Urinary Arsenic Methylation Species, Arsenic Methylation Efficiency during Pregnancy and Birth Outcomes

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Abstract

Arsenic exposure from drinking water has been associated with symptoms during pregnancy, as well as negative birth outcomes. The individual's capability to methylate arsenic modifies the risk of arsenic-related health outcomes. Bangladesh is facing the world's most severe arsenic crisis, as well as a high prevalence of low birth weight. However, little is known about determinants of arsenic metabolism during pregnancy and its relationship to birth outcomes. Thus, we conducted this study in Bangladesh to identify determinants of arsenic methylation capability during pregnancy, and its relationship with birth outcomes.

The study was based on a Bangladesh prospective reproductive cohort (Project Jeebon) of 1,613 pregnant women recruited between 2008 and 2011. We quantified participants' urinary arsenic methylation species, including inorganic arsenic (iAs), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) repeatedly at the early gestational period and the mid-to-late gestational period. In the first paper, we analyzed the association between arsenic metabolism during pregnancy and potential determinants including demographic, exposure, nutritional variables. Our findings suggested that weeks of gestation was strongly associated with arsenic methylation efficiency only in the early gestational period. Daily protein intake was negatively associated with arsenic metabolism in the early gestational period, controlling for energy intake level.

In the second paper, we examined the genetic variants associated with arsenic methylation during pregnancy as well as its SNP-arsenic interaction effect. We have identified SNPs in *As3MT* were associated DMA% in both early and mid-to-late pregnancy period. The individual associations between SNPs in *GSTO2*, *As3MT*, and *CNNM2* gene were modified by arsenic exposure levels in both gestational periods. And we have identified the SNP-arsenic interaction of *N6AMT1* SNPs only in the mid-to-late gestational period. However, these SNP-arsenic interactions effects were not significant after adjusting for false discovery rate.

In the third study, we used causal mediation models to assess maternal arsenic metabolism by gestational periods to identify a sensitive exposure time window linked to adverse birth outcomes. We have found less efficient arsenic metabolism in the mid-to-late gestational period was associated with a lower birth weight that was mediated through the gestational age at birth.

Abbreviations:

SNP: Single nucleotide polymorphisms

As3MT: Arsenite methyltransferase

GSTO2: Glutathione S-transferase omega 2

CNNM2: Gyclin and CBS domain divalent metal cation transport mediator 2

N6AMT1: N-6 Adenine-Specific DNA Methyltransferase 1

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CHAPTER ONE

Determinants of arsenic methylation efficiency and total arsenic excretion of pregnant women in Bangladesh

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Abstract

Background: Epidemiological studies suggest that prenatal inorganic arsenic (iAs) exposure is associated with low birth weight, while the inter-individual variability of metabolizing inorganic arsenic may influence the vulnerability of exposed pregnant women; the factors associated with arsenic methylation are unclear.

Objectives: To examine arsenic methylation and excretion profile during pregnancy and identify determinants of arsenic metabolism efficiency.

Method: We followed 1613 pregnant women in Bangladesh since early pregnancy and took two repeated urine samples at visit 1 (4-16 weeks of pregnancy) and visit 2 (21-37 weeks of pregnancy). We analyzed the concentrations of urinary creatinine and urinary arsenic metabolites, including arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). Creatinine-adjusted total urinary arsenic (U-As) is a biomarker of total arsenic excretion, and the proportions of each urinary arsenic metabolite over total urinary arsenic [iAs^{III}%, iAs^{V}%, iAs%(iAs^{III}% + iAs^{V}%), MMA%, and DMA%] are biomarkers of arsenic methylation efficiency. We examined the trends of arsenic methylation and excretion using paired t-test. Dietary protein and folate intake were assessed using a locally validated semi-quantitative food frequency questionnaire administered at visit 2. Linear regressions were used to examine the determinants of arsenic metabolism, including age, BMI at enrollment, education, income of the financial provider, urinary arsenic exposure, daily folate intake, and daily protein intake, adjusted for daily energy intake.

Results: Comparing visit 2 to visit 1, median iAs% decreased 1.40% (mean decrease = 1.08%, $p = 0.0021$), and U-As increased 21% (95% CI: 15%-26%, $p<0.0001$). Drinking water

arsenic concentration was positively associated with iAs% in both visits. And daily protein intake was positively associated with iAs% at visit 1, adjusted for energy intake.

Conclusion: Our findings indicate that arsenic metabolism and excretion both increased during pregnancy. Daily protein intake is negatively associated with arsenic metabolism in the first visit, adjusting for energy intake.

Introduction

Inorganic arsenic is ubiquitous naturally occurring environmental toxicant [1, 2]. It is a serious public health concern, as two-hundred million people worldwide are exposed to arsenic through drinking water above the World Health Organization (WHO) recommended standard of 10μg/L [3]. In addition to being identified as a Class 1 carcinogen by the WHO [4], arsenic in drinking water has been associated with increased risk to cardiovascular conditions [5], pregnancy complications [6] and developmental impairments [7, 8]. Pregnant women and developing fetuses are especially susceptible. The arsenic-related adverse health conditions during pregnancy include anemia, nausea, vomiting and abnormal cramping [6, 9]. Arsenic can easily cross the placenta, and epidemiological studies have linked prenatal arsenic exposure to reduced gestational weeks, low birth weight, spontaneous abortion, stillbirth, neonatal mortality, and infant mortality [10-18].

The arsenic problem is the most severe in Bangladesh, which has more than 19 million people exposed to >5 times the standard arsenic level in drinking water [19]. This poses a significant risk for maternal and infant health. Low birth weight, defined as birthweight < 2,500 g, affects 36% of newborns in Bangladesh [20]. In addition to avoiding exposure to high levels of arsenic from drinking water, identifying factors that influence susceptibility to arsenic toxicity

to mothers and children can provide knowledge on risk assessment and guide effective interventions in underserved arsenic-endemic areas.

The human body mainly metabolizes arsenic in the liver. The ingested arsenate (iAs^{V}) is reduced to arsenite (iAs^{III}) by arsenate reductase, then react with glutathione (GSH), and enzymatically methylated to monomethylarsonous acid (MMA^{III}) by the methyl-donor S-Adenosyl methionine (SAM). MMA^{III} is either rapidly oxidized to monomethylarsonic acid (MMA^{V}), or involved in another cycle of methylation reaction to form dimethylarsinous acid (DMA^{III}), then oxidized to dimethylarsinic acid (DMA^{V}) [21]. Among the organic forms of arsenic, the trivalent forms (MMA^{III} and DMA^{III}) are highly toxic and reactive intermediates in the arsenic methylation pathway, and their pentavalent forms (MMA^{V} and DMA^{V}) are less toxic and more readily excreted in the urine [22]. The valence of organic arsenics can barely be detected accurately in human urine sample [23] but the proportions of urinary excretion of iAs, monomethyl- forms of arsenic (MMA) and dimethyl- forms of arsenic (DMA) have often used in the evaluation of the *in vivo* arsenic methylation [24].

The inter-individual variability of arsenic methylation is an important modifier of arsenic-related risk. Several human studies have found evidence that the lower proportion of DMA and a higher proportion of iAs may indicate a lower rate of arsenic methylation, which may lead to more iAs being retained in the body [25-28]. Arsenic methylation efficiency can modify the risk of low birth weight to the exposed pregnant women, as greater proportions of inorganic arsenic in urine have been shown to impaired fetal growth [29, 30]. Nevertheless, it is difficult to capture the precise amounts of iAs, MMA, and DMA from inhalation or ingestion.

Many factors influence the methylation and excretion processes of arsenic species, including the level of arsenic exposure, age, BMI, sex, smoking, genetic factors, ethnicities,

pregnancy, and breastfeeding [31, 32]. In addition, the level s of MMA and DMA are differently associated with these factors [31].

There is growing evidence to suggest that nutritional status influences arsenic metabolism and the risk of arsenic-related morbidity. Folate has been associated with the proportion of arsenic species in urine; and since arsenic metabolism pathway involves one-carbon metabolism, folate may play a role in arsenic methylation process by synthesizing the methyl-donor, SAM [33]. An epidemiological study found folate level in plasma is positively related to the percentage of DMA in total urine arsenic (DMA%) and negatively associated with iAs% and MMA%, hence with better arsenic methylation efficiency [33]. In another study, folate supplementation to a folate-deficient cohort was associated with increased urinary DMA%, from 72% to 79% [34], as well as increased excretion of arsenic from blood [35]. A 12 and 24 weeks of treatment of 800 μg folate per day to the folate-deficient cohort significantly lowered blood arsenic as compared to the placebo group, which was sustained for 12 weeks after the cessation of folate [36]. However, literature about folate and arsenic metabolism in pregnant women is limited. Only one epidemiological study has studied this topic and found that arsenic metabolism during pregnancy is independent of blood folate level [37].

Studies about protein intake and arsenic metabolism have not reached a consensus. One epidemiological study in Taiwan found evidence to suggest that a low-protien diet is related to increased arsenic-related skin cancer risk [38]. Animal studies have shown a low protein and methionine diet was an effect modifier of the association between arsenic exposure and arsenic-related health effect [39, 40]. Epidemiology studies have reported that dietary protein intake was associated with higher arsenic metabolism or higher total urinary arsenic excretion [41-43]. But another study just found non-significant results [42].

Pregnant women undergo complex hormonal changes. Studies have shown arsenic methylation efficiency increases remarkably during pregnancy, and that arsenic excretion is mostly in the form of DMA, with a smaller amount as MMA and iAs [37, 44, 45]. The patterns and determinants of arsenic metabolism need to be studied separately in pregnant women, not only because of their changed metabolism, but because their developing children are especially vulnerable to external toxicants [37, 46]. However, little is known about the determinants of arsenic metabolism in pregnant women.

In our study, we analyzed the profile of arsenic methylation and excretion efficiency at different gestational peiords: early and mid-to-late pregnancy. We also investigated the determinants that influence arsenic metabolism and excretion including gestational weeks, water arsenic exposure, socioeconomic variables, dietary folate and protein intakes, to provide a better understanding of arsenic metabolism during pregnancy.

Methods

Study Population

Between 2008 and 2011, we recruited 1613 pregnant women from two study centers in Bangladesh. One is located in Sirajdikhan, a suburban upazila, located 29 km south of the capital city Dhaka, and the other is Pabna, a rural upazila, located 122 km northwest from Dhaka [6, 47]. The eligible participants were adult women (≥ 18 years old) in their first trimester of pregnancy with a single fetus; who had a complete drinking water history that included tube well usage up to 6 months before her pregnancy. These women participated in Dhaka Community Hospital's (DCH) prenatal health program and planned to deliver at home with a DCH-trained midwife or at DCH hospital or clinic. At enrollment (visit 1), trained DCH's women community healthcare

workers, who lived in the local area, administered questionnaires to collect information on demographics, lifestyle, and medical condition.

The follow-up visit (visit 2) occurred at around 28 weeks of pregnancy. The healthcare workers administered a locally validated food frequency questionnaire (FFQ) to acquire their habitual dietary intake information from the previous 12 months. At the time of visit 2, 1438 participants remained in the study, and the reasons of loss-of-follow-up include loss of contact (N= 6), participant dropout (N= 18), miscarriage (N=131), stillbirth (N= 11), sample failure (N=2), missing samples (N=2) and twin pregnancy (N=5).

At recruitment (visit 1), we collected a drinking water sample, a urine sample and toenail sample from each participant. We provided all participants with daily prenatal multi-vitamin with folate (400 μg) upon recruitment. Due to ethical responsibility, we advised the participants to avoid using contaminated water sources. We collected another urine sample at around 29 weeks of gestation, and administered the questionnaire for dietary assessment We followed the participants until one-month post-partum, and collected their drinking water samples and toenail samples again to assess the stability of arsenic exposure.

All procedures of this study were approved by Institutional Review Boards of both Harvard T.H. Chan School of Public Health and the Dhaka Community Hospital Trust.

Urinary arsenic metabolites concentration

For each participant, we collected two spot urine samples at visit 1 and at visit 2, in order to assess the concentrations of the arsenic methylation species, including iAs^{III}, iAs^{V}, MMA and DMA. At the time of each visit, the healthcare workers provided each participants a urine cup and instructed them to obtain enough urine for analysis. Urine samples were sealed appropriately and brought to the local laboratory in iceboxes, then stored in a -20 ºC freezer. Frozen urine

samples were shipped to the Department of Public Health, School of Medicine at Taipei Medical University, Taiwan. The urine samples were then thawed at room temperature, sonicated for dispersion, filtered through Sep-Pak C18 column (Mallinckrodt Baker Inc., NJ, USA), and transferred into 200 μL aliquots. Arsenic species were fractioned by high-performance liquid chromatography (HPLC; Waters 501, Waters Associates, Milford, MA, USA) with columns obtained from Phenomenex (Nucleosil, Torrance, CA, USA). The concentrations of the 4 urinary arsenic metabolites were determined by hydride generator atomic absorption spectrometry (HG-AAS, Perkin Element). The standard reference material (SRM) No. 2670 was obtained from National Institute of Standard and Technology (NIST, Gaithersburg, MD, USA). The recovery rates for iAs^{III}, iAs^{V}, MMA and DMA ranged from 93.8% to 102.2%, and the detection limits for each were 0.02 μg/L, 0.06 μg/L, 0.07 μg/L, and 0.10 μg/L, respectively. We kept records below the limit of detection at their original value in our statistical analyses [48, 49]. In order to control for the dilution of the urine, we analyzed the concentration of urinary creatinine by the colorimetric assay (Roche Modular P800 instrument, Roche Inc., Mannheim, Germany).

Drinking water arsenic concentrations

To assess the drinking water arsenic exposure level (DW-As) and its stability during the follow-up period, two repeated drinking water samples were collected at visit 1 and one-month post-partum.

The protocol for drinking water collection is described elsewhere [6]. Briefly, tube well water samples were collected from the participant's primary water source using well pumps after a one-minute purge. We transferred samples to acid-washed polyethylene bottles and acidified them with nitric acid for storage. The samples were then shipped to the laboratory and analyzed using a hybrid-generation technique of high-resolution inductively coupled process spectrometer

(ICP-MS), following US EPA method 200.8 (Environmental Laboratory Services, North Syracuse, New York). The machine was sensitive to <1 μg/L of total arsenic concentration. Records lower than the detection limit were assigned the half value of the detection limit in statistical analyses.

Toenail samples

To assess long-term arsenic level in human body, we collected two maternal toenail samples for each participant at visit 1 and at one-month post-partum. The protocol is described elsewhere [10, 50]. Briefly, toenail samples were digested using optima nitric acid, and analyzed using ICP-MS for total arsenic concentration [51].

Dietary assessment

To obtain the recalled food consumption frequency during the previous 12 months, we administered a locally-validated semi-quantitative food frequency questionnaire (FFQ) at visit 2 [52]. We included 42 common food items in Bangladesh from five categories: (1) cereal and bread; (2) vegetables; (3) legumes, pulses, and seeds; (4) fish, poultry, meat, and eggs; and (5) milk-based food items. The scale of consumption frequency and portion sizes are described elsewhere [52]. Trained technicians entered the FFQ data. Daily protein intake (g/day), folate intake (μg /day) and energy intake (kCal/day) were estimated using the 2013 food composition table for Bangladesh [53]. For dish types that were not available in the Food Composition Table, the nutrient compositions were calculated based on average weighted recipes provided by local dietitians at DCH using the nutrient retention factors and yield factors provided in the 2013 Bangladesh Food composition table.

Statistical analysis

We adjusted urinary arsenic metabolites concentration (mg/g-creatinine) by their absolute concentration (μg/L) divided by urinary creatinine concentration (mg/dL). The adjusted total urinary arsenic (U-As) was the sum of adjusted urinary arsenic metabolites. The proportion of each urinary arsenic metabolite (iAs^{III}%, iAs^{V}%, MMA%, and DMA%) was its arsenic metabolite concentration divided by the sum of all urinary arsenic metabolite concentrations. The proportion of inorganic arsenic (iAs%) was the sum of iAs^{III}% and iAs^{V}%.

We used paired t-tests to assess the change of arsenic metabolism during the period between visits 1 and 2. We also assessed the changes of arsenic metabolite proportions, the change of log-transformed U-As were assessed, as well as the change of log-transformed DW-As and T-As. We calculated the Spearman correlations of urinary arsenic metabolites.

Linear regression models were used to examine the associations of arsenic metabolism biomarkers and potential determinants. Each urinary arsenic metabolite proportion variable was treated as an outcome variable, respectively. The independent variables were age, body mass index (BMI) at enrollment, environmental arsenic exposure continuous), education level (lower than secondary education/secondary education or higher), income of the financial provider, daily protein intake, daily folate intake, and daily energy intake tertile groups(low/medium/high). We fit the model for the two visits separately.

As no significant differences in demographic or exposure levels were found between the complete cases and whole study population, we did a complete case analysis for all the above assessments. We performed the analysis in SAS software program (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Almost all study participants were Bangladeshi homemakers (99%), non-smokers (100%), and did not chew tobacco or betel nut (99%). Most (99%) reported their physical activity level during pregnancy as "on their feet all day but in a stationary position or only spend half the day moving around on their feet." Detailed information about participants' demographics, lifestyle and exposure information is shown in Table 1.1. Participants from Pabna had a higher average DW-As exposure level (79.5 ± 131.3 μg/L) compared to Sirajdikhan (12.2 ± 47.4 μg/L). All participants reported taking prenatal vitamins with folate every day from visit 1 to visit 2.

Table 1.1: Characteristics of Study Participants in Sirajdikhan and Pabna upazila, Bangladesh.

	Sirajdikhan(N=879)	Pabna (N=727)	All mothers
Age, visit 1[†]			
	22.8 ± 4.1	23.0 ± 4.2	22.9 ± 4.2
Education Level[††]			
< Secondary education	446 (51%)	335 (46%)	781 (49%)
>= Secondary education	431 (49%)	389 (54%)	820 (51%)
BMI, visit 1			
	21.0 ± 3.3	20.0 ± 2.9	21.0 ± 3.0
Financial provider's income (Taka)			
unknown	9 (1%)	23 (3%)	32 (2%)
0 – 2000	5 (1%)	10 (1%)	15 (1%)
2001 – 3000	39 (4%)	197 (27%)	236 (15%)
3001 – 4000	178 (20%)	222 (31%)	400 (25%)
4001 – 5000	328 (37%)	167 (23%)	495 (31%)
5000 – 6000	190 (22%)	63 (9%)	253 (16%)
> 6000	129 (15%)	44 (6%)	173 (11%)
Gestational weeks, visit 1, Range(4-16)			
	11.1 ± 3.1	11.4 ± 3.0	11 ± 3.0
Gestational weeks, visit 2, Range (21-37)			
	28.6 ± 1.8	29.3 ± 1.9	28.9 ± 1.9
Environmental smoke exposure			
No	555 (63%)	370 (51%)	925 (58%)
Yes	322 (37%)	356 (49%)	678 (42%)
Number of glass of water drinking per day			
	7.7 ± 2.3	7.7 ± 2.4	8 ± 2.0
Drinking water arsenic exposure categories			
<=0.89 µg/L	309 (35%)	92 (13%)	401 (25%)
0.89-2 µg/L	397 (45%)	24 (3%)	421 (26%)
2-33 µg/L	103 (12%)	278 (38%)	381 (24%)
>33 µg/L	69 (8%)	332 (46%)	401 (25%)

Table 1.1 (Continued): Characteristics of Study Participants in Sirajdikhan and Pabna.

	Sirajdikhan(N=879)	Pabna (N=727)	All mothers
Drinking water arsenic (µg/L), visit 1			
	12.1 ± 47.4	79.5 ± 131.3	42.6 ± 100.7
Median	1.4	27.0	2.0
Drinking water arsenic (µg/L), one-month post-partum[b]			
	6.8 ± 32.5	78.9 ± 133.7	42.7 ± 103.6
Median	1.0	26.0	1.8
Maternal toenail arsenic (µg/g), visit 1			
	2.2 ± 3.2	4.8 ± 5.9	3.4 ± 4.9
Median	1.1	2.5	1.7
Maternal toenail arsenic (µg/g), one-month post-partum[b]			
	1.8 ± 3.6	3.6 ± 4.2	2.7 ± 4.0
Median	0.7	2.0	1.2
Daily dietary folate intake (µg)			
	268.0 ± 96.7	395.6 ± 102.8	326.8 ± 118.1
Daily dietary protein intake (g)			
	130.7 ± 47.9	219.3 ± 60.0	171.6 ± 69.6
Daily energy intake (kcal)			
	3195.3 ± 977.9	3234.7 ± 763.1	3213.5 ± 885.3

† The continuous variables were all presented as mean ± standard deviation [and median (25th–75th percentile)].

†† The categorical variables were all presented as the numbers (percentages) of participants in the category. The percentages were calculated within each study site.

a Pearson's correlation of drinking water arsenic concentration between the first visit and one-month post-partum is 0.72 ($p<0.0001$).

b Pearson's correlation (Corr) of toenail arsenic concentration between the first visit and one-month post-partum is 0.84.

*limit of detection

Arsenic metabolism profile

Table 1.2 shows the medians and 25th-75th percentiles of the proportions and concentrations of urinary arsenic metabolites in early and mid-to-late gestational periods (visit 1 and visit 2). Table 1.3 shows the paired t-test results for the change between two visits. The proportions of arsenic metabolites in urine are highly correlated with each other in both study visits, as shown in Table 1.4 and 1.5. The median iAs% decreased from 8.5% to 6.6% during the follow-up ($p<0.01$), which mainly due to the decrease of iAs^{III}% (mean change=-1.59%, $p<0.01$). The median DMA% increased from 85.7% to 87.9% from visit 1 to visit 2 ($p <0.01$). There is no change observed in MMA%.

Arsenic excretion profile

The median of U-As increased from 77.6 mg/g-creatinine to 90.4 mg/g-creatinine from visit 1 to visit 2. The mean change of log-transformed U-As from visit 1 to visit 2 was 0.2 ($p-<0.01$), which means that the average ratio of U-As of visit 2 over visit 1 is 1.2. The log-transformed drinking water arsenic from visit 1 to one-month post-partum remains unchanged ($p=0.06$, average ratio=1.08). The log-transformed T-As from visit 1 to one-month post-partum decreased, and the average ratio is 0.70 ($p<0.01$).

Table 1.2: Proportions and concentrations of urinary arsenic metabolites of the study participants in Sirajdikhan and Pabna at Visits 1 and 2

	Sirajdikhan		Pabna		All Mothers	
	visit 1	**visit 2**	**visit 1**	**visit 2**	**visit 1**	**visit 2**
N	879	783	727	662	1606	1445
iAs^{III}%††	0 (0, 4.8)	0 (0, 3.2)	3.1 (0, 10.3)	0 (0, 5.6)	0 (0, 7.8)	0 (0, 4.4)
iAs^{V}%	1.1 (0, 6.3)	1.8 (0, 6.7)	3.8 (0.6, 10)	4 (0.4, 9)	2.1 (0, 8.3)	2.6 (0.1, 7.8)
iAs%	5.7 (0.4, 10.8)	5.3 (1.4, 9.1)	11.3 (7.7, 15.2)	8 (4.9, 11.5)	8.5 (3.6, 13.6)	6.6 (2.8, 10.4)
MMA%	3.5 (0, 7.3)	4.3 (1.8, 6.9)	6 (3.6, 8.9)	5.6 (3.2, 7.9)	4.9 (1.6, 8.2)	4.8 (2.4, 7.5)
DMA%	89.5 (82.1,	89.6 (84.2, 94.5)	81.9 (76.6, 87.1)	85.8 (81.4, 90.5)	85.7 (78.9, 92.5)	87.9 (82.4, 92.8)
iAs^{III}	0 (0, 2.2)	0 (0, 2.1)	3.5 (0, 16)	0 (0, 8.2)	0 (0, 6.5)	0 (0, 3.5)
iAs^{V}	0.6 (0, 3.8)	1.3 (0, 5.2)	5.5 (0.6, 17.6)	6.1 (0.5, 19.8)	1.8 (0, 9.5)	2.5 (0.1, 11)
iAs	2.7 (0.1, 8)	3.1 (0.8, 7.8)	15.2 (7, 37.4)	12.6 (4.8, 35.8)	6.5 (1.6, 20.6)	5.7 (1.6, 17.6)
MMA	1.7 (0, 5.3)	2.6 (1, 5.5)	8.2 (3.3, 21.6)	8.3 (3.6, 23.3)	3.8 (0.8, 12.3)	4.3 (1.6, 11.4)
DMA	43.2 (27.6,	52.8 (36.2, 87.8)	113.5 (64.2,	126 (75.4, 296.2)	65.2 (36.2,	76.4 (45.7,
U-As	48.6 (31.3, 88)	61.7 (41.5,	139 (78.8, 291.6)	153.1 (88.8,	77.6 (41.7,	90.4 (51.6,

† The concentrations of urinary arsenic metabolites are all creatinine-adjusted (mg/g-creatinine) and presented as median (25^{th} percentile, 75^{th} percentile).

†† The proportions of urinary arsenic metabolites were all presented as median (25^{th} percentile, 75^{th} percentile).

* Values marked star are significantly changed from visit 1 to visit 2. The p-values of paired t-tests are lower than 0.05.

** Values marked double star are significantly changed from visit 1 to visit 2. The p-values of paired t-tests are lower than 0.0001.

Table 1.3: the paired t-test results for the change of the percentages of urinary arsenic metabolites

	Sirajdikhan			Pabna			All Mothers		
	Median (25th-75th	Mean change	p-value	Median (25th-75th	Mean change	p-value	Median change (25th-75th	Mean change	p-value
$iAs^{III}\%_2$- $iAs^{III}\%_1$	0.0 (-2.4, 0.3)	-0.72	<.01	-0.1 (-6.8, 0)	-2.61	<.01	0.0 (-4.6, 0.1)	-1.59	<.01
$iAs^{V}\%_2$-$iAs^{V}\%_1$	0.0 (-2.5, 3.6)	0.77	0.08	0.0 (-5.1, 4)	0.2	0.72	0.0 (-3.5, 3.9)	0.51	0.14
$iAs\%_2$-$iAs\%_1$	0.0 (-5.5, 4.5)	0.04	0.92	-2.7 (-7.6, 1.3)	-2.41	<.01	-1.4 (-6.6, 3.2)	-1.08	<.01
$MMA\%_2$-$MMA\%_1$	0.3 (-2.6, 3.7)	0.47	0.03	-0.3 (-3.4, 2.1)	-0.61	<.01	0.0 (-3, 2.9)	-0.03	0.85
$DMA\%_2$-$DMA\%_1$	-0.1 (-7.4, 6.8)	-0.51	0.32	3.3 (-2.8, 9.6)	3.02	<.01	1.8 (-5, 8.2)	1.11	<.01
log(U-As_2)-log(U-	0.2 (-0.3, 0.7)	0.19	<.01	0.1 (-0.2, 0.6)	0.19	<.01	0.2 (-0.3, 0.6)	0.19	<.01
log(DW-As_4)-	0.0 (-0.5, 0.9)	0.06	0.45	0.0 (-0.1, 0.5)	0.19	<.01	0.0 (-0.3, 0.6)	0.13	0.01
log(T-As_4)-log(T-As_1)	-0.4 (-0.8, 0.1)	-0.4	<.01	-0.3 (-0.6, 0)	-0.31	<.01	-0.4 (-0.7, 0)	-0.35	<.01

† p-values are significant levels of paired t-test.

The footnote 1 and 2 means visit 1 and visit 2 respectively. The footnote 4 means one-month post partum.

* change between visit 1 and one-month post-partum Siraj N=577 Pabna N=573 All mothers N=1150

** change between visit 1 and one-month post-partum Siraj N=574 Pabna N=551 All mothers N=1125

Table 1.4: Spearman correlation coeffieicnts (p-values) for arsenic metabolites, in the early gestational period (visit 1, N=1605)

		iAs^{III}%	iAs^{V}%	iAs%	MMA%	DMA%	U-As
iAs^{III}%	ρ	1	-0.27	0.47	0.21	-0.40	0.22
	p-value		<.0001	<.0001	<.0001	<.0001	<.0001
iAs^{V}%	ρ	-0.27	1	0.60	0.30	-0.58	0.36
	p-value	<.0001		<.0001	<.0001	<.0001	<.0001
iAs%	ρ	0.47	0.60	1	0.39	-0.91	0.43
	p-value	<.0001	<.0001		<.0001	<.0001	<.0001
MMA%	ρ	0.21	0.30	0.39	1	-0.69	0.44
	p-value	<.0001	<.0001	<.0001		<.0001	<.0001
DMA%	ρ	-0.40	-0.58	-0.91	-0.69	1	-0.48
	p-value	<.0001	<.0001	<.0001	<.0001		<.0001
U-As	ρ	0.22	0.36	0.43	0.44	-0.48	1
	p-value	<.0001	<.0001	<.0001	<.0001	<.0001	

Table 1.5: Spearman correlation coefficients (p-values) for arsenic metabolites, in the mid-to-late gestational period (visit 2, N=1442)

		$iAs^{III}\%_2$	$iAs^{V}\%_2$	$iAs\%_2$	$MMA\%_2$	$DMA\%_2$	$U\text{-}As_2$
$iAs^{III}\%_2$	ρ	1	-0.30	0.35	0.11	-0.28	0.10
	p-value		<.0001	<.0001	<.0001	<.0001	0.00
$iAs^{V}\%_2$	ρ	-0.30	1	0.70	0.20	-0.65	0.22
	p-value	<.0001		<.0001	<.0001	<.0001	<.0001
$iAs\%_2$	ρ	0.35	0.70	1	0.22	-0.88	0.27
	p-value	<.0001	<.0001		<.0001	<.0001	<.0001
$MMA\%_2$	ρ	0.11	0.20	0.22	1	-0.59	0.22
	p-value	<.0001	<.0001	<.0001		<.0001	<.0001
$DMA\%_2$	ρ	-0.28	-0.65	-0.88	-0.59	1	-0.28
	p-value	<.0001	<.0001	<.0001	<.0001		<.0001
$U\text{-}As_2$	ρ	0.10	0.22	0.27	0.22	-0.28	1
	p-value	0.00	<.0001	<.0001	<.0001	<.0001	

The determinants of arsenic metabolism

The determinants of proportions of urinary arsenic metabolites and U-As at visit 1 are shown in Table 1.6. At visit 1, iAs% is positively associated with daily protein intake. Every one hundred gram daily dietary protein is associated with 2% increase of iAs% in urine, adjusting for total energy intake and other covariates. The average iAs% for low, medium and high tertile groups of protein intake are 8.8%, 9.3% and 11.8% respectively. The energy adjustment in the model showed higher daily energy intake group has 2.7% lower iAs% in urine, but the average iAs% for low, medium and high tertile groups of daily energy intake are 9.6%, 10.4% and 9.5%, which didn't show a clear trend. Gestational age is marginally negatively associated with iAs%. Maternal age and higher education level are negatively associated with iAs% in urine. DW-As is positively associated with iAs%. At visit 2, iAs% is positively associated with DW-As, and negatively associated with the income of financial provider. On the other hand, we found that only DW-As is a determinant of U-As, for both visits; no other determinant had consistent associations with U-As across the two visits.

Table 1.6: Determinants of the proportion of arsenic metabolites in urine, in the early gestation period.

	iAs%		**MMA%**		**DMA%**	
	β	p-value	β	p-value	β	
Gestational age	-0.15 (-0.31, 0.02)	0.08	-0.19 (-0.26, -0.12)	<0.01	0.34 (0.15, 0.53)	<0.01
Maternal age	-0.16 (-0.28, -0.03)	0.02	0 (-0.06, 0.06)	0.98	0.15 (0.01, 0.3)	0.04
BMI at enrollment	-0.02 (-0.18, 0.15)	0.82	-0.05 (-0.12, 0.02)	0.16	0.07 (-0.12, 0.26)	0.47
Education level						
< Secondary education	ref		ref		ref	
>= Secondary education	-1.17 (-2.21, -0.12)	0.03	0.33 (-0.13, 0.79)	0.16	0.84 (-0.36, 2.04)	0.17
Income of financial provider						
< 3000 taka	ref		ref		ref	
>= 3000 taka	-0.61 (-1.67, 0.45)	0.26	0.09 (-0.38, 0.55)	0.72	0.52 (-0.69, 1.74)	0.4
Drinking water arsenic exposure	0.01 (0.01, 0.02)	<0.01	0.01 (0.01, 0.01)	<0.01	-0.02 (-0.03, -0.01)	<0.01
Daily protein intake (g)	0.02 (0.01, 0.04)	<0.01	0 (0, 0.01)	0.28	-0.03 (-0.04, -0.01)	<0.01
Daily energy intake (kCal)						
low	ref		ref		ref	
medium	-0.77 (-2.08, 0.55)	0.25	-0.2 (-0.78, 0.37)	0.49	0.97 (-0.54, 2.48)	0.21
high	-2.66 (-4.22, -1.1)	<0.01	-0.3 (-0.98, 0.39)	0.39	2.96 (1.17, 4.75)	<0.01
Daily folate intake (µg)	0 (-0.01, 0.01)	0.53	0 (0, 0.01)	0.07	-0.01 (-0.02, 0)	0.21

Discussion

Our findings suggest that arsenic methylation efficiency and total urinary arsenic excretion both increase during pregnancy. Furthermore, the determinants of arsenic methylation efficiency are different in the early and mid-to-late gestational periods.

The association between drinking water arsenic and arsenic methylation efficiency was consistent during pregnancy. Higher doses of arsenic were related to increased iAs% and MMA%, as well as decreased DMA%. Several studies have found similar trends [54-56]; and methylation threshold hypothesis has been proposed to explain this, briefly the methylation capacity may reach saturation status under excessive dose of inorganic arsenic, and it is possibly due to the inhibition of the second methylation step (from MMA to DMA) [57-59]. However, epidemiological studies in Chile and Finland do not support this hypothesis, as some people under high exposure of arsenic still had high arsenic methylation efficiency [54, 55, 60, 61]. There may be multiple determinants affecting arsenic metabolism, but the remaining inter-individual variability was not explained in previous studies or in our study.

Pregnant women usually have higher arsenic metabolism, and usually excrete 70%-100% of DMA to urine, compared to an average of 60-80% for men and non-pregnant women [32]. Arsenic metabolism efficiency usually increases during pregnancy, particularly in the first trimester [37, 44]. The same pattern was shown in our cohort, with DMA% increasing during the first trimester, and remaining at a high level at the mid-to-late gestational period (Figure 1.1). From our separate linear regression analyses by study visits, arsenic methylation efficiency was associated with gestational age only at visit 1; no association was found at visit 2. The increase of arsenic methylation efficiency is possibly due to the role of estrogen excretion during pregnancy: estrogen up-regulates the synthesis of choline, which donates its methyl group to

homocysteine to produce methionine, and then produces SAM to methylate iAs^{III} and MMA^{III} [32, 62-64].

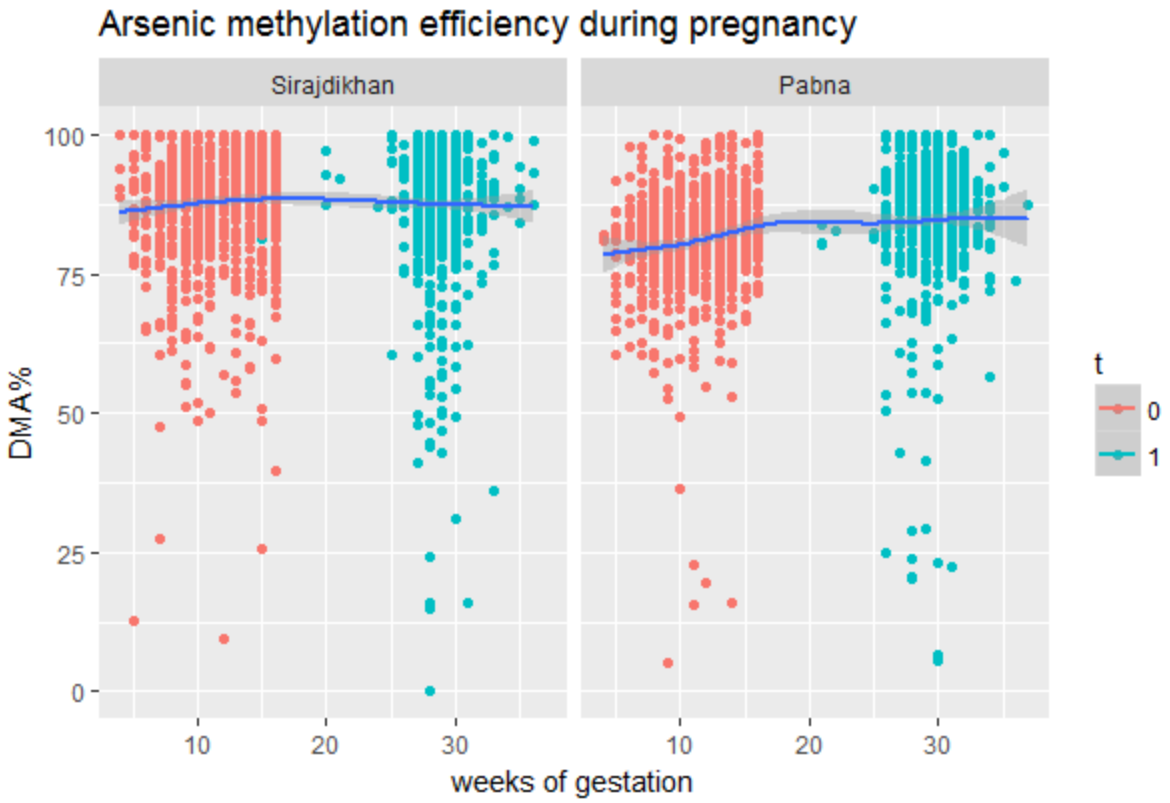

Figure 1.1: The scatter plot of the repeated measures of DMA% during pregnancy, by study sites.

Arsenic methylation over the course of pregnancy, the percentage of DMA% are plotted over gestational age, determined by ultrasound examination. The solid line denotes a Loess local polynomial regression fitting with the shade of standard error.

DMA: dimethylarsinic acid

On the other hand, the U-As also increased during the follow-up, while the DW-As remained unchanged. Toenail arsenic levels can represent the long-term internal dose of inorganic arsenic over the past 3-6 months [22, 50]; thus, the two measurements at visit 1 and one-month post-partum cover the time periods before visit 1, and the mid-to-late gestational periods. The decreased T-As also indicates an up-regulation of arsenic clearance from human body during pregnancy. However, the mechanism of the greater arsenic excretion during pregnancy is not clear yet. Previous studies hypothesized that the increased U-As is partially contributed by the increased DMA%, as DMAs are more readily excreted to urine [37, 44]. In our study, we found no association between DMA% and the change of U-As in linear regression, adjusted for DW-As. Thus, we cannot link the increase of arsenic excretion to the higher DMA% in our cohort.

We hypothesized that dietary folate intake is positively associated with arsenic methylation efficiency; but we did not find an association between them in either visit. At visit 1, when they did not received folate supplements, their source of folate was all from diet. Our data shows the average daily folate intake of participants are over 300 μg. The reason for the null association may because our participants had sufficient folate from diet. This finding agreed with previous findings that folate level during pregnancy has a marginal influence on arsenic methylation [37, 43, 46]. At visit 2, every participant had been taking folate supplements, thus there is less variation on participants' folate level to show any effect.

We found dietary intake of protein was associated with greater iAs% in the first trimester, which indicates less methylation efficiency. This finding is not consistent with previous studies, which found low protein and amino acid diets increased risk of arsenic-related health effects (citation). Some human studies have found worse arsenic-associated health effects among those

consuming lower amounts of meat, eggs, and vegetables [39, 40]. Kurzius-Spencer et al. found that higher protein intake was associated with a lower proportion of inorganic arsenic in urine [42]. Heck et al. found that greater intakes of protein, methionine, and cysteine were associated with a 10-15% greater total urinary arsenic excretion, after controlling for total energy intake, body weight, sex, age, tobacco use, and intake of some other nutrients [41]. However, the complete biological role of was not properly addressed in these studies. Some other studies of protein, amino acids, and arsenic-related skin lesions found little or no effect. There is inconsistent of study results that may be due to inaccurate measures or uncontrolled confounders. In addition, pregnant women appear to have differing arsenic metabolism compared with non-pregnant adults [46]; and factors affecting arsenic metabolisms of non-pregnant women may not be applicable here.

There are several potential confounders. The association between DMA% and protein intake may be slightly confounded by some high-protein dishes, as Lin *et al.* found that toenail arsenic is positively associated with fish and meat items in the same cohort [65].

The effect of dietary protein and folate intake may also be confounded by body size, as participants with different body sizes may proportionally consume less or more food. BMI was adjusted for in our multiple linear regression models. We found similar results in our analyses with and without adjusting for total energy intakes, and had similar results.

Arsenic methylation efficiency during pregnancy may vary by geographical area; and we found different urinary arsenic profiles at our two study centers. The differences may be due to different exposures, diets, or socio-economic status. but this difference was not fully understood yet.

In this study, we used the proportions of arsenic metabolites in urine to represent arsenic methylation efficiency, which is commonly used in epidemiology studies. The ratios between arsenic metabolites are not used here, as many pregnant women have very low proportions of iAs and MMA that resulted in many extreme values.

Although studies showed the percentage of arsenic metabolite was found remarkably stable over time [24, 66], there is also criticism about the reproducibility of urinary arsenic metabolite proportions, as the time in a day may affect the observation of MMA% and iAs% [24, 67]. To minimize this confounding effect, health care workers in our study generally collect urine sampled at s similar time of a day. We recognize the possibility of bias from this confounder that was not controlled for, but it should not significantly bias the associations of arsenic methylation efficiency and its determinants. Also, we did a sensitivity analysis on adjusted iAs concentrations (mg/g-creatinine) and its determents, but found a similar result. Daily protein intake was positively associated with adjusted iAs concentration ($p=0.07$), and the high tertile group of daily energy intake has a lower average adjusted iAs concentration ($p=0.69$), adjusted for total urinary arsenic, age, BMI at enrollment, gestational age, education and the income of financial provider.

Our study has several limitations. One is that there may be measurement error of arsenic profile from a spot urine sample. There is still controversy about the stability of arsenic profile during one day [67]. Another limitation is that participants' dietary intake was self-reported and there may be reporting bias. The protein intake and energy intakes were very high, compared to the U.S. Dietary Reference Intake for pregnant women [68, 69]. FFQs may not be accurate in capturing the absolute amount of dietary intake or active dose of nutrients in human body; however, the FFQ has been shown to have strong validity in correctly ranking the level of

nutrition intake compared to a food diary that does not rely on recall and memory. Also the information on intakes of other nutrients related to one-carbon metabolism was limited, including cysteine, methionine, choline, and vitamin B-12 [70]. The intakes of these nutrients may influence arsenic metabolism along with folate and protein.

Generalizability and public health implications

This is the first study evaluate the association between arsenic methylation efficiency and dietary intake of protein and folate in pregnant women. The patterns of arsenic methylation efficiency and total urinary excretion during pregnancy may vary by geographical areas. People in different geographic areas have variations in demographics, lifestyle and dietary habits. However, methylation efficiency is also possibly influenced by uncontrolled for confounders. Dietary change is a potential approach to improve the health outcome in populations exposed to arsenic. But our study did not find evidence to support this approach in pregnant women.

Conclusions

Our findings suggest that arsenic methylation efficiency and total urinary arsenic excretion both increased during pregnancy. The patterns of arsenic methylation efficiency and total arsenic excretion varied by geographic areas. Daily protein intake was negatively associated with arsenic metabolism in the first trimester.

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CHAPTER TWO

Gene-environment interaction in *As3MT* polymorphisms and maternal arsenic metabolism during pregnancy

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Abstract

Background: Genetic polymorphisms may influence arsenic metabolism. But the role of genetic polymorphisms in arsenic methylation efficiency remain unknown.

Objective: We aimed to investigate the interactions between arsenic exposure and genetic variants on urinary arsenic metabolite profile.

Method: In a group of 1613 pregnant women in Bangladesh, we used the proportion of dimethylarsinic acid over total urinary arsenic excretion (DMA%) as the biomarker of arsenic metabolism. We genotyped 63 SNPs of genes that encode enzymes and proteins involved in arsenic metabolism, including the *As3MT, N6AMT1, GSTO2, CNNM2,* and other genes. Two models were used to assess the SNP-arsenic interaction of arsenic-related SNPs at the early and in the mid-to-late gestational periods.

Result: We found intron variants of *As3MT* gene were associated with DMA% at both early and mid-to-late gestational periods. The genetic variants of *As3MT* and *GSTO2* has SNP-arsenic interaction in both gestational periods , and *N6AMT1* variants only has SNP-arsenic interaction in mid-to-late gestational period; all these interaction effects were significant before adjusting for false discovery rate (FDR), but not significant after adjusting for FDR. We have identified *As3MT* and *As3MT/CNNM2* haplotypes are associated with DMA% in both early and mid-to-late gestational period but we did not detect effect modification in haplotype-based analyses.

Conclusion: We found that genetic polymorphisms are associated with arsenic methylation in the early and the mid-to-late gestational periods. Arsenic exposure level may modify the association between arsenic methylation efficiency and arsenic-related health risk.

Abbreviation:

As3MT: Arsenic methyltransferase

SNP: Single nucleotide polymorphisms

DNMT: DNA methyltransferase

GSTO2: Glutathione S-transferase Omega 2

PNP: Purine nucleoside phosphorylase

N6AMT1: N-6 Adenine-Specific DNA Methyltransferase 1

CNNM2: Cyclin And CBS Domain Divalent Metal Cation Transport Mediator 2

Introduction

Inorganic arsenic is a naturally occurring metalloid that has been classified as a Class 1 carcinogen by the World Health Organization [1-3]. Arsenic has many negative health effects on multiple organs, and it also poses particular risk to pregnant women and their developing fetuses (citation). Prenatal arsenic exposure has been linked to maternal symptoms, including anemia, nausea, vomiting and abnormal cramping [4, 5], while epidemiologic studies have demonstrated associations with negative birth outcomes, including reduced gestational weeks, low birth weight, spontaneous abortion, stillbirth, neonatal mortality, and infant mortality [6-14].

The risk of arsenic toxicity for pregnant women is modified by inter-individual variability of arsenic metabolism [15, 16]. The methylation pathway of inorganic arsenic has been described by Vahter and colleagues [17]; in brief, the ingested arsenate (As^{V}) from drinking water or food is reduced to arsenite (As^{III}) by arsenate reductase; it then reacts with glutathione (GSH) and is methylated to monomethyl- forms of organic arsenic (MMA) by arsenite methyltransferase

(*As3MT*); and MMA will subsequently, MMA enzymetically methylated to dimethyl- forms of organic arsenic (DMA) [17-19]. The arsenic methylation pathway is regarded as a detoxification process, and higher arsenic methylation efficiency is associated with less arsenic-related health risks (citation).

Genetic variants in genes encoding important enzymes and proteins in the arsenic methylation pathway are hypothesized to be major causes of inter-individual variability of arsenic metabolism capability [20-22]. Arsenic methyltransferase and DNA methytrasferases are the major enzymes that catalyzes the methylation reaction of arsenic metabolism in both animal and human studies; and *As3MT* SNPs have been associated with both arsenic methylation efficiency and arsenic-related disease risk [23-28]. Purine nucleoside phosphorylase (PNP) and glutathione-S-transferase (GST) family enzymes participate in the reduction of arsenate and pentavalent arsenic metabolite [29-36]. Heritable variation in these genes has been found to affect disease risk by altering arsenic metabolism efficiency [25, 37-39].

Pregnant women have altered hormone levels, as well as increased arsenic metabolism [40, 41]; thus, it is important to investigate the relationship between genetic variant and arsenic methylation efficiency in pregnant women separately. Studies on genetic variants and arsenic metabolism during pregnancy are limited. Only one study by Gardner *et al.* has found that SNPs in *As3MT* and DNA-methyltransferases 1a (*DNMT1a*) affected arsenic metabolism during pregnancy independently of the effect of biological changes during pregnancy, although the effect is not as strong as pregnancy [26].

In addition, we need to further understand whether the above associations would be modified by arsenic exposure to assess the susceptibility to pregnancy complications and negative birth outcomes. It is challenging to identify the SNP-arsenic interaction due to

insufficient power, and previous studies have provided limited information, mostly on skin disease risk [20, 38, 42, 43].

Bangladesh has a severe problem of arsenic exposure in drinking water as well as a high prevalence of low birth weight [44, 45]. To protect mothers and children from arsenic-related health problems in this low-resource settings, we need to determine their susceptibility to arsenic toxicity during pregnancy. We therefore to determine the gene-environment interaction on arsenic methylation efficiency in exposed pregnant women. In addition, we investigated this topic in the early and the mid-to-late pregnancy periods, in order to increase our knowledge of the relationship among genetic variants, environmental arsenic exposure, and arsenic metabolism during pregnancy.

Methods

Study population

The Bangladesh reproductive cohort study (Project Jeebon) is a community-based observational prospective study of adult pregnant women, to investigate arsenic-related reproductive health problems and birth outcomes. We recruited 1,613 participants during the period between 2008 and 2011 (visit 1), in two Dhaka Community Hospital (DCH) study centers in Pabna upazila and Sirajdikhan upazila in Bangladesh. All participants are in the first trimester of pregnancy; other criteria, protocol and procedures of this cohort study have been published elsewhere [5]. Trained healthcare workers collected maternal urine and blood samples, drinking water samples, as well as administered questionnaires in visit 1. The follow-up visit (visit 2) occurred during the mid-to-late gestational period (21-37 weeks of gestation) and we collected another urine samples.

All protocols were approved by the Institutional Review Boards (IRB) at both the Harvard School of Public Health and the Dhaka Community Hospital Trust. Informed consent was obtained from each participant prior to the study.

Water arsenic exposure

The quantification methods of drinking water arsenic and urinary arsenic metabolites were described in Chapter 1. Briefly, drinking water samples were collected at visit 1 from the participant's primary water source to assess the drinking water arsenic exposure level (DW-As). The samples were then shipped to the laboratory and analyzed the total arsenic concentration by inductively coupled process spectrometer (ICP-MS) following US EPA method 200.8 (Environmental Laboratory Services, North Syracuse, New York). The detection limit of total arsenic concentration is <1 μg/L for the instrument. The records lower than detection limit were assigned 0.5 μg/L in statistical analysis.

Arsenic metabolism

We quantified arsenic methylation metabolites in two repeated spot urine samples separately for each participant in Department of Public Health, School of Medicine at Taipei Medical University, Taiwan. Urine samples were thawed at room temperature, sonicated for dispersion, filtered through Sep-Pak C18 column (Mallinckrodt Baker Inc., NJ, USA), and made 200 μL aliquots. Arsenic metabolites were fractioned by high-performance liquid chromatography (HPLC; Waters 501, Waters Associates, Milford, MA, USA) with Phenomenex columns (Nucleosil, Torrance, CA, USA). The concentration of each arsenic metabolites in urine (iAs^{III}, iAs^{V}, MMA, and DMA) was quantified by hydride generator atomic absorption spectrometry (HG-AAS, Perkin Element). We used SRM 2670 from National Institute of Standard and Technology as standard reference material (NIST, Gaithersburg, MD, USA). We

have good recovery rates for iAs^{III}, iAs^{V}, MMA and DMA ranging from 93.8% to 102.2% with the detection limits of 0.02 μg/L, 0.06 μg/L, 0.07 μg/L, and 0.10 μg/L, respectively. The records below the limit of detection was unchanged as the original value in statistical analysis [46, 47].

Genotyping

Maternal blood samples were collected at prenatal visit 1. The trained healthcare workers drew a 6 ml blood sample to a 7 ml vacutainer and transported it to the laboratory in an icebox within six hours, and then were stored in -80 °C freezers. We extracted DNA from thawed blood in Harvard T.H.Chan School of Public Health, Boston, MA, USA.

A panel of candidate SNPs was selected for genotyping because they were reported related to arsenic methylation pathway or were a proxy of the functional SNP (Table 2.S1). We used TaqMan method with the ABI Prism 7900HT Sequence Detection System for genotyping (Applied Biosystems, Foster City, CA, USA). We randomly selected 5% of the samples as validation duplicates in genotyping procedures.

Our QC criteria include minor allele frequencies (MAF)>0.02, sample call rate>0.95, SNP call rate>0.95, and Hardy-Weinberg equilibrium (HWE) p-value > 0.000001. The QC procedure is shown in Figure 2.1.

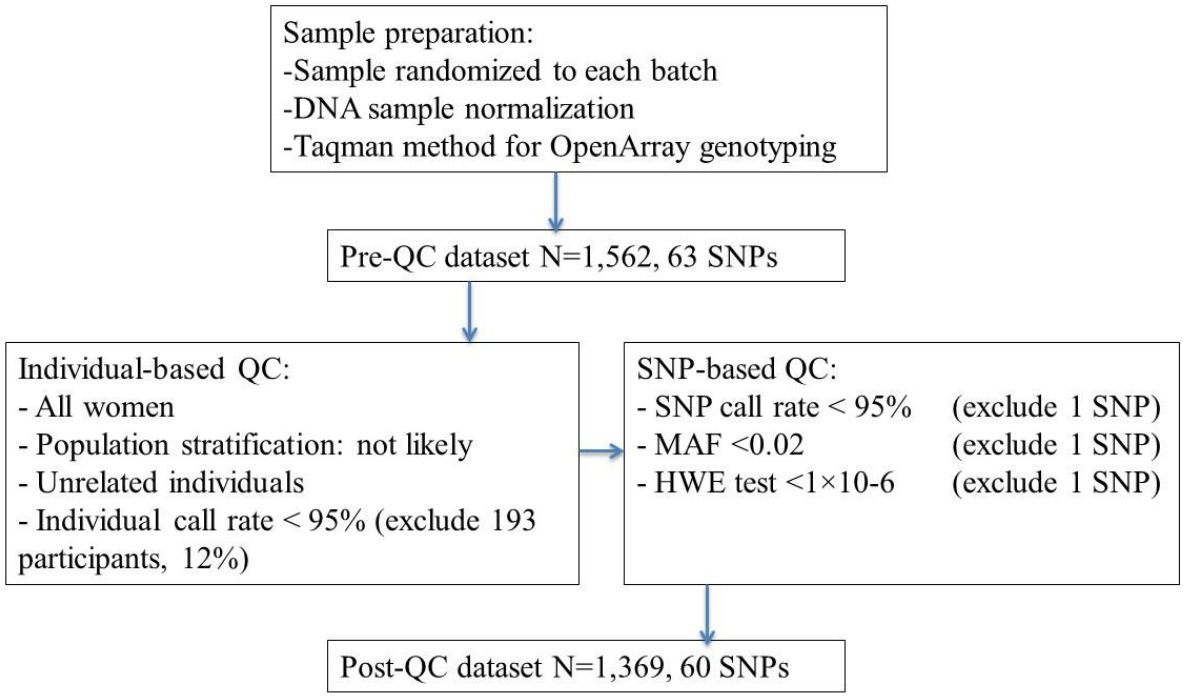

Figure 2.1: The scatter plot of the repeated measures of DMA% during pregnancy, by study sites.

Statistical analysis

The proportions of these metabolites in urine are regarded as biomarkers of arsenic metabolism [48]. The percentage of DMA (DMA%) in urine was treated as the outcome variable, which is calculated as the DMA concentration divided by the sum of iAs^{III}, iAs^{V}, MMA and DMA concentrations.

We conducted a genetic association analysis using linear regression model controlling for DW-As (Model 1) using PLINK [49, 50]. The genetic allelic model was assumed to be additive. Since linkage disequilibrium pattern in the candidate SNPs may cause inflation of p-values of the analyses; the variance inflation factors were calculated using R 2.14.2 [51], and we used Benhamini & Hochberg (1995) step-up false discovery rate (FDR) control to adjust for it [52].

We used two models to assess the SNP-arsenic interactions (Model 2 and 3). In model 2, we performed linear regression models on DMA% and SNPs conditional on high and low DW-As exposure groups (DW-As > 2 μg/L or DW-As ≤ 2 μg/L) using PLINK. We applied the same analyses for DMA% at visit 1 and 2, respectively. We evaluated effect modification of DW-As by comparing the two regression coefficients with Z-scores. In model 3, we used continuous DW-As variable in the linear regression model and added an interaction term, while adjusting for age, BMI, high/low income and education level (secondary education or above/lower education). The analyses of model 3 were performed with SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA); and all the above models were adjusted for FDR.

The linkage disequilibrium (LD) between SNPs was evaluated by HAPLOTYPER [53]. We used Haploview to generate 10 haplotype with $R^2 > 94\%$ [ref]. Haplotype-based association analysis with DMA% was conducted in PLINK.

Results

The participants' characteristics of the Project Jeebon is shown in Table 2.1. Of 1,613 participants in this study, 1,562 participants was genotyped for maternal DNA, and 1,369 participants' genotypes met quality control (QC) criteria for the genetic association analysis. Participants with missing drinking water arsenic concentration, missing urinary arsenic profile data were excluded. Hence, the analyzed sample at visit 1 included 1,364 participants. Until visit 2, we lost some participants due to loss of contact, miscarriage, and stillbirth; at visit 2 the analyzed sample includes 1,228 pregnant women.

The median and 25th - 75th percentiles of DW-As are 2.2 (1.0-34.0) μg/L. The median and 25th - 75th percentiles of adjusted total urinary arsenic for visit 1 and 2 are 79.9 (43.2-185.1) and 92.2 (52.2-202.2) mg/g-creatinine, respectively.

Table 2.1: Study participant characteristics in the Bangladesh reproductive study

	Study sample
N	
Visit 1	1363
Visit 2	1228
age, mean(SD)	22.9(4.2)
BMI at entollment, mean (SD)	20.5 (3.2)
Gestational age, mean (SD)	
Visit 1	11.2 (3.1)
Visit 2	29.0 (1.9)
Adjusted total urine arsenic, median (25th-75th percentiles)	
Visit 1	79.9 (43.2-185.1)
Visit 2	92.2 (52.2-202.2)
Drinking water arsenic, median (25th-75th percentiles)	
Study sample	2.2 (1.0-34.0)
Overall sample	2.0 (0.9-33.0)

Abbreviation: SD, Standard Deviation

Table 2.2 showed SNPs that are associated with DMA% in early gestational period and in mid-to-late gestational period (Model 1). Among 60 SNPs that passed QC process, we identified SNPs that are associated with DMA%. After controlling for FDR, rs9527 and rs1046778 remained significantly associated with DMA% at visit 1; rs3740393 and rs1746778 remained significant associated with DMA% at visit 2. All the above SNPs are intron variants of gene *As3MT*.

In model 2 and model 3 that testing for SNP-arsenic association effect on arsenic methylation efficiency, we found slightly different result in early and mid-to-late gestational periods.

In early gestational period, the individual effect of rs1046778 (*As3MT*) and rs156697 (*GSTO2*) has significant SNP-arsenic interaction (Table 2.3). The association between rs1046778 and DMA% was stronger in those participants in higher exposure group (β =2.53 vs 0.69, p=0.04).

In the analyses for the mid-to-late gestational period (Table 2.4), we observed the same effect modification on the association between rs1046778 and DMA% (β= 2.97 vs 0.86, p=0.04). Conversely, the association between rs156697 and DMA% is stronger among participants in lower exposure group, consistent through different gestational period. *N6AMT1* affect DMA% differently by water arsenic level only in mid-to-late gestational period, but not the early gestational period. Unfortunately, these associations were not significant after adjusting for FDR.

The C allele in rs4919694, which was negatively associated with DMA% and higher arsenic exposure. However, this associations was possibly due to its association *As3MT* SNPs.

Table 2.2: Association between each alleles and DMA% during pregnancy Identified in Bangladesh pregnant women, and p-values under multiple testing adjustment, by visits.

Visit	CHR	Gene	SNP	Position	Coded /non-coded allele	Coded allele freq.	β*	$P_{unadjusted}$	P_{FDR}
Visit 1	10	As3MT	rs9527	102863821	T/C	0.08	-2.98	0.0002	0.0083
	10	As3MT	rs1046778	102901727	C/T	0.35	1.64	0.0003	0.0083
	10	As3MT	rs3740393	102876898	C/G	0.18	1.56	0.0046	0.0928
	10	CNNM2	rs4919694	102939221	C/T	0.1	-1.94	0.0072	0.1085
	10	As3MT	rs11191439	102878966	C/T	0.06	-2.23	0.0130	0.1554
	12	SLCO1B1	rs2291075	21178691	T/C	0.23	1.02	0.0479	0.4786
	10	As3MT	rs10748835	102900499	A/G	0.44	0.74	0.0855	0.7327
	10	GSTO	rs156697	104279427	G/A	0.33	0.76	0.1011	0.7583
Visit 2	10	As3MT	rs3740393	102876898	C/G	0.18	2.54	0.0000	0.0022
	10	As3MT	rs1046778	102901727	C/T	0.35	1.97	0.0001	0.0026
	10	As3MT	rs10748835	102900499	A/G	0.44	1.25	0.0099	0.1979
	10	As3MT	rs3740400	102869708	G/T	0.44	1.18	0.0181	0.2712
	10	As3MT	rs9527	102863821	T/C	0.08	-1.99	0.0278	0.2797
	10	As3MT	rs11191439	102878966	C/T	0.06	-2.17	0.0280	0.2797
	10	CNNM2	rs4919694	102939221	C/T	0.1	-1.43	0.0785	0.5906
	8	GSR/GR	rs2253409	30689449	G/C	0.3	-0.91	0.0838	0.5906
	10	GSTO	rs11509438	104267301	A/G	0.3	1.38	0.0886	0.5906
	12	SLCO1B1	rs2291075	21178691	T/C	0.23	0.84	0.1438	0.7064

*β is in the unit of (%).

Abbreviation: freq., frequency; Chr, chromosome; SNP, single nucleotide polymorphism; SE, standard error; $P_{unadjusted}$, Original p-values; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

Table 2.3: SNP-arsenic interactions on DMA% during early pregnancy period identified in Bangladesh pregnant women, and p-values under multiple testing adjustment.

		MODEL 2				MODEL 3				
SNP	**CA**	**β_{Low_As} (SD)**	**β_{high_As} (SD)**	**P_{GxE}**	**P_{GxE_FDR}**	**β_{SNP} (SD)**	**P_{SNP}**	**β_{GxE} (SD)**	**P_{GxE}**	**P_{GxE_FDR}**
rs156697	G	1.9 (0.8)	-0.2 (0.6)	0.022	0.269	1.7 (0.6)	0.002	-0.6 (0.2)	0.005	0.312
rs1046778	C	0.7 (0.7)	2.5 (0.5)	0.042	0.401	0.8 (0.6)	0.139	0.5 (0.2)	0.01	0.312
rs2297235	G	1.7 (0.9)	-0.5 (0.7)	0.047	0.401	1.4 (0.7)	0.029	-0.5 (0.2)	0.027	0.359
rs4919694	C	0.4 (1.3)	-3.6 (0.8)	0.008	0.243	-0.5 (0.9)	0.601	-0.7 (0.3)	0.034	0.359
rs897453	T	-2.4 (1)	0.6 (0.7)	0.014	0.269	-1.7 (0.7)	0.02	0.5 (0.3)	0.054	0.391
rs11111979	C	-1.5 (0.7)	1.2 (0.5)	0.003	0.179	-0.7 (0.5)	0.183	0.4 (0.2)	0.063	0.391
rs3740393	C	0.5 (0.9)	2.6 (0.7)	0.068	0.496	0.9 (0.7)	0.194	0.5 (0.2)	0.068	0.391
rs11191439	C	0.5 (1.6)	-4 (1)	0.019	0.269	-0.7 (1.2)	0.559	-0.7 (0.4)	0.098	0.515

*β is in the unit of (%).

Abbreviation: SNP, single nucleotide polymorphism; CA, coded allele; As, arsenic; GxE, gene-environment interaction; FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

Table 2.4: SNP-arsenic interactions on DMA% during mid-to-late pregnancy period identified in Bangladesh pregnant women, and p-values under multiple testing adjustment.

		MODEL 2				MODEL 3				
SNP	**CA**	**β_{Low_As} (SD)**	**β_{high_As} (SD)**	**P_{GxE}**	**P_{GxE_FDR}**	**β_{SNP} (SD)**	**P_{SNP}**	**β_{GxE} (SD)**	**P_{GxE}**	**P_{GxE_FDR}**
rs1048546	T	-1.8 (0.7)	1.4 (0.7)	0.001	0.075	-1.2 (0.7)	0.077	0.6 (0.2)	0.005	0.359
rs11191439	C	0.1 (1.5)	-3.7 (1.3)	0.062	0.289	0.1 (1.3)	0.932	-1 (0.5)	0.019	0.375
rs9527	T	0.1 (1.3)	-3.8 (1.3)	0.029	0.289	-0.2 (1.2)	0.837	-0.9 (0.4)	0.028	0.375
rs2705671	G	-2.4 (1.4)	1.4 (1.3)	0.048	0.289	-2 (1.2)	0.1	1 (0.4)	0.032	0.375
rs156697	G	1.9 (0.7)	-0.4 (0.7)	0.028	0.289	1.5 (0.7)	0.018	-0.5 (0.2)	0.033	0.375
rs4919694	C	0.4 (1.2)	-2.7 (1.1)	0.063	0.289	0.2 (1.1)	0.854	-0.7 (0.4)	0.039	0.375
rs1997605	G	-2.1 (1.2)	0.9 (1.1)	0.055	0.289	-1.7 (1)	0.092	0.7 (0.4)	0.057	0.375
rs16983411	G	-1.2 (0.8)	1.3 (0.8)	0.025	0.289	-0.6 (0.7)	0.365	0.5 (0.3)	0.059	0.375
rs11191527	T	-1.1 (0.9)	2.2 (0.9)	0.011	0.289	-0.3 (0.8)	0.69	0.5 (0.3)	0.070	0.400
rs1805087	G	0.8 (0.8)	-1.5 (0.8)	0.044	0.289	0.4 (0.7)	0.581	-0.5 (0.3)	0.082	0.400

*β is in the unit of (%).

Abbreviation: SNP, single nucleotide polymorphism; CA, coded allele; As, arsenic; GxE, gene-environment interaction; FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

Among the 10 haplotypes identified from the candidate SNP panel, we identified *As3MT* (rs9527|rs3740400) and *As3MT/CNNM2* (rs3740393|rs11191439|rs10748835|rs1046778|rs4919694|rs11191527) haplotype is associated with arsenic methylation (Table 2.S4 and 2.S5); but we did not find a haplotype-arsenic interaction effect.

Discussion

In this study, we assessed the gene-environment interaction for two key determinants of arsenic metabolism: 1) genetic variants and 2) drinking water exposure level. We found suggestive evidence that the effect of arsenic metabolism is modified by water arsenic exposure; and the effects may vary by the period of pregnancy. Our findings on the association between genetic variants and arsenic metabolism are important to understand the risk of arsenic-related negative health effects.

The gene-environmental interaction of *AS3MT* polymorphisms on arsenic toxicity has been investigated in both animal models and human cohort studies [28, 32, 38, 39, 54]. Amont them, the Health Effects of Arsenic Longitudinal Study (HEALS) and Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohorts are two major longitudinal epidemiological studies evaluating the effect of arsenic exposure to human. Both have found the evidence that SNPs related to arsenic methylation pathway are associated arsenic toxicity, and the association may be affected by effect modifiers. Pierce *et al.* had found that the individual effect of rs9527 and rs11191527 on skin lesion was modified by arsenic exposure, and participants with higher arsenic exposure had stronger association [38]. In our study, we found that As3MT SNPs or its tag SNPs have gene-environment interaction on arsenic methylation efficiency, which predicted arsenic toxicity. Drobna *et al.* have found that rs3740393 was significantly associated with

placental weight and marginally associated with outer birth outcomes/measures, and this association was modified by infant sex [55]. A 7-years follow-up study in 1137 individuals selected from HEALS had found that the rs3794624 and well water As has GxE interaction on annual pulse pressure (a blood pressure outcome measure) [42].

To our knowledge, our study is the first to identified SNP-arsenic interaction on arsenic methylation efficiency. And this is very likely to be the underlying mechanism of the modified health outcomes. As a result, we had identified SNPs in As3MT were associated DMA% in both early and mid-to-late pregnancy period. The individual effect of rs156697 (*GSTO2*), rs1046778 (*As3MT*), rs2297235 (*GSTO2*) and rs4919694 (*CNNM2*) had SNP-arsenic interaction in early pregnancy period. In mid-to-late pregnancy, rs1048546 (*N6AMT1*), rs11191439 (*As3MT*), rs9527 (*As3MT*), rs2705671 (*N6AMT1*), rs156697 (*GSTO2*), rs4919694 (*CNNM2*) had SNP-arsenic interaction.

The mechanism of the effect modification may has been linked to the accumulated intermediates in arsenic metabolism; arsenic metabolism kinetic studies using *in vitro* tissue extracts suggest that *As3MT* can effectively methylate very low concentrations of MMA^{III} in tissues, but high concentrations of methylarsine oxide ($MAs^{III}O$), an intermediate in arsenic metabolism, may inhibit DMA synthesis [56, 57]. Meanwhile, arsenic methylation efficiency is associated with an oxidative stress biomarker in children and adults, which suggests that the health effects related to arsenic metabolism may be induced by the oxidative stress [58].

Our genotyped SNPs in chromosome 10 are in strong LD, thus haplotype-based association test is appropriate here [27]. We found a *As3MT/CNNM2* haplotype is significantly associated with arsenic methylation efficiency, although we did not identify significant GxE effect. The *CNNM2* gene is within 800 kb of *As3MT*. One study implied *CNNM2* may play an

important role in arsenic metabolism [24], but the strong association between *CNNM2* SNP and DMA% is possibly due to its correlation with *As3MT* SNPs. the

Glutathione is also an important determinant of the pattern and extent formation of arsenicals[59-61], thus, *GST* gene family that encodes glutathione S-transferases play important roles arsenic methylation pathway[31, 33-36]. There is a controversy in the evidence of the *GSTO*'s effect on arsenic metabolism and arsenic-related health outcomes, and the SNP-arsenic interaction has not been identified in previous studies [31, 62-65]. One study found that SNPs in *GSTT1* and *GSTM1* were associated with the levels of MMA and DMA, but the evidence was also not concrete [33-36]. One study had found a significant interaction on the multiplicative scale between *GSTT1* wildtype and secondary methylation ratio (DMA/MMA)[33]. No significant interactions were observed for *GSTM1* or *GSTP1* [35]. In our screening of the GST family, we only found the rs156697 of *GSTO2* has association with arsenic methylation efficiency, as well as the SNP-arsenic interaction effect.

Among the SNPs that was found associated with arsenic metabolism [21], only a few remained associated in this reproductive cohort. Our study is the first to screen the genetic determinants of the arsenic metabolism during pregnancy. In this reproductive cohort, we identified that a SNPs in *As3MT*, *CNNM2,* and *GSTO2* are associated with arsenic metabolism during pregnancy, and the SNP-arsenic effect is not significant after controlling for FDR. Folate is one of the co-substrated of the methyl-doner in one-carbon metabolism [66-69]; but we found no association in SNPs involved in the folate metabolism pathway or arsenic reductases. The null association is possibly due to that arsenic metabolism increases to a very high level during pregnancy, which reduced the inter-individual variation of arsenic methylation efficiency and limited the impact of genetic variants [26, 40].

In this study, we have several limitations. Among different forms of MMA and DMA, their trivalent forms monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) are highly toxic but will be rapidly methylated to their less toxic pentavalent forms monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) [70-72]. All the above metabolites appear in the urine, but it is not possible to capture the precise amount of trivalent forms [48]. We can only use the proportions of total MMA and total DMA in urine as biomarkers of arsenic metabolism, which may not capture the more information of arsenic metabolism. Secondly, we may require a greater sample size to perform SNP-arsenic analysis, thus the null-result of the analysis may due to the limited power. Thirdly, the generalizability of this study may be limited due to the homogeneity of the participants' race and demographics.

In spite of these limitations, we are the first study that look at the SNP-arsenic interaction effect in pregnant women, and we have a well established cohort with comprehensive information on their demographic, lifestyle and medical condition during pregnancy. We have individual level measure of the water arsenic exposure, and repeated urinary arsenic metabolites measures covers different time points during pregnancy. Our statistical methods are well-established and we used two models to compare the consistency of the analytical result. The suggestive result is helpful for future research to investigate the potential mechanism of arsenic methylation regulation during pregnancy.

Understanding the determinants of maternal arsenic methylation during pregnancy is important in predicting the arsenic-related birth outcomes. Our study established the association between genetic polymorphisms and arsenic methylation efficiency that filled the gap of SNP-arsenic interaction during pregnancy.

Conclusions

As3MT and *GSTO2* polymorphisms are associated with arsenic methylation in early and mid-to-late pregnancy period. The arsenic exposure level is an effect modifier of the association. *As3MT* and *As3MT/CNNM2* haplotypes are associated with arsenic methylation efficiency in early and mid-to-late pregnancy.

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Supplementary materials

Table 2.S1: The candidate SNPs and the functional and location information

GENE	Gene function	SNP	Chr	position
MTHFR	methylenetetrahydrofolate reductase (folate pathway)	rs1476413	1	11792243
MTHFR	methylenetetrahydrofolate reductase (folate pathway)	rs1801131	1	11794419
MTHFR	methylenetetrahydrofolate reductase (folate pathway)	rs1801133	1	11796321
MTR	5-Methyltetrahydrofolate-Homocysteine Methyltransferase	rs1805087	1	236885200
DNMT3A	DNA methyltransferases (folate pathway)	rs7560488	2	25345952
CHDH	Choline Dehydrogenase	rs9001	3	53823890
GLRX	Glutaredoxin (Thioltransferase)	rs3822751	5	95818506
GLRX	Glutaredoxin (Thioltransferase)	rs871775	5	95823064
MTRR	5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase	rs1801394	5	7870860
GSR/GR	glutathione reductase	rs8190955	8	30708107
GSR/GR	glutathione reductase	rs2253409	8	30689449
GSR/GR	glutathione reductase	rs2978296	8	30717423
As3MT	Arsenic Methyl Transferase	rs9527	10	102863821
As3MT	Arsenic Methyl Transferase	rs1046778	10	102901727
As3MT	Arsenic Methyl Transferase	rs10748835	10	102900499
As3MT	Arsenic Methyl Transferase	rs11191439	10	102878966
As3MT	Arsenic Methyl Transferase	rs3740393	10	102876898
As3MT	Arsenic Methyl Transferase	rs3740400	10	102869708
CNNM2	Cyclin And CBS Domain Divalent Metal Cation Transport Mediator 1	rs11191527	10	103035377
CNNM2	Cyclin And CBS Domain Divalent Metal Cation Transport Mediator 2	rs4919694	10	102939221
GSTO	Glutathione s-transferases family	rs11509438	10	104267301
GSTO1-1	Glutathione s-transferases family	rs4925	10	104263031
GSTO2-2	Glutathione s-transferases family	rs156697	10	104279427
GSTO2-2	Glutathione s-transferases family	rs2297235	10	104274733
FADS1	fatty acid desaturase	rs174556	11	61813163
GSTP1	Glutathione s-transferases family	rs1695	11	67585218
NCAM1	Neural Cell Adhesion Molecule 1	rs2298526	11	1.13E+08
H19	Imprinted Maternally Expressed Transcript (Non-Protein Coding)	rs217727	11	1995678
SLCO1B1	Solute Carrier Organic Anion Transporter Family, Member 1B1	rs1564370	12	21182256
SLCO1B1	Solute Carrier Organic Anion Transporter Family, Member 1B1	rs2291075	12	21178691
TXNRD1	Thioredoxin Reductase 1	rs11111979	12	1.04E+08
APEX1	Multifunctional DNA Repair Enzyme	rs1130409	14	20456995
MTHFD1	methylenetetrahydrofolate reductase	rs2236225	14	64442127
PNP	purine nucleoside phosphorylase	rs1049562	14	20472356
PNP	purine nucleoside phosphorylase	rs1049564	14	20472447
PNP	purine nucleoside phosphorylase	rs1130650	14	20472467

Table 2.S1 (Continued): The candidate SNPs and the functional and location information

GENE	Gene function	SNP	Chr	position
PNP	purine nucleoside phosphorylase	rs1713420	14	20474585
PNP	purine nucleoside phosphorylase	rs1760940	14	20470092
PEMT	phosphatidylethanolamine N-methyltransferase	rs1531100	17	17561514
PEMT	phosphatidylethanolamine N-methyltransferase	rs4244598	17	17569299
PEMT	phosphatidylethanolamine N-methyltransferase	rs2278952	17	17582270
PEMT	phosphatidylethanolamine N-methyltransferase	rs897453	17	17522317
DNMT1	DNA methyltransferases	rs10854076	19	10137594
DNMT1	DNA methyltransferases	rs16999593	19	10180505
DNMT1	DNA methyltransferases	rs2228611	19	10156401
DNMT1	DNA methyltransferases	rs2228612	19	10162696
DNMT1	DNA methyltransferases	rs7253062	19	10184448
PRDX2	Peroxiredoxin 2	rs10427027	19	12800471
PRDX2	Peroxiredoxin 2	rs12151144	19	12801582
XPD/ERCC2	Excision repair cross-complementation group 2	rs13181	19	45351661
XPD/ERCC2	Excision repair cross-complementation group 2	rs1799793	19	45364001
DNMT3B	DNA methyltransferases	rs2424913	20	32786453
DNMT3B	DNA methyltransferases	rs2424932	20	32808730
DNMT3B	DNA methyltransferases	rs6058896	20	32808346
CBS	Cystathionine-Beta-Synthase	rs234709	21	43066854
CBS	Cystathionine-Beta-Synthase	rs4920037	21	43061781
N6AMT1	N-6 Adenine-Specific DNA Methyltransferase 1	rs1048546	21	28872555
N6AMT1	N-6 Adenine-Specific DNA Methyltransferase 1	rs16983411	21	28877925
N6AMT1	N-6 Adenine-Specific DNA Methyltransferase 1	rs1997605	21	28885096
N6AMT1	N-6 Adenine-Specific DNA Methyltransferase 1	rs2205449	21	28879766
N6AMT1	N-6 Adenine-Specific DNA Methyltransferase 1	rs2705671	21	28879027
SLC19A1	solute carrier family 19 member 1	rs4819130	21	45538385

Abbreviation: Chr, chromosome; SNP, single nucleotide polymorphism.

Table 2.S2: Association Between DMA% and genetic variance by drinking water arsenic level in Bangladesh pregnant women, by gestational periods (Model 2).

		Low DW-As group		High DW-As group				
Visit	SNP	β	s.e.	β	s.e.	Z-score	P_{GXE}	P_{FDR}
Visit 1	rs11111979	-1.46	0.70	1.15	0.53	-2.97	0.0030	0.1794
Low DW-As group	rs4919694	0.40	1.26	-3.59	0.82	2.65	0.0081	0.2425
N=650	rs897453	-2.35	0.95	0.55	0.70	-2.46	0.0138	0.2691
High DW-As group	rs11191439	0.47	1.61	-3.96	1.00	2.34	0.0194	0.2691
N=707	rs156697	1.89	0.75	-0.24	0.55	2.28	0.0224	0.2691
	rs1046778	0.69	0.73	2.53	0.54	-2.03	0.0423	0.4008
	rs2297235	1.66	0.86	-0.50	0.66	1.99	0.0468	0.4008
	rs3740393	0.53	0.88	2.56	0.67	-1.83	0.0676	0.4956
Visit 2	rs1048546	-1.82	0.73	1.44	0.70	-3.23	0.0013	0.0756
Low DW-As group	rs11191527	-1.08	0.89	2.19	0.92	-2.55	0.0109	0.2897
N=584	rs8190955	1.87	1.78	-4.07	1.66	2.44	0.0146	0.2897
High DW-As group	rs16983411	-1.17	0.79	1.28	0.76	-2.24	0.0252	0.2897
N=644	rs156697	1.88	0.74	-0.38	0.71	2.20	0.0276	0.2897
	rs9527	0.11	1.31	-3.84	1.25	2.18	0.0293	0.2897
	rs1046778	0.86	0.72	2.97	0.69	-2.11	0.0350	0.2897
	rs1805087	0.80	0.82	-1.50	0.79	2.02	0.0437	0.2897
	rs2705671	-2.37	1.39	1.44	1.33	-1.98	0.0476	0.2897
	rs1997605	-2.12	1.16	0.92	1.08	-1.92	0.0550	0.2897
	rs2205449	-1.56	0.69	0.28	0.67	-1.92	0.0550	0.2897
	rs11191439	0.07	1.54	-3.67	1.29	1.86	0.0624	0.2897
	rs4919694	0.36	1.24	-2.70	1.08	1.86	0.0628	0.2897

β is in the unit of (%).

Abbreviation: Chr, chromosome; SNP, single nucleotide polymorphism; SE, standard error; GxE, gene-environment interaction; FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

P<0.07 were reported

Table 2.S3: linear regression results on gene-environment interaction analysis (Model 3)[*].

SNP	coded allele	β_{SNP}	s.e.	p-value	β_{GxE}	s.e.	P	P_{FDR}
visit 1 (N=1349)								
rs156697	G	1.74	0.57	0.0024	-0.56	0.2	0.0054	0.3119
rs1046778	C	0.83	0.56	0.1388	0.53	0.2	0.0099	0.3119
rs9001	G	0.79	0.64	0.2218	-0.54	0.24	0.024	0.3591
rs2297235	G	1.43	0.65	0.0291	-0.51	0.23	0.0266	0.3591
rs2228611	T	-0.63	0.54	0.2432	0.44	0.2	0.0288	0.3591
rs4919694	C	-0.49	0.94	0.6008	-0.66	0.31	0.0342	0.3591
rs16999593	C	-4.13	2.19	0.0598	1.47	0.73	0.046	0.3912
visit 2 (N=1213)								
rs1048546	T	-1.15	0.65	0.0775	0.63	0.23	0.0057	0.3591
rs11191439	C	0.11	1.33	0.9328	-1.04	0.45	0.0195	0.3755
rs9527	T	-0.24	1.16	0.8379	-0.89	0.41	0.028	0.3755
rs2705671	G	-2.04	1.24	0.1008	0.95	0.44	0.0321	0.3755
rs156697	G	1.54	0.66	0.0188	-0.5	0.23	0.0338	0.3755
rs4919694	C	0.2	1.08	0.8549	-0.73	0.35	0.0399	0.3755
rs1801394	A	0.92	0.61	0.1331	-0.46	0.23	0.0444	0.3755

[*]The results of $p<0.05$ were reported.

Abbreviation: SNP, single nucleotide polymorphism; GxE, gene-environment interaction; FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

Table 2.S4: Haplotype-based genetic association results for DMA%, in the early gestational period (visit 1).

HAPLOTYPE	β	P	P_{FDR}
rs10854076|rs2228611|rs2228612|rs7253062			
GCTA	0.02	0.971	0.973
CCCG	-0.3	0.571	0.799
GTTG	0.09	0.849	0.973
GCTG	1.45	0.346	0.673
rs1801131|rs1801133			
TA	-0.55	0.43	0.708
GG	0.57	0.21	0.658
TG	-0.34	0.446	0.708
rs10427027|rs12151144			
CC	0.82	0.286	0.658
TA	-0.8	0.28	0.658
rs1531100|rs4244598|rs2278952			
ACA	-0.92	0.238	0.658
ACG	-0.2	0.724	0.973
GCG	-0.21	0.791	0.973
GTG	0.55	0.242	0.658
rs1049564|rs1130650			
AT	0.06	0.923	0.973
GC	-0.09	0.879	0.973
rs2291075|rs1564370			
CG	-0.42	0.345	0.673
TC	0.97	0.076	0.38
CC	-0.33	0.491	0.716
rs1048546|rs16983411|rs2705671|rs2205449|rs1997605			
TAGTG	-0.27	0.763	0.973
TATTG	-0.17	0.895	0.973
TGTTA	0.42	0.415	0.708
GATTA	-0.67	0.301	0.658
GATAA	-0.02	0.973	0.973
rs9527|rs3740400			
TG	-2.97	0	0.01
CG	1.64	0.001	0.01
CT	-0.64	0.166	0.658

Table 2.S4 (Continued): Haplotype-based genetic association results for DMA%, in the early gestational period (visit 1).

HAPLOTYPE	β	P	P_{FDR}
rs3740393|rs11191439|rs10748835|rs1046778|rs4919694|rs11191527			
GTACTT	1.11	0.064	0.373
GCATCC	-2.84	0.002	0.023
GTATCC	-1.36	0.251	0.658
CTACTC	1.46	0.011	0.096
GTGTTC	-0.85	0.055	0.373
rs11509438|rs2297235|rs156697			
GGG	0.49	0.375	0.691
AAG	0.55	0.465	0.708
GAG	-0.14	0.91	0.973
GAA	-0.58	0.224	0.658

Abbreviations: FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

Table 2.S5 (Continued): Haplotype-based genetic association results for DMA%, in the mid-to-late gestational period (visit 2).

HAPLOTYPE	β	P	P_{FDR}
rs10854076|rs2228611|rs2228612|rs7253062			
GCTA	0.16	0.749	0.95
CCCG	-0.68	0.252	0.678
GTTG	0.27	0.59	0.883
GCTG	1.3	0.445	0.865
rs1801131|rs1801133			
TA	1.22	0.113	0.494
GG	-0.3	0.558	0.883
TG	-0.22	0.656	0.883
rs10427027|rs12151144			
CC	-0.46	0.596	0.883
TA	0.16	0.842	0.969
rs1531100|rs4244598|rs2278952			
ACA	-0.04	0.962	0.984
ACG	-0.11	0.858	0.969
GCG	-0.41	0.633	0.883
GTG	0.24	0.641	0.883
rs1049564|rs1130650			
AT	-0.56	0.403	0.865
GC	0.51	0.44	0.865
rs2291075|rs1564370			
CG	-0.59	0.227	0.662
TC	0.76	0.201	0.64
CC	0.01	0.984	0.984
rs1048546|rs16983411|rs2705671|rs2205449|rs1997605			
TAGTG	-0.48	0.625	0.883
TATTG	-0.82	0.541	0.883
TGTTA	0.08	0.889	0.972
GATTA	-1.27	0.07	0.35
GATAA	0.64	0.184	0.64
rs9527|rs3740400			
TG	-2.04	0.027	0.158
CG	1.96	0	0.004
CT	-1.22	0.015	0.131

Table 2.S5 (Continued): Haplotype-based genetic association results for DMA%, in the mid-to-late gestational period (visit 2).

HAPLOTYPE	β	P	P_{FDR}
rs3740393|rs11191439|rs10748835|rs1046778|rs4919694|rs11191527			
GTACTT	0.6	0.361	0.842
GCATCC	-2.3	0.022	0.154
GTATCC	-0.36	0.787	0.95
CTACTC	2.33	0	0.004
GTGTTC	-1.35	0.006	0.07
rs11509438|rs2297235|rs156697			
GGG	0.03	0.958	0.984
AAG	1.09	0.182	0.64
GAG	0.4	0.779	0.95
GAA	-0.5	0.334	0.835

Abbreviations: FDR, false descivery rate; P_{FDR}, Benjamini & Hochberg (1995) step-up FDR control

CHAPTER THREE

Arsenic metabolism in mid-to-late pregnancy affects birth weight through gestational age in a Bangladesh cohort

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Abstract

Background: Arsenic exposure during pregnancy is linked to adverse birth outcomes. Whether inter-individual variability in arsenic metabolism during pregnancy modifies the risk of adverse outcomes is unclear.

Objective: We used causal mediation models to assess maternal arsenic metabolism by gestational age to identify a sensitive exposure time window linked to adverse birth outcomes.

Methods: This prospective birth cohort study included 1,176 Bangladeshi maternal–infant pairs recruited during 2008–2011. We measured repeatedly maternal urinary concentrations of inorganic arsenic (iAs) and its monomethylated and dimethylated metabolites during two visits at a mean of 11 weeks (visit 1) and 29 weeks (visit 2) gestation. Counterfactual mediation analysis was performed while adjusting for potential confounders to assess how much of the association between proportion of iAs (iAs%) and birthweight was mediated by gestational age.

Results: The association between iAs% at visit 2 and birth weight was mainly mediated by gestational age at birth. A 10-unit increase in iAs% at visit 2 was associated with a –15.13 g [bootstrapped 95% confidence interval (CI): –42.95, 19.03] change in birth weight, in which –9.86 g (bootstrapped 95% CI: –20.31, –2.35) was mediated through gestational age, adjusted for potential confounders. We did not find statistically significant exposure–mediation interactions.

Conclusions: This study demonstrates that less efficient arsenic metabolism is associated with lower birth weight that is mediated through gestational age in a Bangladeshi cohort. Further, birth weight is sensitive to arsenic metabolism in mid-to-late gestation, providing a new perspective on risk factors for low birth weight in a susceptible population.

Introduction

Inorganic arsenic (iAs) exposure from drinking water is a global public health concern. Worldwide, 200 million people are exposed annually to arsenic in drinking water at levels above the World Health Organization's recommended guideline of 10 μg/L (Naujokas et al. 2013). Developing fetuses are particularly vulnerable to exposure since arsenic crosses the placenta [1-3]. Studies have demonstrated that prenatal arsenic exposure can adversely affect fetal growth and increase the risk of spontaneous abortion and stillbirth, early childhood mortality and morbidity, and disease risk later in life [4-7].

The detrimental health effects of arsenic are associated with not only the dose of arsenic exposure, but also with the capacity of biotransformation to less toxic metabolites, which varies from person to person [8]. The detoxification process of iAs is hypothesized to involve methylation reactions in the liver, although some transient intermediate products are highly toxic [9]. During this process, ingested iAs is methylated to organic monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA) by the addition of one methyl group and two methyl groups, respectively, which renders arsenic less toxic and more readily excreted in urine [9, 10]. Further, arsenic methylation capability can be individually determined by genetic variants, dose, socioeconomic status, lifestyle, and nutrition [11-13].

Bangladesh has a high prevalence of low birth weight (36%), ranging from 15% in government maternity hospitals to 47% in the Shaharasthi sub-district, according to a 2003–2004 national survey [14]. Low birth weight has been linked with high concentrations of arsenic exposure from underground drinking water [15]. However, many pregnant women likely cannot afford to change their water source. Because several cohort studies report that nutritional supplements appear to promote improved arsenic metabolism [16-20], nutrition supplementation

programs could offer an affordable preventative approach to arsenic-related negative birth outcomes in exposure-endemic areas.

However, the association between arsenic metabolism and birth outcomes is not yet well established. One epidemiological study found that maternal urinary concentration of MMA is negatively associated with birth weight and gestational age and that maternal urinary concentration of iAs is associated with lower mean gestational age and birth length [21]. However, urine samples in this study were collected immediately before birth and therefore may not have captured a critical time window during pregnancy that allows a time lag before manifestation of toxicity.

To bridge these knowledge gaps, we investigated the association between arsenic metabolism and birth outcomes (i.e., weight, length, and head circumference) in a cohort study in Bangladesh. We further evaluated the mediation pathway to test the hypothesis that less efficient arsenic metabolism would adversely affect birth outcomes through reduced gestational age and limited maternal weight gain [22]. The secondary goal of this study was to identify a sensitive time window for arsenic metabolism to affect birth outcomes by comparing associations between arsenic metabolism and birth outcomes at two different time points during pregnancy.

Methods

Study population

This prospectively enrolled cohort recruited 1,613 pregnant women from Pabna and Sirajdikhan Upazila regions of Bangladesh in collaboration with Dhaka Community Hospital (DCH). Participants were included in the study if they were in their first trimester of a singleton

pregnancy; were 18 years or older; had a complete drinking water history, with tube well usage up to 6 months before pregnancy; were planning to participate in DCH's prenatal health program; and were planning to deliver the child at home with a DCH-trained midwife or at a DCH hospital or clinic. In total, the study cohort included 1,176 pregnant women. Trained female DCH community healthcare workers collected participants' medical information and biospecimens and administered questionnaires about participants' demographics and lifestyles. All study procedures were approved by the institutional review boards of Harvard T.H. Chan School of Public Health and Dhaka Community Hospital Trust.

Figure 3.1 summarizes sample acquisition and participant follow-up. Briefly, we collected two urine samples from each participant at visit 1 (at enrollment, 4–16 weeks of gestation as measured by ultrasound) and visit 2 (21–37 weeks of gestation). We followed participants until birth to obtain birth outcomes. At each study period, participants were excluded due to loss-of-contact, participant dropout, miscarriage, and stillbirth. We also excluded participants with twin pregnancies, missing water exposure data, or urine sample failures.

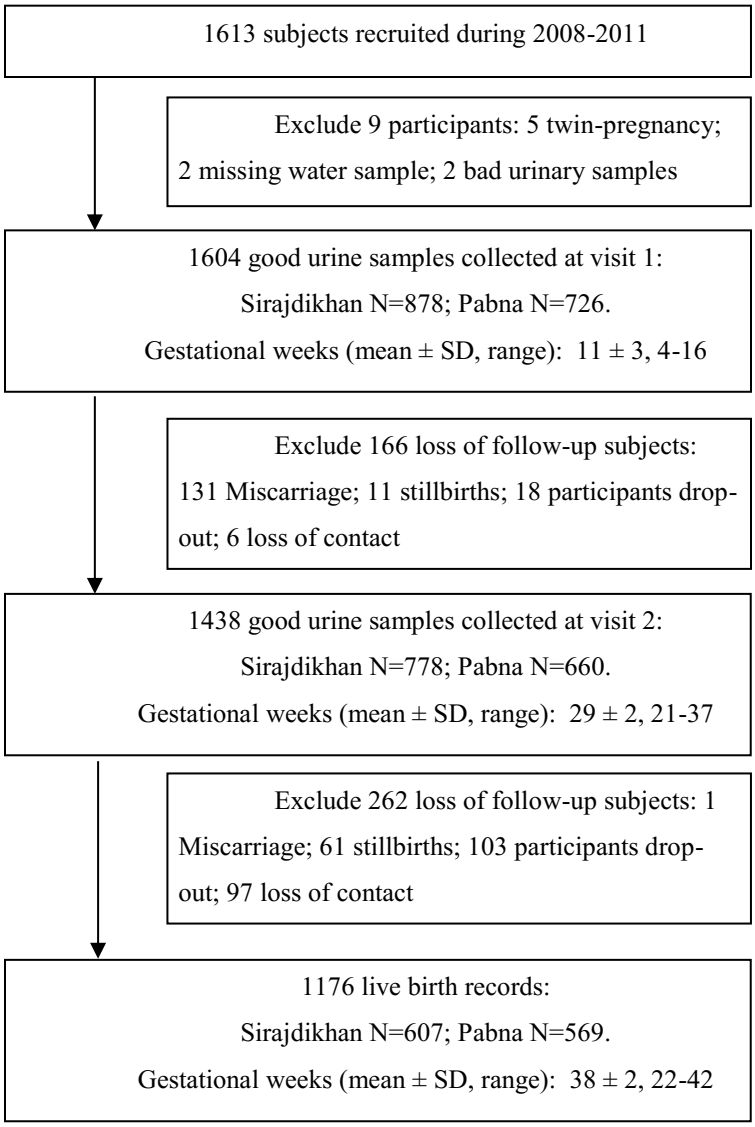

Figure 3.1: Overview of sample acquisition and participant follow-up procedures.

Exposure assessment

The exposure of interest was arsenic methylation efficiency. Urinary arsenic metabolites of arsenite (As^{III}), arsenate (As^{V}), MMA, and DMA were measured by hydride generator atomic absorption spectrometry (HG-AAS, Perkin-Elmer, Waltham, MA, USA). Concentration of iAs in urine was calculated as the sum of iAs^{III} and iAs^{V}. Proportion of each arsenic species (iAs%, MMA%, and DMA%) was calculated as relative concentration of the species divided by total concentration of all three arsenic species in urine. The primary exposure biomarker was urinary iAs%, and a smaller iAs% was indicative of more ingested arsenic that was methylated to less toxic products. Secondary exposure biomarkers were urinary MMA% and DMA%.

Detailed protocols for arsenic metabolite concentration measurements have been previously summarized [23, 24]. Briefly, trained healthcare workers collected urine samples and shipped them to the Department of Public Health, School of Medicine, at Taipei Medical University in Taiwan. The samples were stored in -80 °C freezers immediately after received. Trained lab personnel analyzed urinary concentration of arsenic species, including iAs^{III}, iAs^{V}, MMA, and DMA. Urine samples were thawed at room temperature before analysis, sonicated for dispersion, and filtered through Sep-Pak C18 columns (Mallinckrodt Baker Inc., NJ, USA). We made 200-mL aliquots, fractioned them using high-performance liquid chromatography (HPLC; Waters 501, Waters Associates, Milford, MA, USA), and quantified four arsenic metabolites (iAs^{III}, iAs^{V}, MMA, and DMA) using hydride generator atomic absorption spectrometry (HG-AAS, Perkin-Elmer, Waltham, MA, USA). Our standard reference material was SRM 2670 from the National Institute of Standard and Technology (Gaithersburg, MD, USA). Recovery rates for iAs^{III}, iAs^{V}, MMA, and DMA ranged 93.8%–102.2%, and detection limits were 0.02 μg/L, 0.06 μ g/L, 0.07 μ g/L, and 0.10 μ g/L, respectively. Statistical analysis did not change records below

the limit of detection. We also performed a colorimetric assay on a Roche Modular P800 instrument (Roche Inc., Mannheim, Germany) for each urine sample to determine concentration of urinary creatinine, which allowed us to adjust arsenic species concentration for the density of urine.

Outcomes, potential mediators, and covariates assessment

Birth weight, birth length, and birth head circumference were measured by trained health care workers. Approximately 46% of neonatal anthropometrics were measured at a hospital or clinic; the rest were assessed at the participant's home with identical staff and survey instruments. Potential mediators included gestational age (weeks) and maternal weight gain (kg) during the follow-up period from enrollment to delivery.

Potential covariates included maternal age (years), body mass index (BMI) at enrollment, education level (≤secondary education or >secondary education), income of the financial provider (≤3000 taka or >3000 taka), maternal weight at enrollment (kg), drinking water arsenic exposure level at visit 1 (μg/L), daily protein and energy intakes (classified into tertiles), environmental tobacco smoke exposure (yes or no), betel nut chewing (yes or no), birth type (Caesarean or vaginal), birth location (home, clinic, or hospital), infant sex, and study site (Pabna or Sirajdikhan). Daily protein and energy intakes were based on dietary intake information obtained from the validated dish-based semi-quantitative food frequency questionnaire administered at visit 2 [25] and calculated using the 2013 Bangladesh food composition table [26].

Water arsenic exposure was measured by sampling participants' drinking water at visit 1 per previously published collection and processing protocols [27]. Briefly, health care workers collected tube well water samples from each participant's primary water source using well

pumps after a one-minute purge, and samples were stabilized with nitric acid for room-temperature storage. Samples were shipped to the laboratory for analysis of total arsenic concentration by inductively coupled process spectrometer (ICP-MS) following U.S. EPA method 200.8 (Environmental Laboratory Services, North Syracuse, NY). The instrument was sensitive to 1 μg/L of total arsenic concentration. Records lower than the limit of detection limit were assigned a value of 0.5 μg/L for statistical analysis.

Statistical analysis

We first conducted linear regression analyses with the proportion of each urinary arsenic species predicting birth weight, birth length, and birth head circumference. We conducted separate analyses for visit 1 and visit 2 while adjusting for potential confounders. Next, we conducted linear regression analyses with the proportion of each urinary arsenic species predicting potential mediators of gestational age and maternal weight gain during pregnancy. In the third step, we conducted regression analyses with each potential mediator and each birth outcome. We evaluated potential confounders of these models by statistical relevancy to exposure, mediator, and outcome. We also evaluated covariates based on a review of the literature. Finalized covariates for each model are reported in results tables. We used a significance level of 0.05. All analyses were performed with SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA).

Because iAs% and DMA% were inversely correlated, the effect of iAs% and DMA% had similar magnitudes but opposite directions. Thus, we used iAs% as our primary exposure biomarker to represent arsenic metabolism efficiency, as it indicates proportion of iAs not methylated in the urine. Also, iAs% at visit 2 was associated with gestational age and birth weight, but not maternal weight gain and other birth outcomes. We finalized the mediation

pathway (Figure 3.2) from these analyses and proceeded with iAs% at visit 2 as the exposure variable, gestational age as the only mediator, and birth weight as the outcome variable in a counterfactual approach of mediation analysis [28]. Exposure, mediator, and outcome variables were all continuous. Covariates included maternal age, maternal BMI at enrollment, maternal education level, income of the financial provider, log-transformed water arsenic exposure level, infant sex, and birth type. We used 1000-replicates bootstrap to obtain standard errors with more conservative and accurate inferences. The model of mediation analysis with interaction is shown below.

The effect of iAs% at visit 2 on gestational age and the effect of iAs% at visit 2 and gestational age on birth weight were assessed using linear regression models. We fit a linear regression model for gestational age (M) on iAs% at visit 2 (A) and a set of confounders (C):

$$E[M|A=a,C=c]=\beta_0+\beta_1 a+\beta_2' c$$

We fit a linear regression model for infant birth weight (Y) on iAs% at visit 2 (A), gestational age (M), and confounders (C) with and without interaction of A and M:

$$E[Y|A=a,M=m,C=c]=\theta_0+\theta_1 a+\theta_2 m+\theta_3 am+\theta_4' c$$

Linear regression for infant birth weight was also fit without including gestational age and its interaction with iAs% at visit 2 to assess total effects (TE) of iAs% at visit 2.

Linear regression models for gestational age and for birth weight were combined to obtain natural direct effects and natural indirect effects using STATA (version 14.0; StataCorp, College Station, TX, USA) Paramed package [29]. We ran analyses with and without the exposure–mediator interaction term.

For all mediation models, we assumed that there were no unmeasured confounders among exposure, mediator, or outcome and that there were no mediator–outcome confounders affected by exposure.

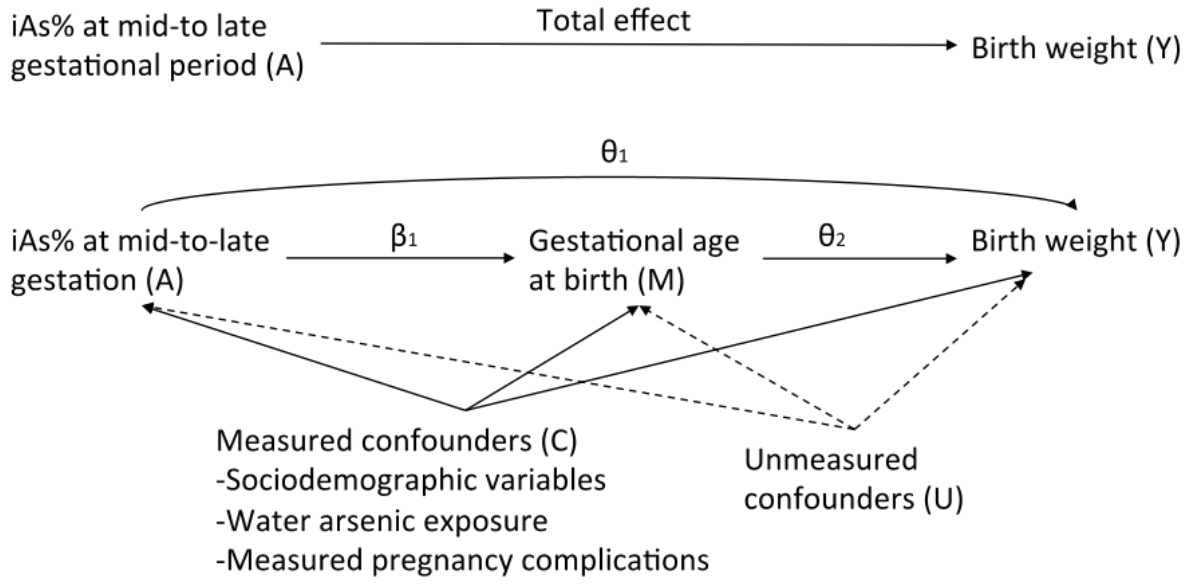

Figure 3.2: Relationships between inorganic arsenic percentage (iAs%) in mid-to-late gestation (A), gestational age at birth (M), and birth weight (Y), with measured confounders (C) and unmeasured confounders (U). Dashed arrows indicates sources of unmeasured confounding.

Results

Study participants Bangladeshi (100%), homemakers (99%), non-smokers (100%), did not chew tobacco (99%), and were active at least for half a day each day (92%). As shown in Table 3.1, average exposure level, anthropometry measures, educational levels, and income of financial provider differed by study site. Participants in Sirajdikhan had higher maternal weight at enrollment, higher body mass index at enrollment, and greater maternal weight gain until birth. Participants in Sirajdikhan had lower arsenic concentration in their drinking water sources (Sirajdikhan median = 1.4 μg/L; Pabna median = 27.0 μg/L) and a lower rate of environmental smoke exposure. In addition, financial providers of participants in Sirahdikhan had higher average incomes. Additionally, 57% of Sirajdikhan participants delivered at a hospital, compared to 19% of Pabna participants.

Table 3.1: Demographic characteristics, exposure levels, and birth outcomes of participants by study site (N = 1176)[a]

	Sirajdikhan (N = 607)		Pabna (N = 569)	
	Birth weight (g)		Birth weight (g)	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal age				
18–22 years old	346 (57.0)	2914 (278.9)	297 (52.2)	2750 (478.7)
23–41 years old	261 (43.0)	2914 (297.6)	272 (47.8)	2769 (508.1)
Maternal BMI at enrollment				
<20 kg/m^2	261 (43.0)	2907 (273.5)	328 (57.6)	2704 (477.2)
≥20 kg/m^2	346 (57.0)	2919 (296.8)	241 (42.4)	2834 (504.2)
Gestational age				
28–37 weeks	83 (13.7)	2687 (393.0)	338 (59.4)	2680 (497.7)
38–42 weeks	524 (86.3)	2950 (248.2)	231 (40.6)	2874 (462.5)
Maternal weight at enrollment				
<47 kg	303 (49.9)	2894 (277.2)	352 (61.9)	2686 (477.9)
≥47 kg	304 (50.1)	2934 (295.2)	217 (38.1)	2878 (493.9)
Maternal weight gain during follow-up				
Missing	22 (3.6)	2800 (155.8)	2 (0.4)	3000 (707.1)
<9 kg	230 (37.9)	2878 (302.4)	396 (69.5)	2699 (478.1)
≥9 kg	355 (58.5)	2945 (278.6)	171 (30.1)	2895 (499.2)
Infant sex				
Male	297 (48.9)	2963 (257.3)	297 (52.2)	2781 (481.4)
Female	310 (51.1)	2867 (305.6)	272 (47.8)	2725 (511.8)

Table 3.1 (Continued): Demographic characteristics, exposure levels, and birth outcomes of participants by study site (N = 1176)[a]

	Sirajdikhan (N = 607)		Pabna (N = 569)	
	Birth weight (g)		Birth weight (g)	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Maternal education				
≤Secondary education	296 (48.8)	2899 (283.4)	253 (44.5)	2695 (492.9)
>Secondary education	311 (51.2)	2928 (289.8)	316 (55.5)	2802 (494.8)
Income of financial provider				
≤3000 taka	159 (26.5)	2892 (245.1)	348 (61.3)	2781 (479.4)
>3000 taka	441 (73.5)	2920 (298.9)	220 (38.7)	2711 (521.6)
Environmental tobacco smoke exposure				
No	392 (64.69)	2928 (297.3)	288 (50.6)	2785 (517.7)
Yes	214 (35.3)	2889 (265.7)	281 (49.4)	2723 (472.3)
Betel nut chewing				
No	596 (99.2)	2916 (286.9)	561 (98.8)	2756 (497.9)
Yes	4 (0.7)	2698 (296.7)	7 (1.2)	2714 (357.3)
Birth place				
Home	225 (37.3)	2889 (209.4)	412 (72.4)	2656 (456.9)
Clinic	34 (5.6)	2919 (185.3)	48 (8.4)	2995 (444.9)
Hospital	345 (57.1)	2929 (334.5)	109 (19.2)	3022 (529.2)
Birth type				
Vaginal	271 (44.6)	2896 (222.9)	492 (86.5)	2702 (481.4)
Cesarean	336 (55.4)	2929 (329.1)	77 (13.5)	3090 (461.9)

Table 3.1 (Continued): Demographic characteristics, exposure levels, and birth outcomes of participants by study site (N = 1176)[a]

	Sirajdikhan (N = 607)		Pabna (N = 569)	
	Birth weight (g)		Birth weight (g)	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Water arsenic exposure[b]				
≤10 ug/L	533 (87.81)	2922 (288.9)	184 (32.17)	2730 (474.8)
10–50 ug/L	32 (5.27)	2884 (234.2)	171 (29.9)	2736 (516.5)
>50 ug/L	42 (6.92)	2837 (290)	217 (37.94)	2790 (498.4)

SD: standard deviation
[a]Study population included women who provided two spot urine samples and were followed until birth.
[b]Cut-off points of water arsenic exposure categories were based on WHO-recommended and Bangladesh-recommended levels.

We found that arsenic metabolism efficiency at visit 2 predicted gestational age, but not maternal weight gain. Table 3.2 reports adjusted estimates from a linear regression model for gestational age at birth based on iAs% at visit 2. A 10-unit increase in iAs% at visit 2 was associated with a significant –0.12 week [95% confidence interval (CI): –0.22, –0.03] change in gestational age (p = 0.013), adjusted for maternal age, BMI at enrollment, education, income, water arsenic level, infant sex, and birth type. Site-specific analyses showed similar results (Table 3.S1).

Arsenic metabolism efficiency at visit 2 predicted birth weight but not birth length and head circumference. In the adjusted linear regression model (Table 3.3), a 10-unit increase in iAs% at visit 2 was associated with a –15.13 g (95% CI: –35.46, 5.19) change in birth weight, although this decrease was not significant even when controlled for age, BMI at enrollment, education, income, water arsenic level, infant sex, and birth type (p = 0.144). Table 3.4 shows

adjusted estimates and CIs of the above model when additionally adjusted for gestational age. When adjusted for gestational age, a 10-unit increase in iAs% at visit 2 was associated with a –5.27 g (95% CI: –24.11, 13.56) change in birth weight, although this decrease still was not significant ($p = 0.58$). In site-specific analysis of the association between birth weight and iAs% at visit 2 (Table 3.S2), a 10-unit increase in iAs% was associated with a significant 21.68 g decrease in birth weight in Sirajdikhan ($p = 0.04$) but an insignificant 11.72 g decrease in Pabna ($p = 0.50$).

Table 3.2: Adjusted estimates of gestational age (N = 1163)[a]

	Gestational age estimate (weeks) (95% CI)	*p*
Intercept	38.25 (37.39, 39.10)	0.000
10-unit increase in $iAs\%_2$	–0.12 (–0.22, –0.03)	0.013
Maternal age	–0.00 (–0.03, 0.03)	0.898
Maternal BMI at enrollment	0.01 (-0.03, 0.04)	0.738
Maternal education (>secondary education)	–0.30 (-0.52, –0.07)	0.010
Income of financial provider (>3000 taka)	0.20 (-0.03, 0.42)	0.089
log(water arsenic exposure)	–0.22 (-0.27, –0.16)	0.000
Female infant (compared to male infant)	0.10 (-0.12, 0.31)	0.381
Cesarean birth (compared to vaginal birth)	0.37 (0.12, 0.61)	0.003

95% CI: 95% confidence interval; $iAs\%_2$: the proportion of inorganic arsenic in total urine arsenic at visit 2; BMI: body mass index

[a]Excluded mothers with incomplete covariate information (n = 13).

Table 3.3: Adjusted estimates of birth weight (N = 1163)[a]

	Birth weight estimate (g) (95% CI)	*p*
Intercept	2554.77 (2373.55, 2735.99)	0.000
10-unit increase in $iAs\%_2$	–15.13 (-35.46, 5.19)	0.144
Maternal age	–3.69 (–9.46, 2.09)	0.211
Maternal BMI at enrollment	18.52 (10.86, 26.19)	0.000
Maternal education (>secondary education)	34.24 (–13.22, 81.70)	0.157
Income of financial provider (>3000 taka)	–37.05 (–85.04, 10.95)	0.130
log(water arsenic exposure)	–6.03 (–17.60, 5.54)	0.307
Female infant (compared to male infant)	–71.93 (–117.16, –26.70)	0.002
Cesarean birth (compared to vaginal birth)	146.33 (94.73, 197.92)	0.000

95% CI: 95% confidence interval; $iAs\%_2$: the proportion of inorganic arsenic in total urine arsenic at visit 2; BMI: body mass index

[a]Excluded mothers with incomplete covariate information (n = 13).

Table 3.4: Estimates of birth weight adjusted for gestational age (N =1163)[a]

	Birth weight estimate (g) (95% CI)	*p*
Intercept	–550.39 (–1014.79, -86.00)	0.020
10-unit increase in $iAs\%_2$	–5.27 (–24.11, 13.56)	0.583
Gestational age (weeks)	81.19 (69.86, 92.51)	0.000
Maternal age	–3.54 (–8.88, 1.80)	0.193
Maternal BMI at enrollment	18.02 (10.94, 25.11)	0.000
Maternal education (>secondary education)	58.25 (14.25, 102.24)	0.010
Income of financial provider (>3000 taka)	–53.00 (–97.42, –8.59)	0.019
log(water arsenic exposure)	11.60 (0.63, 22.58)	0.038
Female infant (compared to male infant)	–79.65 (–121.47, –37.83)	0.000
Cesarean birth (compared to vaginal birth)	116.68 (68.82, 164.55)	0.000

95% CI: 95% confidence interval; $iAs\%_2$: the proportion of inorganic arsenic in total urine arsenic at visit 2; BMI: body mass index

[a]Excluded mothers with incomplete covariate information (n = 13).

Table 3.5 shows estimates from linear regression for iAs% at visit 2 and birth weight mediated through gestational age, with a sensitivity analysis conditional on study site. Adjusting for measured confounders, a 10-unit increase in iAs% at visit 2 was associated with a –15.13 g (bootstraped 95% CI: –42.95, 19.03) change in birth weight, –5.27 g (bootstraped 95% CI: –30.28, –25.67) of which (35%) was natural direct effect, and –9.86 g (bootstraped 95% CI: –20.31, –2.35) of which (65%) was mediated through gestational age. In the adjusted mediation analysis restricted to the Sirajdikhan site, a 10-unit increase in iAs% at visit 2 was associated with a –21.68 (–54.50, 4.92) change in birth weight, –14.67 (–42.44, 8.45) of which (68%) was natural direct effect, and –7.01 (–15.15, 1.88) of which (32%) was natural indirect effect. When restricted to the Pabna site, natural direct effect, natural indirect effects and total effect were –0.18 (–40.25, 41.59), –11.53 (–30.88, 0.96), and –11.72 (–56.74, 30.22), respectively. We found marginally significant indirect effect in site-specific analysis, which was probably due to loss of power. These estimates were subject to unmeasured confounding by common causes of shortened gestational age and low birth weight. Adjusting for the exposure–mediator interaction term yield similar results (Table 3.S3).

Table 3.5: Effect estimates of birth weight with a 10-unit increase in iAs%$_2$[a]

	Birth weight estimate (g) (95% CI)			
Model[b]	Natural direct effect	Natural indirect effect	Total effect	Percent mediated[c]
All mothers (N = 1163)	–5.27 (–30.28, 25.67)	–9.86 (–20.31, –2.35)	–15.13 (–42.95, 19.03)	65
Sirajdikhan (N = 596)	–14.67 (–42.44, 8.45)	–7.01 (–15.15, 1.88)	–21.68 (–54.50, 4.92)	32
Pabna (N = 567)	–0.18 (–40.25, 41.59)	–11.53 (–30.88, 0.96)	–11.72 (–56.74, 30.22)	98

95% CI: 95% confidence interval; iAs%$_2$: the proportion of inorganic arsenic in total urine arsenic at visit 2

[a]Calculated using counterfactual approach without interactions and standard errors generated from 1000-replicates bootstrap.

[b]Adjusted for maternal age, maternal BMI at enrollment, maternal education, higher income of financial provider, log-transformed water arsenic exposure, infant sex, and birth type.

[c]Percent mediated = natural indirect effect/(natural direct effect + natural indirect effect) × 100.

Discussion

We show that arsenic metabolism during mid-to-late pregnancy is associated with gestational age, both of which are associated with birth weight, in a Bangladeshi cohort. Using mediation analysis, we demonstrated within a hypothesized causal framework that the relationship between arsenic metabolism and birth weight is partially mediated by gestational age, which has not been previously demonstrated in human or animal studies.

This finding has biologic plausibility based on the current knowledge of the role of iAs in oxidative stress, as well as the effect of iAs on gestational age [21, 30-32]. Lower arsenic methylation efficiency may result in accumulated iAs in the maternal–fetal interface, and both in vivo and in vitro studies show that iAs plays a role in generating reactive oxygen species and inflammation [21, 30-32]. Thus, increased inflammation at the maternal–fetal interface may trigger early initiation of parturition pathways [33]. Future studies of oxidative stress biomarkers in exposed populations are needed to further examine this suggested pathway.

On the other hand, a previous study found that iAs in urine is associated with leptin, a hormone regulating long-term food intake and energy expenditure that maintains body weight [34]. Reduced food intake may affect fetal growth and thus affect birth weight. However, our study used maternal weight gain as an indicator of fetal growth but did not find an association with arsenic metabolism. Thus, our findings do not support the premise that arsenic methylation efficiency affects fetal growth.

In addition to our primary findings, we conducted a mediation analysis, which allowed testing of possible exposure–mediator interactions. Adjusting for an interaction term reduced the size of natural direct, natural indirect, and total effects of mothers in combined study sites and the Sirajdikhan study site alone. However, adding an interaction term increased the percent of the

effect that was mediated. This suggests that a biologic interaction may exist in these relationships. Specifically, more efficient arsenic metabolism showed a positive indirect effect through gestational age and a positive total effect on birth weight, although the impact was not substantial.

We did not use the average of repeated measures of urinary arsenic metabolites because pregnant women had increased arsenic metabolism, and this change primarily happened in the first trimester. Namely, at the time we collected the first sample, mothers may have been undergoing rapid metabolism changes. This could explain why arsenic metabolism at visit 1 did not predict gestational age or birth weight. Arsenic metabolism tends to be more stable in mid-to-late gestation [35, 36], and thus this period may be more predictive of gestational age and birth weight.

However, we recognize some limitations to our study. First, we measured urinary arsenic metabolites despite the fact that measures from maternal blood or the maternal–fetal interface may be more biologically relevant [37, 38]. However, markers in urine may be indicative, at least in part, of the uterine compartment, and urine collection is less invasive than other fluid samples during pregnancy. Second, to perform causal mediation analysis, we had to make strong assumptions that there was no unmeasured confounding between any combination of exposures, mediators, and outcomes. Further, there should not be any mediator–outcome confounders affected by exposure. We addressed potentially important confounders by adjusting for drinking water arsenic exposure because it is associated with arsenic metabolism (Table 3.S4). A previous study has shown that arsenic in drinking water also affects birth outcomes mediated through gestational age and maternal weight gain per week [22]. Nutritional deficiency is another potential confounder, as one epidemiologic study found low dietary protein intake may decrease

arsenic metabolism in a U.S. population [39]. However, adjusting for maternal daily dietary protein intake, along with maternal daily energy intake tertiles and BMI, did not change our results. Because there may be unknown confounders associated with study sites, we also performed site-specific analyses using linear regression and mediation analysis (Tables 3.S1, 3.S2, and 3.S5). Although the two sites showed different magnitudes of the association, the direction remained the same. Another limitation of this study is generalizability—our cohort was homogeneous in race, socioeconomic status, and lifestyle as compared to the global population. Thus, we are cautious to generalize these results to other populations. Finally, although we had a large sample size for mediation analysis, our interaction tests may have be underpowered.

Nonetheless, this study has several strengths. First, we examined associations and explored the sensitive time window in a prospective cohort with a large number of participants, and we repeatedly measured biomarkers of exposure prior to the outcome assessment. Second, we accurately measured arsenic metabolism biomarkers. Because profiles of urinary arsenic species were stable over repeated sampling within several days, arsenic metabolites from spot urine samples therefore can reliably be used to represent arsenic methylation efficiency [40]. Third, this is the first epidemiological study to attempt to identify mediators in the relationship between inter-individual variability of arsenic metabolism and birth outcomes. Several studies have examined the relationships between arsenic metabolism and birth outcomes, but they are limited by an inability to establish causality [4, 6]. Although mediation analysis still does not concretely establish a causal pathway, our study provides an additional step that no previous studies have been able to take.

Counterfactual mediation analysis provides a strong theoretical basis of causal inference to identify potential causal pathways. Compared to classic regression approaches to mediation

analysis, the counterfactual approach allows testing of exposure–mediator interactions in linear models [41]. These methods can be particularly useful for future epidemiology studies that require flexibility in models that incorporate non-linearity and interactions. Overall, this study provides new perspective on the risk factors for low birth weight. Future studies with more diverse populations are needed to validate these results.

Conclusions

Our approach of systematically building more complex mediation models shows the utility of applying sophisticated analytical approaches to a prospective study design. Conditional on the underlying assumptions, our findings provide causal evidence for mediation between arsenic metabolism during mid-to-late gestation and birth weight by gestational age in a Bangladeshi cohort.

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Supplementary materials

Table 3.S1: Mean change in gestational age associated with a 10-unit change in average urinary arsenic metabolites[a] among Pabna and Sirajdikhan participants

	Gestational age (weeks)		Gestational age (weeks), adjusted[c]	
Arsenic metabolite[b]	β (95% CI)	*p*	β (95% CI)	p
All mothers (N = 1163)[d]				
iAs%$_1$	–0.19 (–0.31, –0.08)	<0.01	–0.09 (–0.20, 0.02)	0.11
MMA%$_1$	–0.32 (–0.58, –0.07)	0.01	0.08 (–0.18, 0.33)	0.54
DMA%$_1$	0.19 (0.09, 0.29)	<0.01	0.06 (–0.04, 0.15)	0.24
iAs%$_2$	–0.19 (–0.29, –0.09)	<0.01	–0.12 (–0.22, –0.03)	0.01
MMA%$_2$	–0.11 (–0.36, 0.14)	0.4	0.02 (–0.22, 0.26)	0.86
DMA%$_2$	0.18 (0.09, 0.27)	<0.01	0.1 (0.01, 0.19)	0.02
Sirajdikhan (N = 596)				
iAs%$_1$	–0.02 (–0.13, 0.09)	0.71	–0.04 (–0.15, 0.07)	0.49
MMA%$_1$	–0.02 (–0.25, 0.21)	0.85	0 (–0.23, 0.23)	0.99
DMA%$_1$	0.02 (–0.07, 0.11)	0.69	0.03 (–0.06, 0.12)	0.56
iAs%$_2$	–0.07 (–0.17, 0.03)	0.15	–0.09 (–0.19, 0.01)	0.07
MMA%$_2$	–0.06 (–0.28, 0.15)	0.57	–0.06 (–0.27, 0.15)	0.57
DMA%$_2$	0.07 (–0.02, 0.16)	0.12	0.09 (0.00, 0.18)	0.06
Pabna (N = 567)				
iAs%$_1$	0.06 (–0.12, 0.24)	0.50	0.09 (–0.10, 0.27)	0.35
MMA%$_1$	0.28 (–0.16, 0.72)	0.22	0.35 (–0.12, 0.82)	0.14
DMA%$_1$	–0.09 (–0.25, 0.07)	0.29	–0.12 (–0.28, 0.05)	0.17
iAs%$_2$	–0.14 (–0.28, 0.01)	0.07	–0.14 (–0.29, 0)	0.06
MMA%$_2$	0.18 (–0.27, 0.63)	0.42	0.23 (–0.23, 0.69)	0.33
DMA%$_2$	0.11 (–0.03, 0.25)	0.13	0.11 (–0.03, 0.26)	0.12

Table 3.S1 (Continued): Mean change in gestational age associated with a 10-unit change in average urinary arsenic metabolites[a] among Pabna and Sirajdikhan participants

95% CI: 95% confidence interval; iAs: inorganic arsenic; MMA: monomethylarsonic acid; DMA: dimethylarsenic acid

[a]A 10-unit change in proportion of urinary arsenic metabolite corresponds roughly to an increase of one standard deviation of the distribution.

[b]Subscripts indicate measurements at visit 1 ($_1$) or visit 2 ($_2$).

[c]Adjusted for maternal age, maternal BMI at enrollment, maternal education, higher income of financial provider, natural log-transformed drinking water arsenic level, infant sex, and birth type.

[d]Excluded mothers with incomplete covariate information (n = 13).

Table 3.S2: Mean change in birth weight associated with a 10-unit change in average urinary arsenic metabolitesa among Sirajdikhan and Pabna participants

	Birth weight (g)		Birth weight (g), adjusted[c]		Birth weight (g), adjusted for gestational age[d]	
Arsenic metabolite[b]	β (95% CI)	*p*	β (95% CI)	*p*	β (95% CI)	*p*
All mothers (N = 1163)[e]						
iAs%$_1$	–16.7 (–40.37, 6.97)	0.17	–5.71 (29.13, 17.71)	0.63	1.59 (–20.06, 23.24)	0.89
MMA%$_1$	–15.49 (–68.53, 37.54)	0.57	11.25 (–42.88, 65.37)	0.68	4.8 (–45.20, 54.81)	0.85
DMA%$_1$	14.6 (–5.73, 34.93)	0.16	2.77 (–17.77, 23.31)	0.79	–1.91 (–20.89, 17.07)	0.84
iAs%$_2$	–20.75 (–41.55, 0.06)	0.05	–15.13 (–35.46, 5.19)	0.14	–5.27 (–24.11, 13.56)	0.58
MMA%$_2$	–3.71 (–56.10, 48.68)	0.89	–1.28 (–52.15, 49.60)	0.96	–3.03 (–50.01, 43.96)	0.90
DMA%$_2$	18.74 (–0.76, 38.25)	0.06	13.59 (–5.54, 32.72)	0.16	5.1 (–12.62, 22.82)	0.57
Sirajdikhan (N = 596)						
iAs%$_1$	–7.21 (–30.05, 15.63)	0.54	–9.97 (–32.69, 12.75)	0.39	–6.97 (–28.05, 14.11)	0.52
MMA%$_1$	12.75 (–35.09, 60.59)	0.60	7.41 (–41.01, 55.84)	0.76	7.39 (–37.51, 52.29)	0.75
DMA%$_1$	3.1 (–16.30, 22.50)	0.75	6.09 (–13.34, 25.52)	0.54	3.9 (–14.12, 21.93)	0.67
iAs%$_2$	–22.04 (–42.87, –1.22)	0.04	–21.68 (–42.22, –1.14)	0.04	–14.67 (–33.80, 4.46)	0.13
MMA%$_2$	24.99 (–20.22, 70.20)	0.28	17.03 (–27.49, 61.55)	0.45	21.83 (–19.44, 63.11)	0.30
DMA%$_2$	14.12 (–5.06, 33.29)	0.15	15.21 (–3.69, 34.11)	0.11	8.41 (–9.18, 26.01)	0.35
Pabna (N = 567)						
iAs%$_1$	11.41 (–32.95, 55.77)	0.61	11.93 (–30.14, 54.00)	0.58	5.03 (–34.50, 44.55)	0.80
MMA%$_1$	31.17 (–76.80, 139.14)	0.57	46.64 (–61.12, 154.41)	0.40	18.53 (–82.87, 119.93)	0.72
DMA%$_1$	–13.38 (–53.10, 26.35)	0.51	–15.59 (–53.65, 22.47)	0.42	–6.44 (–42.24, 29.37)	0.72
iAs%$_2$	–5.23 (–41.01, 30.55)	0.77	–11.72 (–45.79, 22.36)	0.50	–0.19 (–32.29, 31.91)	0.99
MMA%$_2$	–29.53 (–139.56, 80.49)	0.60	–19.79 (–125.83, 86.24)	0.71	–38.00 (–137.56, 61.57)	0.45
DMA%$_2$	7.7 (–26.65, 42.06)	0.66	12.94 (–20.10, 45.97)	0.44	3.88 (–27.21, 34.97)	0.81

Table 3.S2 (Continued): Mean change in birth weight associated with a 10-unit change in average urinary arsenic metabolitesa among Sirajdikhan and Pabna participants

95% CI: 95% confidence interval; iAs: inorganic arsenic; MMA: monomethylarsonic acid; DMA: dimethylarsenic acid

[a]A 10-unit change in proportion of urinary arsenic metabolite corresponds roughly to an increase of one standard deviation of the distribution.

[b]Subscripts indicate measurements at visit 1 ($_1$) or visit 2 ($_2$).

[c]Adjusted for maternal age, maternal BMI at enrollment, maternal education, higher income of financial provider, natural log-transformed drinking water arsenic level, infant sex, and birth type.

[d]Adjusted for maternal age, maternal BMI at enrollment, maternal education, higher income of financial provider, natural log-transformed drinking water arsenic level, infant sex, birth type, and gestational age.

[e]Excluded mothers with incomplete covariate information (n = 13).

Table 3.S3: Effect estimates of birth weight with a 10-unit increase in $iAs\%_2$[a]

Model[b]	Controlled direct effect (95% CI)	Natural direct effect (95% CI)	Natural indirect effect (95% CI)	Total effect (95% CI)	Percent mediated[c]
All mothers (N = 1163)					
0–10%[d]	–2.48 (–25.65, 25.45)	–2.21 (–24.17, 25.51)	–9.85 (–20.17, –2.42)	–12.05 (–36.63, 17.89)	81
10–20%[e]	–2.48 (–24.10, 25.84)	–2.70 (–24.41, 25.66)	–10.34 (–20.59, –1.95)	–13.03 (–37.78, 14.99)	79
Sirajdikhan (N = 596)					
0–10%[d]	–17.89 (–41.46, 11.41)	–9.45 (–27.21, 12.89)	–7.14 (–15.78, 1.43)	–16.58 (–35.81, 7.39)	43
10–20%[e]	–17.89 (–44.15, 11.83)	–10.26 (–31.38, 11.89)	–7.95 (–20.24, 0.90)	–18.21 (–40.29, 6.58)	43
Pabna (N = 567)					
0–10%[d]	9.15 (–35.58, 54.81)	4.63 (–36.73, 46.65)	–11.40 (–28.11, 0.07)	–6.77 (–52.98, 34.89)	NA
10–20%[e]	9.15 (–31.14, 60.48)	3.86 (–34.32, 48.99)	–12.17 (–31.97, 0.50)	–8.31 (–47.59, 39.88)	NA

$iAs\%_2$: the proportion of inorganic arsenic in total urine arsenic at visit 2; 95% CI: 95% confidence interval; NA: not applicable

[a]Calculated using counterfactual approach with interactions and standard errors generated from 1000-replicates bootstrap.

[b]Adjusted for age, BMI at enrollment, higher education, higher income, log-transformed water arsenic exposure, infant sex, and birth type.

[c]Percent mediated = natural indirect effect/(natural direct effect + natural indirect effect) × 100.

[d]Model 2: with interaction; a0–a1: 0–10; mediator: 38.

[e]Model 3: with interaction; a0–a1: 10–20; mediator: 38.

Table 3.S4: Mean urinary arsenic metabolites by drinking water arsenic exposure among Sirajdikhan and Pabna participants

Water arsenic exposure[ab]	iAs%$_1$ Mean (SD)	MMA%$_1$ Mean (SD)	DMA%$_1$ Mean (SD)	iAs%$_2$ Mean (SD)	MMA%$_2$ Mean (SD)	DMA%$_2$ Mean (SD)
Sirajdikhan (N = 607)						
≤10 µg/L[a]	7.9 (10.0)	4.3 (4.7)	87.8 (11.8)	7.6 (11.4)	5.2 (5.3)	87.2 (12.4)
10–50 µg/L	8.1 (10.3)	5.6 (4.4)	86.2 (10.8)	6.3 (4.4)	4.2 (3.1)	89.5 (6.2)
>50 µg/L	9.3 (10.3)	8.2 (5.1)	82.4 (12.2)	8.5 (8.4)	5 (3.7)	86.5 (9.8)
Pabna (N = 569)						
≤10 µg/L	12.5 (13.1)	4.9 (3.6)	82.5 (13.4)	7.8 (10.1)	4.7 (3.3)	87.5 (11.0)
10–50 µg/L	11 (6.6)	6.6 (3.7)	82.4 (8.3)	8.7 (8.0)	5.8 (4.1)	85.5 (9.0)
>50 µg/L	13.6 (6.1)	7.4 (3.6)	79 (7.8)	12.7 (13.8)	6.4 (3.4)	80.9 (13.4)

iAs: inorganic arsenic; MMA: monomethylarsonic acid; DMA: dimethylarsenic acid; SD: standard deviation

[a]Subscripts indicate measurements at visit 1 ($_1$) or visit 2 ($_2$).

[b]Cut-offs for water arsenic exposure categories were based on WHO-recommended and Bangladesh-recommended levels.

Table 3.S5. Mean change in birth outcomes associated with one-week-longer gestational age among Sirajdikhan and Pabna participants

	Unadjusted		Adjusted[a]		Adjusted for maternal weight gain[b]			
	Gestational age (weeks)		Gestational age (weeks)		Gestational age (weeks)		Maternal weight gain (kg)	
Birth measure	β (95% CIft gv)	*p*	β (95% CI)	*p*	β (95% CI)	*p*	β (95% CI)	*p*
All mothers (N = 1163)[c]								
Birth weight (g)	81.28 (70.16, 92.40)	<0.01	81.41 (70.12, 92.71)	<0.01	76.16 (64.13, 88.2)	<0.01	12.79 (5.00, 20.59)	<0.01
Birth length (cm)	0.05 (–0.03, 0.12)	0.22	0.11 (0.03, 0.18)	0.01	0.1 (0.02, 0.18)	0.02	0.02 (–0.04, 0.07)	0.55
Head girth (cm)	0.14 (0.1, 0.17)	<0.01	0.16 (0.12, 0.2)	<0.01	0.15 (0.10, 0.19)	<0.01	0.03 (0.00, 0.05)	0.05
Sirajdikhan (N = 596)								
Birth weight (g)	74.58 (58.66, 90.50)	<0.01	79.63 (63.51, 95.76)	<0.01	75.95 (59.47, 92.43)	<0.01	7.65 (0.22, 15.09)	0.04
Birth length (cm)	0.46 (0.33, 0.59)	<0.01	0.48 (0.35, 0.62)	<0.01	0.46 (0.32, 0.60)	<0.01	0.05 (–0.02, 0.11)	0.14
Head girth (cm)	0.22 (0.14, 0.30)	<0.01	0.22 (0.14, 0.30)	<0.01	0.22 (0.14, 0.30)	<0.01	0.01 (–0.02, 0.05)	0.45
Pabna (N = 567)								
Birth weight (g)	83.33 (64.34, 102.31)	<0.01	80.73 (62.83, 98.64)	<0.01	73.95 (55.52, 92.37)	<0.01	21.5 (6.39, 36.61)	0.01
Birth length (cm)	0.1 (0.00, 0.20)	0.06	0.11 (0.01, 0.21)	0.03	0.1 (0.00, 0.20)	0.05	0.03 (–0.06, 0.11)	0.53
Head girth (cm)	0.13 (0.08, 0.18)	<0.01	0.13 (0.08, 0.18)	<0.01	0.12 (0.07, 0.17)	<0.01	0.05 (0.01, 0.09)	0.02

[a]Adjusted for maternal age, maternal BMI at enrollment, maternal education, income of financial provider, and natural log-transformed drinking water arsenic level.

[b]Adjusted for maternal age, maternal BMI at enrollment, maternal education, income of financial provider, natural log-transformed drinking water arsenic level, and maternal weight gain (missing = 23).

[c]Excluded mothers with incomplete covariate information (n = 13).