that incorporated. The level of methylation for each CpG target region was quantified using the Pyro Q-CpG Software. Human incorporation. The level of methylation for each CpG target

Statistical Analyses

Descriptive statistics were calculated for sample characteristics of mothers and their newborns and by newborns’ sex. Sex differences for continuous variables were assessed using the Wilcoxon rank-sum test. Pyrosequencing data that did not pass Pyro Q-CpG Software quality scores or were not reproducible were excluded from analyses; these included three samples (one maternal Alu, one newborn Alu and one newborn LINE-1). For the methyl-incorporation assay, quality data was available for 98 samples. Spearman correlation coefficients were used to assess bivariate associations between maternal and newborn DNA methylation and sex specific correlations between uCrn and DNA methylation measures. Wald tests were used to evaluate differences in correlations by sex. Linear regression models were used to examine associations between As exposures and DNA methylation measures controlling for uCrn, including a uCrn by sex interaction term as appropriate. An As by sex interaction term was added to test for sex differences in the effect of As on DNA methylation. We also categorized uAs adjusted for uCrn, defined as the residual from regressing log uAs on sex-specific log uCrn, into quartiles, and computed mean levels of global DNA methylation for each quartile. All analyses were performed using SAS (version 9.2; SAS Institute Inc., Cary, NC).

Results

The demographic and clinical data of the study population are presented in Table 1. The mean age of the women was 26.7 ± 5.3 years and mean parity was 1.8 ± 0.9. Of the 101 births, 58 were males. The average birth weight was higher among male compared to female newborns (2,830.5 ± 517.2 g vs 2,647.0 ± 336.5 g; p < 0.05). Gestational age did not differ between...
male and female newborns. Water As concentrations ranged from 0.01 to 661 μg/L; 28% and 39% of tube wells exceeded the Bangladesh standard of 50 μg/L and the World Health Organization guideline of 10 μg/L, respectively. As previously reported [27], there was a strong correlation between cbAs and mbAs concentrations (r = 0.84; p < 0.0001), with the mean cbAs concentration higher than the mean mbAs concentration (15.7 μg/L vs. 11.9 μg/L, respectively; p < 0.0001). There were no significant differences in As exposures by newborn sex. As compared to male newborns, female newborns had higher maternal plasma homocysteine (6.7 ± 1.3 μmol/L vs. 6.1 ± 1.6 μmol/L; p = 0.04) and cord plasma homocysteine (6.1 ± 1.4 μmol/L vs. 5.4 ± 1.3 μmol/L; p = 0.008). None of the As exposure variables were significantly associated with gestational age, birth weight and length, or head circumference; however, male newborns had higher placenta and birth weight and larger head circumference as compared to female newborns (p < 0.05).

Global DNA methylation levels for the methyl-incorporation, Alu, LINE-1 and LUMA assays for mothers and newborns are also provided in Table 1 (full methylation data are available on request for academic or non-commercial purposes). Data are expressed as the percent of DNA methylation for Alu, Line-1 and LUMA. For the methyl-incorporation assay, the data are expressed as disintegrations per minute per microgram DNA (DPM/μg DNA), and are inversely related to endogenous DNA methylation levels. LINE-1 methylation was higher in male compared to female newborns (79.58 ± 1.8 vs. 78.82 ± 1.4; p = 0.001). The associations between mother/newborn pairs for each DNA methylation assay are shown in Table 2. Positive associations between mother/newborn pairs were observed for DNA methylation as measured by methyl-incorporation (Spearman correlation coefficient: r = 0.32, p = 0.001), LUMA (r = 0.22, p = 0.03), and LINE-1 (r = 0.21, p = 0.03). The correlation between mother/newborn pairs for Alu methylation was not statistically significant. In newborn sex-specific analyses, the associations between mother/newborn pairs for the methyl-incorporation assay were similar among male (r = 0.35, p = 0.007) and female (r = 0.31, p = 0.047) newborns. For the three pyrosequencing assays; however, the associations between maternal/newborn DNA methylation levels differed by newborn sex. For example, the correlations between mother/newborn pairs were significant for Alu methylation and LUMA among female newborns (r = 0.42, p = 0.005 and r = 0.37, p = 0.02, respectively), but not among male newborns. In contrast, a significant correlation for LINE-1 methylation was apparent only among male newborns (r = 0.39, p = 0.002).

No significant correlations were observed between As exposure and maternal and newborn DNA methylation (Table S1). However, in linear regression models adjusting for sex-specific effects of maternal uCrns levels, maternal uAs was negatively related with newborn methyl-incorporation levels (Table 3: b = -2.049 ± 1.087; p = 0.06). Figure 1 shows the pattern of the mean level of newborn DNA methylation, measured by the methyl-incorporation assay, among quartiles of maternal uAs adjusted for the sex-specific effect of uCrn. The mean newborn methyl-incorporation levels tended to decrease with increasing maternal uAs, indicating that uAs is associated with an increase in global DNA methylation. Compared to the first and second lowest quartiles of maternal uAs, newborns in the highest quartile of maternal uAs had lower methyl incorporation levels (Q1: 91,331 ± 2,499 DPM/μg DNA and Q2: 92,021 ± 2,436 DPM/μg DNA vs. Q4: 84,745 ± 2,491 DPM/μg DNA [p = 0.06 and 0.04, respectively]. Maternal uAs was not significantly associated with newborn methylation of Alu, LINE-1 and LUMA in the total sample (Table 3). We also found no significant associations between mbAs or cbAs concentrations and measures of newborn DNA methylation in the total sample.

In secondary analyses, we observed sex-specific patterns of association between As exposure and newborn DNA methylation via pyrosequencing (Table 3). Arsenic exposure measurements, while not statistically significant, were negatively related to the DNA methylation measures, Alu, LINE-1, and LUMA, for female newborns (N = 49), whereas these relationships were positive for male newborns (N = 50). Moreover, we observed borderline significant sex differences in the associations between blood As levels, cbAs and mbAs, and Alu methylation (p = 0.03 and 0.09, respectively) and between maternal uAs and LINE-1 methylation (p = 0.07). The methyl-incorporation assay did not display a sex-specific pattern of association with As exposure.

While not part of our a priori hypothesis, we observed an unexpected relationship between maternal uCrn levels and newborn global DNA methylation dependent on newborn sex (Table 4). Among male newborns, maternal uCrn was positively correlated with global DNA methylation (methyl-incorporation: r = -0.12, p = 0.38; Alu: r = 0.34, p < 0.01 and LINE-1: r = 0.28, p < 0.05). In contrast, among female newborns, maternal uCrn was negatively related with DNA methylation (methyl-incorporation: r = 0.49, p < 0.01; Alu: r = -0.06, p = 0.69; and LINE-1: r = -0.14, p = 0.36). We also found that these sex-specific patterns of association between maternal uCrn and newborn DNA methylation measures, i.e. methyl-incorporation, Alu and LINE-1 methylation, were statistically different (p < 0.05). The sex difference in the associations of uCrn with methyl-incorporation and LINE-1 methylation were still evident in linear regression models when uAs was included in the model (p = 0.002 and = 0.07, respectively). A similar sex-specific pattern of association was evident between cord blood homocysteine and newborn DNA methylation measures, although only LINE-1 methylation reached statistical significance (p < 0.05). Sex-specific patterns were not evident between other indicators of one-carbon metabolism and newborn DNA methylation.

Discussion

The objective of this study was to investigate the associations between prenatal As exposure and global methylation of cord blood DNA. In the total sample, our data indicated that maternal uAs was positively related to global methylation of cord blood DNA as assessed by the methyl-incorporation assay. In secondary analyses, we observed sex-specific patterns of association between As exposure and DNA methylation. Among male newborns, there was a consistent positive association between As exposure and DNA methylation. In contrast, As exposure was generally inversely associated with DNA methylation among female newborns. To our knowledge, there are no previous mother/newborn studies in humans investigating the association between prenatal As exposure and global methylation of cord blood DNA.

Growing evidence in human and animal studies suggests that early-life exposure to As increases the incidence of adverse health outcomes later in life, despite a reduction or elimination of exposure during adulthood. For example, inhabitants of Chile’s region II who were born just prior to or during an acute increase in water As concentrations, resulting in early-life and prenatal exposure, had higher mortality rates from malignant and nonmalignant lung disease and acute myocardial infarction as young adults compared to unexposed individuals from a different region [9,10,11]. The data also indicated that sex modified the effects of early-life As exposure and subsequent mortality rates,
Table 1. Demographics and clinical data of the study population.

| Variable                        | Mother* (N = 101) | Newborn* | Male (N = 58) | Female (N = 43) |
|---------------------------------|-------------------|----------|---------------|-----------------|
| Maternal Age (years)            | 26.7 (5.3)        | 27.5 (5.4)| 25.7 (5.0)    |                 |
| Education (years)               | 7.4 (3.6)         | 7.6 (3.5)| 7.2 (3.7)     |                 |
| Parity                          | 1.8 (0.90)        | 1.9 (0.8)| 1.7 (0.9)     |                 |
| Water arsenic (µg/L)            | 90.5 (165.8)      | 92.5 (157.0)| 87.8 (179.2)  |                 |
| Urinary arsenic (µg/L)          | 127.6 (232.9)     | 134.3 (226.1)| 118.7 (244.3)|                 |
| Urinary creatinine (mg/dl)      | 48.6 (35.3)       | 47.6 (32.9)| 50.1 (38.7)   |                 |
| Urinary arsenic (µg/g creatinine)| 271.7 (489.5)  | 282.1 (450.3)| 257.7 (543.1)|                 |
| Blood arsenic (µg/L)            | 11.9 (8.6)        | 12.6 (10.1)| 10.9 (6.0)    |                 |
| Plasma folate (nmol/L)          | 20.3 (9.9)        | 20.7 (79.6)| 19.6 (9.4)    |                 |
| Plasma homocysteine (µmol/L)    | 6.4 (1.5)         | 6.1 (1.6) | 6.7 (1.5)*    |                 |
| Plasma B12 (pmol/L)             | 180.8 (71.5)      | 193.6 (79.6)| 163.2 (54.9)  |                 |

Table 2. Spearman correlation coefficients for global DNA methylation between maternal vs. newborn pairs, combined and stratified by newborn sex.

|          | Methyl-incorporation | Alu | LINE-1 | LUMA |
|----------|----------------------|-----|--------|------|
|          | r (n)                | r (n) |  r (n) | r (n) |
| Total Sample | 0.32 (98)**          | 0.15 (99) | 0.21 (100)** | 0.22 (101)** |
| Male newborns | 0.35 (56)**          | 0.21 (100)** | 0.22 (101)** | 0.23 (101)** |
| Female newborns | 0.31 (42)**          | 0.42 (42)** | 0.39 (58)** | 0.37 (43)** |

*P<0.05; **P<0.01; ***P<0.001; Wilcoxon rank sum test for differences between newborn sex.

doi:10.1371/journal.pone.0037147.t002
Table 3. Estimated parameter for associations between biomarkers of As exposure and newborn global DNA methylation assays combined and stratified by sex.

| DPM/μg DNA | Alu | LINE-1 | LUMA |
|------------|-----|--------|------|
|            | b ± se (p-value) | b ± se (p-value) | b ± se (p-value) | b ± se (p-value) |
| **Total Sample**<sup>1</sup> | | | |
| Water As | -196.1 ± 412 (0.64) | -0.0004 ± 0.04 (0.99) | -0.016 ± 0.05 (0.76) | -0.126 ± 0.10 (0.21) |
| mbAs | -1858.2 ± 2478 (0.46) | -0.035 ± 0.22 (0.87) | 0.189 ± 0.32 (0.55) | 0.267 ± 0.60 (0.66) |
| cbAs | -1627.3 ± 2417 (0.50) | -0.046 ± 0.21 (0.83) | 0.201 ± 0.32 (0.53) | 0.060 ± 0.60 (0.92) |
| uAs | -2049.2 ± 1087 (0.06) | -0.003 ± 0.10 (0.98) | -0.022 ± 0.14 (0.87) | 0.009 ± 0.27 (0.71) |
| **Female cord blood** | | | |
| Water As | 334.9 ± 668 (0.62) | -0.037 ± 0.05 (0.45) | -0.050 ± 0.07 (0.50) | -0.311 ± 0.19 (0.12) |
| mbAs | 2902.5 ± 3834 (0.45) | -0.464 ± 0.29 (0.12) | -0.266 ± 0.43 (0.54) | -0.530 ± 1.12 (0.64) |
| cbAs | 2037.1 ± 3507 (0.56) | -0.458 ± 0.27 (0.09) | -0.053 ± 0.41 (0.90) | -0.445 ± 1.02 (0.66) |
| uAs | 1771.1 ± 1657 (0.29) | -0.164 ± 0.13 (0.21) | -0.314 ± 0.18 (0.09) | -0.218 ± 0.49 (0.66) |
| **Male cord blood** | | | |
| Water As | -416.0 ± 526 (0.43) | 0.035 ± 0.05 (0.48) | 0.047 ± 0.07 (0.53) | 0.005 ± 0.12 (0.97) |
| mbAs | -1063.5 ± 3274 (0.75) | 0.283 ± 0.30 (0.35) | 0.515 ± 0.45 (0.25) | 0.827 ± 0.68 (0.23) |
| cbAs | -1227.7 ± 3379 (0.72) | 0.355 ± 0.31 (0.26) | 0.430 ± 0.47 (0.36) | 0.553 ± 0.71 (0.44) |
| uAs | -2279.0 ± 1454 (0.13) | 0.123 ± 0.14 (0.37) | 0.196 ± 0.20 (0.33) | 0.330 ± 0.30 (0.28) |
| **Total sample**<sup>1,2</sup> | | | |
| Water As x sex | -871.3 ± 842 (0.30) | 0.039 ± 0.07 (0.60) | 0.085 ± 0.11 (0.44) | 0.289 ± 0.20 (0.16) |
| mbAs x sex | 1839.0 ± 5026 (0.72) | 0.747 ± 0.43 (0.09) | 0.781 ± 0.64 (0.23) | 1.426 ± 1.22 (0.25) |
| cbAs x sex | 809.5 ± 4860 (0.87) | 0.813 ± 0.42 (0.05) | 0.482 ± 0.63 (0.45) | 1.032 ± 1.20 (0.39) |
| uAs x sex | -507.9 ± 2195 (0.82) | 0.288 ± 0.19 (0.14) | 0.510 ± 0.28 (0.07) | 0.553 ± 0.47 (0.24) |

<sup>1</sup>Sex- specific effect of uCrn was controlled in the models for DPM/DNA, Alu and Line-1.  
<sup>2</sup>Interaction by newborn sex.

doi:10.1371/journal.pone.0037147.t003

Figure 1. Newborn global DNA methylation and maternal urinary As exposure. Mean (SE) newborn global methylation levels as measured by the methyl-incorporation assay by quartiles of maternal uAs exposure adjusted for the sex-specific effect of uCrn. * Q1 and Q2 vs. Q4; p = 0.06 and 0.04, respectively.
doi:10.1371/journal.pone.0037147.g001
such that males had higher mortality rates from lung cancer and acute myocardial infarction than females. Likewise, animal models have shown that brief prenatal exposure produced tumor formation in adulthood in a sex-specific manner [6]. Interestingly, tumor-free newborn mice, subjected to the same prenatal exposure protocol, exhibited similar changes in hepatic gene expression as observed in adult mice with hepatic tumors as well as reduced hepatic DNA methylation within GC-rich regions [8]. These studies indicate that epigenetic processes mediate persistent changes in tissues/cells analyzed and duration and/or magnitude of exposure [21].

Our previous studies also found that plasma folate modified the incidence of adult-onset diseases in response to early-life As exposure. Our data is consistent with the possibility that sex-specific epigenetic effects of As exposure could induce differential health outcomes between males and females later in life.

Overall, our results indicate that maternal uAs is positively associated with newborn global DNA methylation as assessed by the methyl-incorporation assay. Previous studies from our group have demonstrated that uAs concentrations were associated with an increase in global DNA methylation among Bangladeshi adults [21,22]. Similarly, As-associated increases in global DNA methylation were found among adults from West Bengal, India [23]. While animal and cell culture experiments have shown that As exposure is associated with global DNA hypomethylation [8,15,16,17,18,19], our contradictory findings may be a result of differences in tissues/cells analyzed and duration and/or magnitude of exposure [21].

Our previous studies also found that plasma folate modified the association between As exposure and global DNA methylation, such that only participates with adequate folate status ($\geq 9$ nmol/L) showed a positive association [21,22]. In the current study, most of the women in the study were taking prenatal vitamin supplements; therefore, plasma concentrations of folate and B12 are reflective of recent supplement use rather than long-term nutritional status. While B12 was found to be inversely correlated with LINE-1 methylation, we consider the direction of the correlation to be opposite to what one might have predicted and may have been a chance observation. Adjusting for folate and other covariates did not influence the magnitude of this effect (data not shown).

In secondary analyses, we found that newborn sex modified the association between As exposure and global methylation of cord blood DNA. Although the associations did not reach statistical significance, arsenic exposure and DNA methylation were positively associated in males and inversely associated in females.

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**Table 4.** Spearman correlation coefficients for newborn global DNA methylation vs. urinary creatinine and indicators of one-carbon metabolism in plasma, combined and stratified by newborn sex.

|                          | Methyl-incorporation | Alu          | LINE-1        | LUMA         |
|--------------------------|----------------------|--------------|---------------|--------------|
| Total Sample             |                      |              |               |              |
| Maternal urinary creatinine | $-0.16$ (98)         | 0.16 (100)   | 0.14 (100)    | $-0.16$ (101) |
| Maternal folate          | 0.06 (96)            | $-0.04$ (98) | $-0.11$ (98)  | 0.08 (99)    |
| Maternal B12             | $-0.05$ (92)         | $-0.09$ (94) | $-0.03$ (94)  | 0.05 (95)    |
| Maternal homocysteine    | 0.06 (98)            | 0.06 (100)   | $-0.01$ (100) | 0.07 (101)   |
| Cord folate              | 0.03 (93)            | $-0.18$ (95) | $-0.16$ (95)  | 0.02 (96)    |
| Cord B12                 | 0.02 (89)            | $-0.09$ (90) | $-0.23$ (90)* | $-0.003$ (91) |
| Cord homocysteine        | 0 (97)               | 0.03 (99)    | $-0.07$ (99)  | $-0.05$ (100) |
| Male newborns            |                      |              |               |              |
| Maternal urinary creatinine | $-0.12$ (56)         | 0.34 (57)**  | 0.28 (58)*    | $-0.12$ (58) |
| Maternal folate          | 0.23 (56)            | $-0.10$ (57) | $-0.19$ (58)  | 0.11 (58)    |
| Maternal B12             | $-0.02$ (53)         | $-0.20$ (54) | $-0.19$ (55)  | $-0.11$ (55) |
| Maternal homocysteine    | $-0.04$ (56)         | 0.10 (57)    | 0.08 (58)     | 0.14 (58)    |
| Cord folate              | 0.09 (52)            | $-0.31$ (53)*| $-0.25$ (54)  | 0.03 (54)    |
| Cord B12                 | 0.08 (53)            | $-0.21$ (53) | $-0.32$ (54)* | $-0.10$ (54) |
| Cord homocysteine        | $-0.08$ (55)         | 0.12 (56)    | 0.14 (57)     | 0.03 (57)    |
| Female newborns          |                      |              |               |              |
| Maternal urinary creatinine | 0.49 (42)**          | $-0.06$ (41)* | $-0.14$ (42)* | $-0.15$ (43) |
| Maternal folate          | $-0.17$ (40)         | 0.02 (41)    | 0.01 (40)     | 0.10 (41)    |
| Maternal B12             | $-0.04$ (39)         | 0.05 (40)    | 0.12 (39)     | 0.32 (40)*   |
| Maternal homocysteine    | 0.14 (42)            | 0.04 (42)    | $-0.04$ (42)  | $-0.07$ (43) |
| Cord folate              | $-0.08$ (41)         | $-0.01$ (42) | 0.01 (41)     | 0.01 (42)    |
| Cord B12                 | $-0.03$ (36)         | 0.10 (37)    | $-0.27$ (36)  | 0.20 (37)    |
| Cord homocysteine        | 0.07 (42)            | $-0.12$ (43) | $-0.27$ (42)* | $-0.28$ (43) |

*p<0.05; **p<0.01; *p<0.005; **p<0.001 from Wald test for sex differences in correlations.
doi:10.1371/journal.pone.0037147.t004
These results were unanticipated and were not part of our a priori hypothesis. However, a growing body of evidence in humans suggests that sex differences may arise in epigenetic responses to prenatal environmental exposures [41]. For example, the Dutch Hunger Winter Families Study found prenatal exposure to famine was associated with sex-specific DNA methylation changes that persisted into adulthood [42]. It has been proposed that sex-specific epigenetic differences may be due to sex hormones and their effects on organ development [41]. Although limited data exists on hormonal regulation of epigenetic processes during the neonatal period, progesterone and estrogen have been shown to downregulate DNA methyltransferase expression in women [43], and higher methylation of repetitive sequences has been reported in adult men as compared to women [44,45]. We also observed that male newborns had significantly higher LINE-1 methylation in cord blood as compared to female newborns. Interestingly, a recent study in adult mice reported sex-specific changes in hepatic global DNA methylation. In response to As exposure and a methyl-deficient diet, female mice had a significant increase in DNA methylation whereas male mice had a decrease in DNA methylation [46].

Our findings for sex-specific associations between maternal uCrn and global DNA methylation in cord blood were unanticipated. Numerous studies from our group have showed that uCrn is a strong predictor of As methylation [27,47,48,49]. Moreover, uCrn was found to be an independent predictor of the risk for development of As-induced skin lesions, such that every doubling of uCrn concentrations was associated with 60% reduced risk for skin lesion development [22]. The final step in creatine biosynthesis is a methylation reaction which consumes roughly 50% of all SAM [50,51], and endogenous creatine synthesis is downregulated by dietary creatine intake. Creatine, together with several isoforms of creatine kinase, plays critical roles in many aspects of energy metabolism in tissues with high energy requirements [52]. Because creatine is nonenzymatically degraded to creatinine, which is excreted in urine at a constant rate, uCrn is commonly used to normalize for fluctuations in the concentration of urine. However, uCrn concentrations are strongly influenced by other factors such as dietary creatine intake from protein sources, age, sex, and muscle mass [53,54]. Although little is known about fetal requirements for creatine, animal studies indicate that the creatine transporter, responsible for cellular creatine uptake, is widely expressed very early in development, whereas fetal creatine biosynthesis doesn’t occur until very late in pregnancy, suggesting that the developing fetus is dependent on transport of maternal creatine across the placenta [55]. It is unclear why we observed sex-specific patterns of association (e.g., positive in male and negative in female newborns) between DNA methylation and maternal uCrn levels. Creatine biosynthesis is differentially influenced by estrogen and testosterone [52], and maternal circulating sex hormone concentrations are influenced by the sex of the fetus [56]. Thus, although speculative, it is possible that maternal creatine biosynthesis is influenced by fetal hormone levels. Because estrogen levels may be associated with both creatinine and DNA methylation, it is possible that the sex-specific association between maternal uCrn and cbDNA methylation could be explained by estrogen. However, we cannot rule out the possibility that this was simply a chance observation, and additional studies are needed to replicate this unexpected finding.

An advantage of this study is the use of multiple assays to estimate global DNA methylation levels. Although the four methods have been used widely as indicators of global DNA methylation [32,38,57], the genomic targets for each assay are distinct. They can be classified as either genome-wide assays (methyl-incorporation and LUMA) or as repetitive element assays (Alu and LINE-1). Of the four assays, the methyl-incorporation assay provides the greatest genomic coverage, as it measures all unmethylated CpG sites across the genome. Of note, this reaction (using unlabeled S-adenosylmethionine) is routinely used to generate 100% methylated DNA standards for other DNA methylation techniques, such as Pyrosequencing [58]. Using the methyl-incorporation assay, DNA methylation was positively associated with maternal uAs levels in our total study sample and also showed the strongest correlation between mother and newborn DNA methylation levels. LUMA is another genome-wide assay that employs the methyl-sensitive and insensitive enzymes, HpaII and MspI, respectively, to interrogate the methylation status of the estimated 2.2 million CpG dinucleotides contained within CCGG sequences in the human genome [59]. LUMA has been previously shown to be associated with environmental exposures [36]. However, only roughly 8% of total genomic CpGs are located within CCGG HpaII recognition sequence [59], thus the leaner coverage may explain why our results for LUMA were not as robust as our findings for the methyl incorporation assay.

Alu and LINE-1 are highly repetitive elements. Approximately 1.5 million copies of Alu and 0.5 million copies of LINE-1 are dispersed throughout the genome. Together they comprise around 25% of the genome and > 40% of methylated CpG domains [34,60]. As such, Alu and LINE-1 methylation are considered to be surrogates for global DNA methylation and have been found to be associated with a number of environmental exposures [35,61,62]. These methylation analyses are based on PCR amplification of consensus LINE-1 and Alu sequences, which amplifies a representative subset of elements (e.g. approximately 1% of Alu elements [57]). Downstream sequencing is then limited to three (Alu) and four (LINE-1) consecutive CpG dinucleotides. In our study, Alu and LINE-1 methylation display a distinct sex difference in association with As exposure. It is unclear if these differential associations are influenced by sex hormones of the developing fetus or other unidentified factors.

An unanticipated finding was that the associations between maternal DNA methylation and that of her newborn as measured by Pyrosequencing differed by newborn sex, such that LINE-1 methylation was correlated only among male newborn/maternal pairs, whereas Alu methylation was correlated among female newborn/maternal pairs. These inconsistencies complicate the interpretation of Alu and LINE-1 as surrogates for global DNA methylation. A potential explanation for these differences may be related to the fact that Alu and LINE-1 use different types of internal RNA polymerase promoters, suggesting that these repetitive elements may be under different selective pressures [63]. Differences in copy numbers within sex chromosomes may also contribute to these inconsistencies. It has also been reported that higher annealing temperatures produced larger differences in LINE-1 methylation levels between sexes [45].

A limitation to this study is our use of a convenience sample of participants drawn from a study that was originally designed to characterize the As metabolite profiles among only 101 mother/newborn pairs [27]. This resulted in low statistical power for the current study, especially after stratifying our data by sex or testing for interactions. Although this impaired our ability to detect statistically significant associations, the data are strongly suggestive of an influence of maternal As exposure on cord blood DNA methylation that may differ by sex.

In conclusion, the results of this study suggest that newborn sex may modify the association between prenatal As exposure and global methylation of cord blood DNA. Our data offer a plausible
epigenetic mechanism by which prenatal arsenic exposure may contribute to observed sex differences in As-induced health outcomes later in life. Additional studies are needed to confirm these results and to examine the persistence of DNA methylation marks across the lifespan.

Supporting Information

Table S1 Spearman correlation coefficients between As exposures and maternal and newborn DNA methylation.

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Acknowledgments

This work was a collaboration between the Mailman School of Public Health and The International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B). We acknowledge with gratitude the support of these organizations for this research.

Author Contributions

Conceived and designed the experiments: MY JR HJG JRP MVC. Performed the experiments: JRP VS VI. Analyzed the data: JRP MNH PFL XL. Wrote the paper: JRP MVC. Data management: DL.

Table S1 Spearman correlation coefficients between As exposures and maternal and newborn DNA methylation. (DOCX)
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