

Arsenic Metabolism in Children Differs From That in Adults

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ABSTRACT

Arsenic toxicity in adults is associated with methylation efficiency, influenced by factors such as gender, genetics, and nutrition. The aim of this study was to evaluate influencing factors for arsenic metabolism in children. For 488 children (9 years), whose mothers participated in a study on arsenic exposure during pregnancy (nested into the MINIMat trial) in rural Bangladesh, we measured urinary concentrations of inorganic arsenic (iAs) and its metabolites methylarsonic acid (MMA) and dimethylarsinic acid (DMA) by HPLC-HG-ICPMS. Methylation efficiency was assessed by relative amounts (%) of the metabolites. We evaluated the impact of factors such as maternal urinary metabolite pattern, arsenic exposure, gender, socioeconomic status, season of sampling, and nutritional factors, including erythrocyte selenium (Ery-Se), and plasma folate and vitamin B12.

Children had higher %DMA and lower %iAs in urine compared to their mothers, unrelated to their lower exposure [median urinary arsenic (U-As) 53 vs 78 µg/l]. Surprisingly, selenium status (Ery-Se) was strongly associated with children's arsenic methylation; an increase in Ery-Se from the 5–95th percentile was associated with: +1.8 percentage points (pp) for %iAs ($P = .001$), +1.4 pp for %MMA ($P = .003$), and –3.2 pp for %DMA ($P < .001$). Despite this, Ery-Se was positively associated with U-As (5–95th percentile: +41 µg/l, $P = .026$). As expected, plasma folate was inversely associated with %iAs (5–95th percentile: –1.9 pp, $P = .001$) and positively associated with %DMA (5–95th percentile: +2.2 pp, $P = .008$). Children methylated arsenic more efficiently than their mothers. Also influencing factors, mainly selenium and folate, differed. This warrants further research.

Key words: arsenic; children; metabolism; methylation; selenium; folate

Chronic exposure to inorganic arsenic (iAs) through drinking water and food increases the risk of cancer and numerous non-cancer effects (IARC, 2012), but the susceptibility varies markedly between individuals and populations (Faita et al., 2013). Recent findings indicate that early-life is a critical period for arsenic exposure, giving rise to highly elevated health risks later in life, including cancer and overall morbidity and mortality (Smith et al., 2012, 2013).

A well-established susceptibility factor for arsenic-related adverse health effects in adults is inefficient arsenic

metabolism. Arsenic is methylated in the body via the 1-carbon metabolism, and the main metabolites excreted in the urine are methylarsonic acid (MMA) and dimethylarsinic acid (DMA), besides some remaining unmethylated inorganic arsenic. Higher fractions of iAs and MMA in the urine have been associated with higher retention of arsenic (Vahter, 2002) and higher risk of adverse health effects (Chen et al., 2005; Lindberg et al., 2008b; Tseng, 2009). Multiple factors have been shown to influence the metabolism of arsenic in adults, including polymorphisms in the arsenic (+III oxidation state) methyltransferase (AS3MT)

gene (Antonelli *et al.*, 2014; Engstrom *et al.*, 2011), gender (Lindberg *et al.*, 2008a), nutrition (Howe *et al.*, 2014; Li *et al.*, 2008), smoking (Lindberg *et al.*, 2010), and pregnancy (Gardner *et al.*, 2012). In addition, selenium has been associated with the methylation of arsenic in adults (George *et al.*, 2013; Huang *et al.*, 2008; Pilsner *et al.*, 2011), but the results are inconsistent.

Although several recent studies indicate severe health effects of elevated arsenic exposure in children, mainly in the form of impaired immune function (Ahmed *et al.*, 2014; Soto-Pena *et al.*, 2006), growth (Gardner *et al.*, 2013), and cognitive function (Hamadani *et al.*, 2011; Wasserman *et al.*, 2007), little is known about potential susceptibility factors, including the efficiency of arsenic metabolism. In a couple of studies, factors such as plasma homocysteine levels and gender appeared to influence the urinary arsenic metabolite fractions differently in young children (1.5–6.5 years) compared to adults (Fangstrom *et al.*, 2009; Hall *et al.*, 2009). The aim of the present study was to further clarify children's metabolism of arsenic by assessing influencing factors such as past and present arsenic exposure, gender, socioeconomic status (SES), season of sampling, and nutritional factors including selenium, folate and vitamin B12, in 9-year old children.

METHODS

Study design

This prospective cohort study is part of our ongoing research concerning effects of arsenic on pregnancy outcomes and child health and development (Vahter *et al.*, 2006), which was nested in a randomized population-based food and micronutrient supplementation trial in pregnancy (MINIMat) in Matlab, a rural area 53 km southeast of Dhaka in Bangladesh (Persson *et al.*, 2012). Between November 2001 and October 2003, the trial enrolled a total of 4436 pregnant women with a wide variation of arsenic in drinking water. Despite extensive mitigation activities, our follow-up of about half of the children at 5 (Gardner *et al.*, 2011a) and 10 years of age (Kippler *et al.*, 2016) indicated persistent elevated exposure to arsenic.

In a subcohort of 640 children, born from June 2003 to June 2004, we have prospectively evaluated the effects of arsenic exposure on the children's immune function (Ahmed *et al.*, 2012). Because we barely found any impact of arsenic metabolism on various outcomes (unpublished data; Ahmed *et al.*, 2013; Gardner *et al.*, 2013; Hamadani *et al.*, 2011), we now evaluate factors influencing the metabolism of arsenic in the present study. Details on the selection of these children and the follow-ups have been described in detail previously (Ahmed *et al.*, 2012, 2014). In total, 488 children had both prenatal and concurrent (at 9 years of age) exposure data. We have exposure data for the children also at 4.5 years, but we restricted this study to the follow-up at 9 years as 34% of the children had no blood analyzed at 4.5 years. Importantly, the methylation patterns were very similar at 4.5 and 9 years, and there were no major differences in other characteristics either (Supplementary Table S1).

The study was approved by the Ethical Committees at icddr,b, Bangladesh, and Karolinska Institutet, Sweden. Informed consent was obtained from all the guardians. Participants were free to refrain partially or totally from participation at any time of the study.

Arsenic exposure and metabolism

We assessed individual arsenic exposure based on the sum concentration of iAs and its methylated metabolites (MMA and

DMA) in urine, hereinafter referred to as U-As, which reflects exposure from all sources, ie, both drinking water and food. Arsenic methylation efficiency was evaluated based on the relative concentrations (%) of the different arsenic metabolites in urine (Vahter, 2002).

Spot urine samples were collected in early and late pregnancy [gestational week (GW) 8 and 30] as previously described (Vahter *et al.*, 2006). For the purpose of the present study, we have used the urine data from early pregnancy. These provide a more representative pattern of the women's usual urinary arsenic metabolites, since the methylation of arsenic changes markedly during pregnancy (Gardner *et al.*, 2012; Li *et al.*, 2008). At the follow-up of the children we collected spot urine samples at the health care facilities using plastic urine collection cups tested to be free from trace elements. Urine was immediately transferred to 24 ml polyethylene bottles, which were kept in refrigerator until they, at the end of the day, were sent to the Matlab hospital laboratory for storage at -20°C . Every week, urine samples were transported to the Nutritional Biochemistry laboratory in Dhaka, where they were stored at -80°C . At the end of the sampling period, urine samples were transported in cooling boxes on ice to Karolinska Institutet in Stockholm, Sweden, for analysis of arsenic metabolites and other elements.

The concentrations of arsenic metabolites [iAs (III), iAs (V), MMA (V), and DMA (V)] in maternal and child urine were measured using high-performance liquid chromatography on-line coupling with hydride generation inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS), as described previously (Fangstrom *et al.*, 2008; Gardner *et al.*, 2011a). In short, the urine samples (0.5 ml) were filtered on a $0.2\ \mu\text{m}$ hydrophilic syringe filter (Sarstedt, Germany) and $10\ \mu\text{l}$ was injected on the anion exchange column (Hamilton PXP-X100, $10\ \mu\text{m}$, $250 \times 4.6\ \text{mm}$, Reno, Nevada) of the HPLC system (Agilent 1100 series; Agilent Technologies, Waldbronn, Germany). The limit of detection (LOD) was $0.2\ \mu\text{g/l}$ for inorganic As (III), MMA and DMA, and $0.5\ \mu\text{g/l}$ for inorganic As (V). Nine samples were below LOD for AsIII, 5 for MMA, 21 for AsV, but none for DMA. We used the measured concentrations for these samples, and replacing them with $\text{LOD}/\sqrt{2}$ made no difference to the results. The intra- and inter-assay coefficients of variation were both approximately 4%, based on repeated measurements of the quality control material NIES CRM No.18 human urine (National Institute for Environmental Studies, Tsukuba City, Japan; $n = 49$) during 8 non-consecutive days. The reference value of DMA was $36 \pm 9\ \mu\text{g/l}$ (mean \pm SD) and our measured mean DMA concentrations were $42\text{--}43\ (\pm 1.2\text{--}1.6\ \mu\text{g/l})$, which was in agreement with values obtained previously (Ahmed *et al.*, 2014; Gardner *et al.*, 2011a).

Total arsenic in urine (U-As_{tot}) was measured using ICP-MS (Agilent 7700x, Agilent Technologies, Tokyo, Japan), with the collision/reaction cell in helium mode to minimize interferences (Lu *et al.*, 2015). Before the measurements, the urine samples were diluted 1:10 with 1% nitric acid (65% w/w, Scharlau, Scharlab S.L., Sentmenat, Spain). The LOD was $<0.042\ \mu\text{g/L}$ (Supplementary Table S2 and S3), and no samples were below this concentration. The correlation between children's U-As_{tot} and U-As (sum of iAs metabolites), measured with the 2 methods (HPLC-HG-ICPMS and ICP-MS, respectively) was $r_s = 0.98$ ($P < .001$), providing additional support for good analytical quality. However, some differences between U-As_{tot} and U-As were found, why we additionally measured arsenobetaine in urine of a subsample of children from the same study population ($n = 20$) using HPLC-ICPMS, at the University of Graz, Austria. Urine was filtered through $0.2\ \mu\text{m}$ Nylon filters (Markus Bruckner Analysentechnik, Linz, Austria) and $10\ \mu\text{l}$ were

injected onto a cation-exchange column (Ionospher 5C, 100 × 3 mm, Agilent Technologies) placed in an Agilent 1100 series HPLC system. The mobile phase was 10 mM pyridine pH 2.8 (adjusted with formic acid), the flow rate was 1 ml/min, and the column temperature was 30 °C. The outlet of the column was connected to the nebulizer of an Agilent 7500ce ICP-MS. To enhance the arsenic signal, 1% CO₂ was added to the argon gas. The LOD for arsenobetaine was 0.5–3 µg As/l, depending on the intensity of the DMA peak eluting before the arsenobetaine.

To compensate for the variation in dilution of the sampled urine, which has a considerable effect on the measured urinary concentrations, we adjusted the obtained concentrations for specific gravity, measured by a digital refractometer (RD 712 Clinical Refractometer, EUROMEX microscopes, Holland). We previously found that adjustment by specific gravity in this population was less affected by age, body size, SES and arsenic exposure, than the more commonly used creatinine adjustment (Nermell *et al.*, 2008). Also, the urinary DMA was found to correlate significantly with urinary creatinine excretion, both being influenced by the 1-carbon metabolism. All urinary concentrations were adjusted to the overall mean specific gravity, which was 1.012 for both maternal and child urine (range 1.002–1.031). In a subsample of the studied mothers (*n* = 30), we also measured the osmolality by a digital cryoscopic osmometer (OSMOMAT 030, Gonotec Gesellschaft für Meß- und Regeltechnik mbH, Berlin, Germany) and found a strong correlation with the specific gravity at GW8 ($r_s = 0.98$, $P < .001$), supporting appropriate adjustment for the variation in dilution.

Potential factors influencing arsenic metabolism

Maternal anthropometry, parity, education and SES of the families were collected in early pregnancy at enrollment in the MINIMat trial (Persson *et al.*, 2012). Socioeconomic status was estimated via an asset index, generated through principal component analysis of household assets (on the basis of household ownership of different items) including land, dwelling characteristics, and household sanitation. At the follow-up at 9 years of age, the children's body weight was measured to the nearest 0.1 kg with a digital scale (TANITA HD - 318, Tanita Corporation, Japan), and height was measured with a locally manufactured wooden stadiometer (precise to 0.1 cm). The measured height and weight were converted to height-for-age (HAZ), weight-for-age (WAZ), and BMI-for-age z-scores (BAZ; SD scores), using the WHO growth reference for school-aged children and adolescents (<http://www.who.int/growthref/tools/en/>). Stunting was defined as HAZ < -2 (below minus 2 standard deviations from median HAZ of the reference population), and underweight was defined as WAZ < -2 (below minus 2 standard deviations from median WAZ of the reference population). Season of urine and blood sampling was defined as pre-monsoon (January–May), monsoon (June–September), or post-monsoon (October–December). As additional markers of the nutritional status of the children, we measured selenium, zinc, copper, and manganese in blood (erythrocyte fraction) and folate, and vitamin B12 in plasma. Venous blood was collected in Na-heparin treated trace element free tubes (Vacuette, Greiner Bio-One International AG, Kremsmünster, Austria) at the health care facilities and transported to the Matlab hospital laboratory for separation of plasma and erythrocytes.

Concentrations of the elements in erythrocytes were measured using ICP-MS (Agilent 7700x, Agilent Technologies, Tokyo, Japan) after dilution of the erythrocytes in a 1:25 alkali solution consisting of 1-butanol 2% (w/v; anhydrous, 99.8%, Sigma-

Aldrich, Schnellendorf, Germany), EDTA 0.05% (w/v; H₄-EDTA, 99.995%, Sigma-Aldrich, Schnellendorf, Germany), triton X-100 0.05% (w/v; BioXtra, Sigma-Aldrich, Schnellendorf, Germany), and NH₄OH 1% (w/v; Merck, Darmstadt, Germany) (Lu, *et al.*, 2015). For comparison, we also measured selenium in urine (U-Se) using ICP-MS (as described above for U-As_{tot}) but with the collision/reaction cell in hydrogen mode, and erythrocyte arsenic (Ery-As). For selenium, the LOD in both erythrocytes and urine was below 0.006 µg/l (Supplementary Tables S2 and S3). Obtained values for selenium in reference samples were in good agreement with reference values (Supplementary Tables S2 and S3). Preferably, selenium measured in blood should be expressed per g hemoglobin (Hb) (Skröder *et al.*, 2015; Stefanowicz *et al.*, 2013) and was done so in the present study. Still, we found a strong correlation between Hb-adjusted and unadjusted erythrocyte selenium (Ery-Se) in the children ($r_s = 0.83$, $P < .001$; $n = 488$).

Folate and vitamin B12 was measured in plasma by electrochemiluminescence immunoassay (ECLIA) using an automated immunoassay analyzer (Cobas e601, Roche Diagnostics, Mannheim, Germany). Quality control serum samples (PreciControl Varia 1 and 2, Roche Diagnostics, Mannheim, Germany) were analyzed in each run to check accuracy and precision. The relative coefficient of variation was <5%.

Statistical analyses

Statistical analyses were conducted using the software Stata/IC, version 12.1 (StataCorp, Texas). Data distribution was evaluated by scatter plots and histograms (Figure 1). Associations between the different arsenic metabolites (iAs, MMA, and DMA) and covariates (mother's age, education, BMI, and family SES, children's height, weight, HAZ, WAZ, and BAZ, gender, season of sampling, U-As, Hb, erythrocyte zinc, copper, manganese, Ery-Se, U-Se, and plasma vitamin B12, and folate) were initially evaluated using Spearman's rank correlation coefficients (for continuous variables) and Mann-Whitney *U* test or Kruskal-Wallis test (for categorical variables).

We further assessed the associations between the metabolites (as well as U-As and Ery-As) and the covariates using multi-variable-adjusted linear regression analyses. The limit for collinearity was set at $r_s = 0.60$, and all regression models were checked with normality and residual versus fitted plots. Covariates included in the final model were factors reported to affect arsenic methylation that were also significantly associated with any of the metabolites in the bivariate analysis, creating the best fitted model for the majority of the outcomes (ie forward selection and backward elimination for each metabolite). These covariates were maternal education, age, SES and % of the assessed metabolite, children's age, HAZ, season of sampling, Ery-Se, U-Se, U-As, and plasma folate. Children's HAZ was added as stunted/normal height, since there appeared to be an association between HAZ and MMA only in stunted children (Supplementary Figure S1). Maternal % of the assessed metabolite in early pregnancy was added to assess the extent of heritability of arsenic methylation capacity. Concentrations of U-As were added to the model with a spline knot at 58 µg/L, below which the arsenic mainly originates from food (mainly rice) (Lindberg *et al.*, 2008a; Vahter *et al.*, 2006), known to be partly in the form of DMA (Meharg *et al.*, 2008). Thus, the metabolism of iAs can only be properly evaluated at exposure levels >58 µg/l in this population. The value for the U-As spline knot was set at 58 µg/l as this was implied both by the turning point of the scatter plot for %DMA and U-As (Figure 2) and by the intercept for the association between U-As and water arsenic at 9 years ($n = 221$;

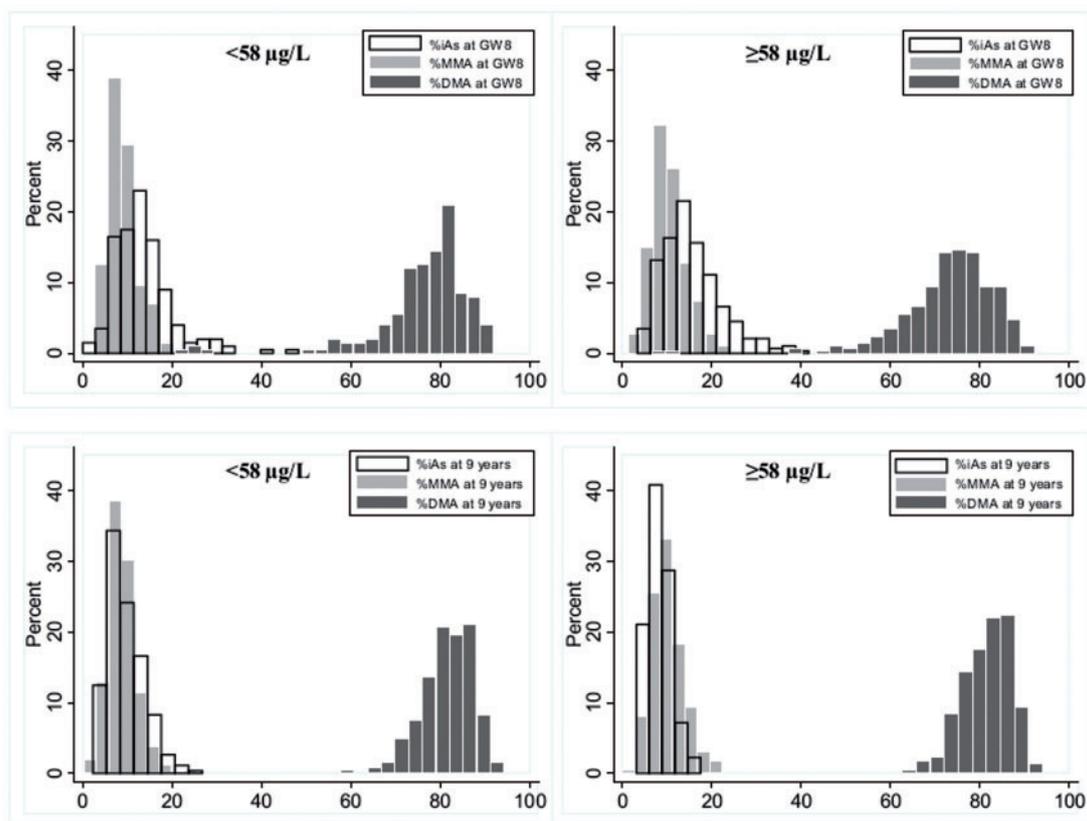


FIG. 1 Histograms of metabolite distribution in pregnant women [top; gestational week (GW) 8] and their children at 9 years (bottom), stratified by urinary arsenic concentrations of 58 µg/l. Abbreviations: DMA, dimethylarsinic acid; iAs, inorganic arsenic; MMA, methylarsonic acid.

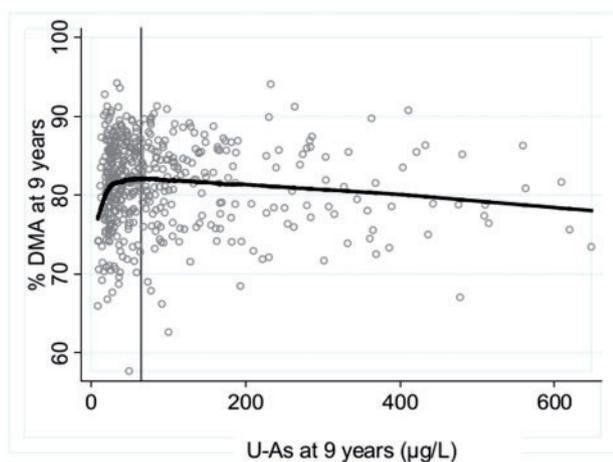


FIG. 2 Scatter plots with smoothed lowest lines for children's % dimethylarsinic acid (%DMA) and urinary arsenic (U-As; below 1000 µg/l to fit scale) at 9 years of age ($n=488$). The black vertical line shows the turning point of the lowest line, at U-As 58 µg/l.

data not shown). We did not include other elements measured in erythrocytes (zinc, copper, manganese), or Hb, in the models as they had no impact on the arsenic methylation (data not shown). To assess potential differences in influential factors depending on the source of arsenic, we also stratified the analyses on U-As below and above 58 µg/l. We also assessed the influence of all factors on the total arsenic concentrations, using U-As and Ery-As as the outcomes instead of the metabolites.

In sensitivity analyses, we additionally adjusted for prenatal U-As and urinary selenium (U-Se at GW8; Ery-Se in pregnancy was not used as this was only available for 136 mothers). Also, we tried adjusting for Ery-As instead of U-As. Since the percentages of the metabolites follow a beta distribution, we also applied Dirichlet multivariate regression. This is an extension of the beta regression which includes all proportions (in this case the %iAs, MMA, and DMA) in the same model.

Based on experimental studies, it has been suggested that selenium and arsenic could form a complex (Berry and Galle, 1994; Levander, 1977), excreted via bile or urine. To assess the potential existence of such a complex, we tested whether the ratio of total urinary arsenic (U-As_{tot}; measured with ICP-MS) over the sum of metabolites of iAs (U-As; measured with HPLC-ICPMS) was associated with the concentration of urinary selenium.

RESULTS

The basic characteristics and arsenic metabolite pattern of the studied children at 9 years and their mothers during pregnancy are described in Table 1. There was no difference in the U-As concentration or the relative amounts of the metabolites between boys and girls. Arsenic in children's erythrocytes and urine were strongly correlated ($r_s = 0.79$, $P < .001$). Although the children's concentrations of U-As were positively correlated with those of the mothers in early pregnancy (Table 2), the median U-As concentration for the children (53 µg/l, range 9–1268 µg/l) was lower than their mothers' concentration (77 µg/l, range 2–2063 µg/l; $P < .001$). The children also had significantly lower urinary %iAs and higher %DMA compared to their mothers at

Table 1 Basic Characteristics and Urinary Arsenic Metabolites of the Studied Children and Mothers (n = 488)

Characteristic	GW8 ^a	9 years ^a
Age (years)	26 ± 5.9	8.9 ± 0.11
Height (cm)	150 ± 5.3	124 ± 5.4
Weight (kg)	46 ± 7.7	22 ± 3.6
BMI (kg/m ²)	21 ± 2.9	14 ± 1.7
SES	0.88 (-3.6, 3.0)	-0.15 (-1.4, 2.0)
Parity	1.4 ± 1.3	-
Sex (%male/female)	0/100	49/51
HAZ (z-score)	-	-1.4 ± 0.89
WAZ (z-score)	-	-1.7 ± 1.1
BAZ (z-score)	-	-1.3 ± 1.1
U-As (µg/l) ^b	78 (20–679)	53 (19–361)
Ery-As (µg/kg)	4.8 (1.5–20) ^c	3.3 (1.3–19)
U-Se (µg/l) ^b	9.4 (5.0–19)	14 (7.9–27)
Ery-Se (µg/kg)	158 (117–218) ^c	172 (139–213)
Ery-Se (µg/g Hb)	0.47 (0.35–0.69) ^d	0.48 (0.38–0.61)
Ery-Zn (µg/kg)	9035 (5602–10 994) ^c	9344 (6856–11 618)
Ery-Cu (µg/kg)	924 (718–1319) ^c	646 (560–754)
Ery-Mn (µg/kg)	18 (10–29) ^c	23 (15–37)
Folate (µg/l)	—	10 ± 2.5
Vitamin B12 (µg/l)	—	639 ± 241 ^f
%iAs	14 (6.2–30)	8.2 (4.5–17)
%MMA	9.5 (4.7–17)	8.9 (4.6–16)
%DMA	76 (56–87)	82 (72–90)

Abbreviations: BAZ, BMI-for-age z-score; BMI, body mass index; DMA, dimethylarsinic acid; Ery-As, erythrocyte arsenic; Ery-Cu, erythrocyte copper; Ery-Mn, erythrocyte manganese; Ery-Se, erythrocyte selenium; Ery-Zn, erythrocyte zinc; HAZ, height-for-age z-score; iAs, inorganic arsenic; MMA, methylarsonic acid; SES, Socioeconomic status; U-As, Urinary arsenic; WAZ, weight-for-age z-score.

^aValues are shown as mean ± SD, median (5–95 percentiles), or %.

^bAdjusted to average specific gravity of 1.012 g/ml.

^cGW14; n = 136.

^dGW14; n = 132.

^eGW14; n = 122.

^fn = 486.

GW8 ($P < .001$; Figure 1), and the differences were less obvious at the lower exposure levels (U-As < 58 µg/l with exposure mainly through food). Also, the mothers' U-As was inversely correlated with their urinary %DMA and positively correlated with their %iAs and %MMA. The corresponding correlations in the

children were much weaker (Table 2). In fact, the children in the highest quartile of U-As (median 192 µg/l) had significantly higher %DMA (82%) compared with the mothers in the lowest quartile of U-As (median 27 µg/l; 78% DMA, $P < .001$).

Both the children's plasma folate and U-Se concentrations were inversely correlated with their urinary %iAs and positively associated with %DMA, but not associated with %MMA (Table 2). In contrast, Ery-Se was positively associated with both %iAs and %MMA, and inversely associated with %DMA (Figure 3). The concentrations of Ery-Se and U-Se were poorly correlated ($r_s = 0.090$, $P = .046$). Other essential elements measured in erythrocytes (manganese, zinc, copper), plasma vitamin B12, and Hb, did not correlate with any of the metabolites and were thus not included in the following analyses.

In the multivariable-adjusted linear regression models with a spline knot for U-As at 58 µg/l, the children's urinary %iAs was found to be higher at the monsoon season than at pre-monsoon, and positively associated with their Ery-Se and maternal %iAs (Table 3). An increase in Ery-Se from the 5th to the 95th percentile (0.38–0.61 µg/g Hb) increased %iAs by almost 2 percentage points (pp), corresponding to about 0.5 SD. On the contrary, the associations with U-As (below spline knot at 58 µg/l), U-Se (µg/l), and folate (µg/l) were inverse. An increase in U-Se and plasma folate from the 5th to the 95th percentile (7.9–27 µg/l for U-Se and 6.4–15 µg/l for plasma folate) was associated with a ~2 pp decrease in %iAs ($P < .001$ for both). When stratifying by low/high U-As (below or above 58 µg/L), it appeared that the season of sampling (monsoon: +3.3 pp; $P < .001$, compared to pre-monsoon) and U-As (-0.66 pp per 10 µg/l; $P = .001$) had a considerably stronger influence on %iAs below 58 µg/l (mainly reflecting exposure to iAs and DMA through food) than above (Supplementary Table S4). The main influencing factor above 58 µg/l (mainly reflecting exposure to iAs through drinking water) was Ery-Se (+2.5 pp from the 5th to the 95th percentile; $P < .001$). U-As (range 58–1268 µg/L in the high stratum) was not significantly associated with %iAs (Supplementary Table S4).

The %MMA was positively associated with Ery-Se (Figure 3), SES, maternal education, and child U-As (above spline knot at 58 µg/l; Table 3). Again, selenium was strongly influential; an increase in Ery-Se from the 5th to the 95th percentile increased %MMA by 1.4 pp (=0.4 SD). The increase in %MMA for the same increase in SES (5th to 95th percentile) also corresponded to about 0.4 SD. For U-As (above the spline knot at 58 µg/l;

Table 2 Spearman Correlation Coefficients (P-value) for Arsenic Metabolites in Pregnancy and at 9 Years (n = 488)

	GW8				9 years			
	% iAs	%MMA	%DMA	U-As	% iAs	% MMA	% DMA	U-As
GW8								
% MMA	0.36 (<0.001)							
% DMA	-0.91 (<0.001)	-0.67 (<0.001)						
U-As	0.21 (<0.001)	0.26 (<0.001)	-0.28 (<0.001)					
9 years								
% iAs	0.091 (0.044)	0.059 (0.19)	-0.096 (0.035)	0.0022 (0.96)				
% MMA	0.17 (<0.001)	0.14 (0.0015)	-0.19 (<0.001)	0.12 (0.0061)	0.31 (<0.001)			
% DMA	-0.17 (<0.001)	-0.12 (0.0059)	0.18 (<0.001)	-0.064 (0.16)	-0.82 (<0.001)	-0.76 (<0.001)		
U-As	0.15 (<0.001)	0.12 (0.0063)	-0.18 (<0.001)	0.36 (<0.001)	-0.14 (0.0025)	0.19 (<0.001)	-0.0021 (0.96)	
Ery-As	0.14 (0.0018)	0.14 (0.0015)	-0.18 (<0.001)	0.37 (<0.001)	0.018 (0.69)	0.23 (<0.001)	-0.13 (0.0037)	0.79 (<0.001)
U-Se	0.025 (0.58)	-0.026 (0.57)	-0.027 (0.55)	-0.030 (0.51)	-0.20 (<0.001)	0.038 (0.40)	0.11 (0.016)	0.010 (0.82)
Ery-Se	0.053 (0.24)	0.025 (0.58)	-0.063 (0.16)	0.029 (0.53)	0.19 (<0.001)	0.18 (<0.001)	-0.22 (<0.001)	0.12 (0.0059)
Folate	-0.029 (0.39)	-0.0081 (0.86)	0.039 (0.39)	-0.027 (0.56)	-0.15 (<0.001)	-0.0067 (0.88)	0.093 (0.041)	0.017 (0.71)

Abbreviations: DMA, dimethylarsinic acid; Ery-As, erythrocyte arsenic; Ery-Se, erythrocyte selenium; GW, gestational week; iAs, inorganic arsenic; MMA, methylarsonic acid; U-As, urinary arsenic; U-Se, urinary selenium.

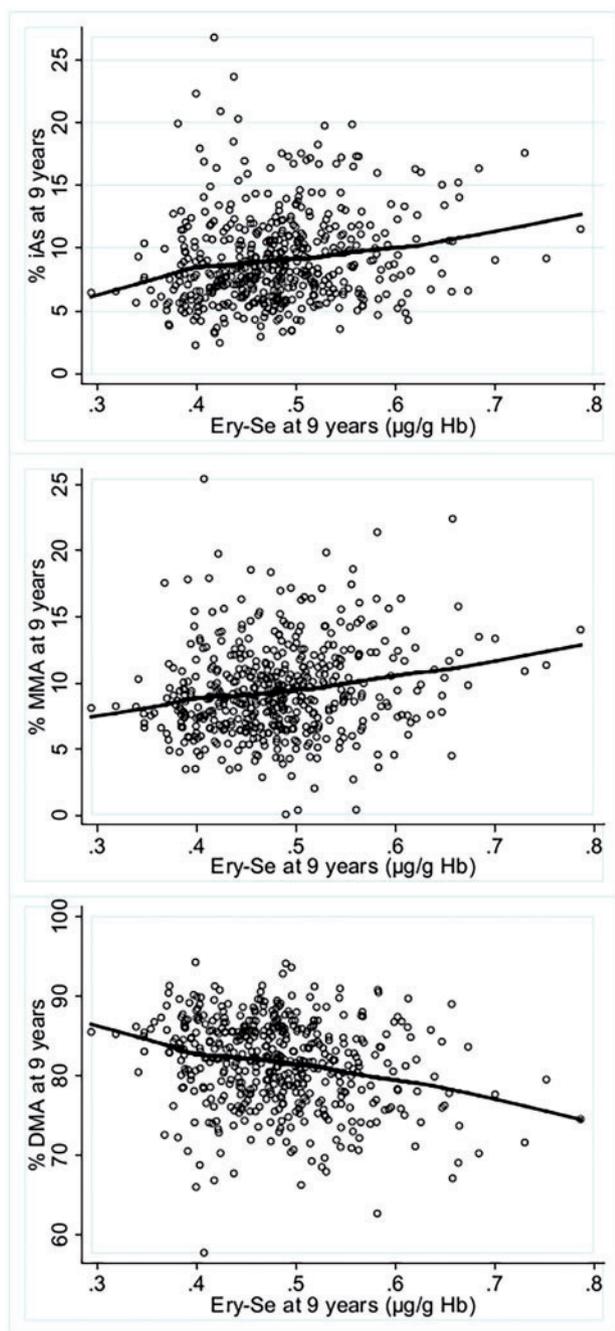


FIG. 3 Scatter plots with smoothed lowess lines for the relative amounts of the arsenic metabolites (%iAs, %MMA and %DMA) and erythrocyte selenium (Ery-Se; $\mu\text{g/g Hb}$) at 9 years of age. Abbreviations: DMA, dimethylarsinic acid; Ery-Se, erythrocyte selenium; Hb, hemoglobin; iAs, inorganic arsenic; MMA, methylarsonic acid.

range: 59–1268 $\mu\text{g/l}$), the increase in %MMA was 0.46 per 100 $\mu\text{g/l}$. When stratifying on U-As at 58 $\mu\text{g/l}$, there were no major differences in the influencing factors for %MMA between strata (Supplementary Table S4).

The children's urinary %DMA was influenced by very much the same factors as the %iAs, although in the opposite direction (Table 3); ie, the %DMA decreased about 3 pp ($=0.6$ SD) when Ery-Se increased from the 5th to the 95th percentile, whereas the corresponding increase in U-Se and plasma folate was associated with an increase in %DMA of about 2 pp ($=0.4$ SD).

Stratifying by U-As at 58 $\mu\text{g/l}$ showed that U-As ($+0.78$ pp per 10 $\mu\text{g/l}$; $P = .005$) and season of sampling (-4.0 pp at monsoon compared to pre-monsoon; $P < .001$) were strong influencing factors mainly in the low U-As stratum, while Ery-Se was the strongest influencing factor above 58 $\mu\text{g/l}$ (-4.1 pp from 5th to 95th percentile of Ery-Se; $P < .001$; Supplementary Table S4).

In the sensitivity analyses, the addition of maternal U-As and U-Se at GW8 to all the models had no large impact on any of the factors associated with children's %iAs, %MMA or %DMA, and neither maternal U-As nor U-Se were by themselves associated with any of the children's metabolites. Replacing U-As in the models with Ery-As showed that this was associated with all metabolites in the same directions and similar strengths as U-As (data not shown). The Dirichlet regression model showed similar results as the linear regression models for each metabolite (Supplementary Table S5).

Additionally, we assessed the factors influencing child U-As and Ery-As (Table 4). This showed that U-As was significantly higher at monsoon season of sampling ($+54$ $\mu\text{g/l}$, $P < .001$) compared to pre-monsoon, and positively associated with Ery-Se (5–95th percentile: $+41$ $\mu\text{g/l}$, $P = .026$; corresponding to 0.3 SD), whereas maternal age was inversely associated with child U-As ($B = -2.4$ $\mu\text{g/l}$ per year; 95% CI: -4.3 , -0.46 ; $P = .015$). For Ery-As, the influencing factors were even more obvious than for U-As (Table 4).

The difference between the concentration of U-As_{tot} (all forms of arsenic) and U-As (iAs + MMA + DMA), observed for some of the children, was not explained by arsenobetaine, as we were unable to detect this in a subsample of children ($n = 20$) from the same study population with a high ratio of U-As_{tot}/U-As. There appeared to be more children with larger difference between U-As_{tot} and U-As in the highest quartile of U-Se (range 18–55 $\mu\text{g/l}$) compared to the lowest (range 2.2–11 $\mu\text{g/l}$; mean difference 5.7 $\mu\text{g/l}$ vs 0.53 $\mu\text{g/l}$ in highest and lowest quartile of U-Se, respectively; $P = .0024$), and the overall positive correlation between U-Se and the ratio U-As_{tot}/U-As was significant, but weak ($r_s = 0.16$, $P < .001$).

DISCUSSION

This study suggests that children's metabolism of iAs differs from that in adults, which might explain the lack of data on arsenic metabolism as a susceptibility factor for arsenic toxicity in children. The main influencing factor for the urinary arsenic metabolites at 9 years of age was the selenium status, assessed as Ery-Se, which unexpectedly was associated with increasing %iAs and %MMA, but decreasing %DMA. This was particularly obvious at higher arsenic exposure levels, where the influence from ingested DMA (with the rice-based diet) had little impact on the urinary metabolite pattern. Plasma folate was associated with lower %iAs and higher %DMA. Factors of minor importance for the children's arsenic methylation included the maternal arsenic metabolite pattern (suggesting genetic predisposition), and maternal education and SES (for %MMA only). Other nutritional markers (zinc, copper, and manganese, Hb, vitamin B12, and child anthropometry) had no apparent impact, and neither had children's age or gender.

The children had higher %DMA and lower %iAs compared to their mothers during pregnancy, as found at younger age (Fangstrom et al., 2009). As the %DMA increases during pregnancy (Gardner et al., 2011b), the differences are even more pronounced when compared to adults in general (Hall et al., 2009; Lindberg et al., 2008a; Sun et al., 2007). Possibly, the number of factors impairing the methylation of iAs to DMA increases with

Table 3 Multivariable-Adjusted Linear Regression Analyses of Children's % Inorganic Arsenic (iAs), % Methylarsonic Acid (MMA), % Dimethylarsinic Acid (DMA), and Influencing Factors (n = 488)

Influencing Factors	%iAs	P value	%MMA	P value	%DMA	P value
Age (years)	-0.96 (-3.8, 1.9)	0.51	2.0 (-0.78, 4.8)	0.16	-0.77 (-5.2, 3.7)	0.73
Stunting	0.065 (-0.66, 0.79)	0.86	0.30 (-0.40, 1.0)	0.40	-0.41 (-1.5, 0.70)	0.47
Pre-monsoon	Reference		Reference		Reference	
Monsoon	2.1 (1.2, 2.9)	<0.001	0.95 (0.14, 1.8)	0.022	-3.1 (-4.4, -1.8)	<0.001
Post-monsoon	0.87 (-0.086, 0.26)	0.33	0.47 (-0.30, 1.2)	0.23	-1.3 (-2.5, -0.11)	0.033
U-As < 58 µg/l	-0.066 (-0.088, -0.044)	<0.001	0.016 (-0.0053, 0.037)	0.14	0.052 (0.019, 0.086)	0.002
U-As ≥ 58 µg/l	-0.00041 (-0.0033, 0.0024)	0.78	0.0046 (0.0018, 0.0073)	0.001	-0.0043 (-0.0087, 0.00014)	0.058
U-Se (µg/l)	-0.11 (-0.16, -0.058)	<0.001	0.0037 (-0.046, 0.053)	0.88	0.11 (0.028, 0.19)	0.008
Ery-Se (µg/g Hb)	7.8 (3.5, 12)	<0.001	6.3 (2.1, 10)	0.003	-14 (-21, -7.2)	<0.001
Folate (µg/l)	-0.22 (-0.35, -0.098)	<0.001	-0.045 (-0.16, 0.075)	0.46	0.26 (0.067, 0.45)	0.008
%metabolite GW8	0.050 (0.018, 0.083)	0.002	0.10 (0.030, 0.18)	0.006	0.081 (0.038, 0.12)	<0.001
SES GW8	0.087 (-0.086, 0.26)	0.33	0.19 (0.027, 0.36)	0.023	-0.28 (-0.55, -0.014)	0.039
Maternal education (years)	-0.042 (-0.14, 0.052)	0.38	0.16 (0.066, 0.25)	0.001	-0.12 (-0.26, 0.029)	0.12
Maternal age (years)	0.018 (-0.036, 0.071)	0.52	0.054 (0.0026, 0.10)	0.039	-0.073 (-0.15, 0.0089)	0.081

Estimates are shown as B (95% CI).

Abbreviations: iAs, inorganic arsenic; DMA, dimethylarsinic acid; Ery-Se, erythrocyte selenium; GW, gestational week; MMA, methylarsonic acid; SES, Socioeconomic status; U-As, Urinary arsenic; U-Se, urinary selenium.

Table 4 Multivariable-Adjusted Linear Regression Analyses of Children's U-As and Ery-As, and Influencing Factors (n = 488)

Influencing factors	U-As	P-value	Ery-As	P-value
Age (years)	85 (-18, 188)	0.11	3.3 (-1.5, 8.1)	0.17
Stunting	-12 (-38, 14)	0.37	-0.55 (-1.8, 0.66)	0.38
Pre-monsoon	Reference		Reference	
Monsoon	54 (24, 84)	<0.001	2.7 (1.4, 4.1)	<0.001
Post-monsoon	7.9 (-20, 36)	0.58	0.33 (-0.99, 1.7)	0.62
U-Se (µg/l)	-1.4 (-3.2, 0.46)	0.14	-0.13 (-0.21, -0.044)	0.003
Ery-Se (µg/g Hb)	177 (22, 333)	0.026	13 (5.4, 20)	0.001
Folate (µg/l)	-2.0 (-6.5, 2.4)	0.37	-0.15 (-0.36, 0.053)	0.15
SES GW8	-0.21 (-6.5, 6.0)	0.95	-0.020 (-0.31, 0.27)	0.89
Maternal education (years)	-2.6 (-5.9, 0.79)	0.13	-0.16 (-0.31, 0.000040)	0.050
Maternal age (years)	-2.4 (-4.3, -0.46)	0.015	-0.11 (-0.20, -0.027)	0.011

Estimates are shown as B (95% CI).

Abbreviations: Ery-As, erythrocyte arsenic; Ery-Se, erythrocyte selenium; MMA, methylarsonic acid; GW, gestational week; SES, Socioeconomic status; U-As, urinary arsenic; U-Se, urinary selenium.

age (Lindberg et al., 2008a), such as smoking (Lindberg et al., 2010) and decreased liver function (Schmucker, 2005). Also, the fact that the metabolite pattern in the children was not markedly influenced by higher exposure levels indicates that their methylation of arsenic is not as sensitive to inhibition as that in adults. For the %MMA, the main susceptibility factor for arsenic toxicity in adults (Lindberg et al., 2008b), the increase in %MMA was 0.46 pp MMA per 100 µg/l in U-As for the presently studied children, compared to 0.78 pp MMA/100 µg/l in adults in the same study area (Lindberg et al., 2008a). A previous study on children of the same study population found a positive association between higher %MMA and growth (Gardner et al., 2013), and another study found no impact of %MMA on the inverse associations between arsenic exposure and children's neurodevelopment (Hamadani et al. 2011), supporting that %MMA is not a measure of susceptibility to arsenic-induced toxicity. There was no difference in the methylation pattern between boys and girls, suggesting hormonal influences later in life (Lindberg et al., 2008a).

The positive associations between Ery-Se and %iAs and %MMA, and inverse association with %DMA, all appeared linear and robust towards additional adjustments. The results are in contrast to previous findings in the mothers (Li et al., 2008) and

other adults (Hsueh et al., 2003; Pilsner et al., 2011), in whom the arsenic metabolite pattern in urine was not influenced by plasma or serum selenium. To note, Ery-Se is considered a more long-term marker of the selenium status than is plasma selenium (Thomson, 2004). The observed associations might be related to competition for methyl groups and glutathione, both involved in the 1-carbon metabolism. Similar to arsenic, selenium is methylated to selenosugars and the trimethylselenonium ion, which are excreted via urine (Francesconi and Pannier, 2004; Jackson et al., 2013; Kuehnelt et al., 2015). One hypothesis is therefore that a poor 1-carbon metabolism would result in lower selenium excretion and higher Ery-Se, as well as lower %DMA. An efficient 1-carbon metabolism and availability of methyl-groups is likely particularly important in growing children to meet the demands for production of e.g. creatine, proteins, phospholipids, and DNA, which might explain the particularly strong association between blood selenium and arsenic metabolism in the children. Another explanation for the inverse association between Ery-Se and %DMA could be that selenium inhibits arsenic methylation through binding to and modification of AS3MT, the main arsenic methyltransferase (Geng et al., 2009). In primary human hepatocytes, the inhibition of arsenic methylation seemed to depend on the selenium species;

selenite exhibited the strongest effect, and this was obvious already at sub- μM concentrations (Walton *et al.*, 2003).

Surprisingly, the urinary excretion of arsenic increased with increasing Ery-Se, despite the fact that Ery-Se was associated with lower %DMA in urine. In adults, a higher %DMA is usually associated with a higher rate of overall arsenic excretion up to a certain exposure level where the arsenic starts inhibiting its own methylation (Vahter, 2002). An increase from the 5th to the 95th percentile in the children's Ery-Se corresponded to 41 $\mu\text{g/l}$ higher U-As, ie, quite a substantial change considering that the median U-As was 53 $\mu\text{g/l}$. On the other hand, Ery-Se showed a strong positive association also with Ery-As (5–95th increase in Ery-Se: +3 $\mu\text{g/kg}$ in Ery-As), why reverse causality cannot be excluded, i.e. that elevated arsenic exposure (high U-As and Ery-As) might alter the selenium kinetics. However, a study in Bangladeshi adults ($n=287$) with quite high arsenic exposure through drinking water found a strong inverse association between plasma selenium (mean $87.6 \pm 1.8 \mu\text{g/l}$) and total arsenic in urine (mean 173 $\mu\text{g/l}$; but no association with the urinary arsenic metabolites) and a weaker inverse association with blood arsenic (at the highest plasma selenium only) (Pilsner *et al.*, 2011). Another study from the same study area found much weaker inverse associations of blood selenium with total urinary arsenic in both adults and children (mean 91 and 78 $\mu\text{g As/l}$, respectively), and surprisingly, the largest decrease was between the lowest quintiles of selenium (George *et al.*, 2013).

The positive association of total U-Se excretion with %DMA, and inverse association with %iAs, were similar to findings in the mothers (unpublished data) and other adults (Hsueh *et al.*, 2003). The association might be explained by coexcretion of methylated metabolites of arsenic and selenium, and, as mentioned above, a similar influence from 1-carbon metabolism. Previously, a similar positive correlation has been observed for %DMA and urinary creatinine, which is produced from creatine, a major product of 1-carbon metabolism through the methylation of guanidinoacetate (Nermell *et al.*, 2008; Peters *et al.*, 2014). Obviously, an efficient 1-carbon metabolism is essential for methylation and excretion of both arsenic (Hall *et al.*, 2009; Marafante and Vahter, 1984) and selenium (Jackson *et al.*, 2013). The positive influence from plasma folate on %DMA is also likely related to the 1-carbon metabolism, as better folate status is important for the remethylation of homocysteine (Bailey *et al.*, 2015).

An antagonistic relationship between arsenic and selenium, where one would reduce the toxicity and affect the biotransformation of the other, has been suggested (Levander, 1977; Pilsner *et al.*, 2011; Zeng *et al.*, 2005). In rats, such a complex $[(\text{GS})_2\text{AsSe}]^-$ has been shown to be transported into bile through the Multidrug Resistance-associated Protein 2 (MRP2) (Kala *et al.*, 2000). The biliary excretion of arsenic in humans is negligible in contrast to certain experimental animals (Anundi *et al.*, 1982; Vahter, 2002), and the existence of this conjugate in humans remains unknown. However, a study on mice have shown that the MRP-family transporters are involved in arsenic elimination through urine (Kala *et al.*, 2004), the major eliminating pathway also in humans. An *in vitro* study recently showed that human embryonic kidney cells transfected with MRP2 can also transport $[(\text{GS})_2\text{AsSe}]^-$ (Carew and Leslie, 2010). Similarly, a study on rat kidney cells found that arsenic and selenium may form a complex in the lysosomes (As_2Se), which was hypothesized to be excreted with urine (Berry and Galle, 1994). Thus, complex formation and excretion via urine could be a mechanism also in humans, although such a complex has never been identified in human urine. We found that more U-As (about 10%) in other form(s) than the measured arsenic metabolites (total arsenic

minus sum of iAs metabolites) was excreted in the highest quartile of U-Se compared to the lowest, possibly suggesting the excretion of an arsenic-selenium complex. As we were unable to detect arsenobetaine in a subsample of children from the same study population (with high ratio total arsenic/sum of metabolites) the difference could not be due to co-excretion of selenium and organic arsenicals commonly occurring in seafood. As we have not explored the potential presence of a selenium-arsenic complex, its presence in the children's urine is merely speculations that need to be addressed further. The absence of arsenobetaine in the children's urine is consistent with a relatively low consumption of fish [37–55 g/d in children living in similar areas (Khan *et al.*, 2009)]. Also, fish collected from a nearby village contained only trace amounts of arsenic (Das *et al.*, 2004), and freshwater fish (most likely the type of fish consumed in the present area) contains less arsenobetaine compared to marine fish (Caumette *et al.*, 2012).

The association between maternal and child metabolite pattern is in line with a genetic influence on the metabolism of arsenic, and previous studies have also shown a family-related arsenic metabolite pattern (Chung *et al.*, 2002; Tellez-Plaza *et al.*, 2013). Arsenic methylation rate is influenced by single nucleotide polymorphisms (SNPs) in the gene encoding for AS3MT, and our previous studies showed that the genotype associated with less efficient arsenic methylation is fairly prevalent among the studied mothers (Engstrom *et al.*, 2013). In a previous study, the arsenic metabolite patterns seemed more similar between siblings than between parents and their children (Chung *et al.*, 2002), which is also in line with the present findings of differences in methylation efficiency between children and adults. Also, the source of arsenic seems to affect the metabolite pattern. At fairly low exposure levels ($<58 \mu\text{g/l}$ in urine), when the ingested arsenic is largely from food (Vahter *et al.*, 2006), %iAs decreased and %DMA increased with increasing exposure, probably because a part of arsenic in rice is present as DMA (Meharg *et al.*, 2008). Indeed, increasing intake of rice has been found to increase the %DMA in urine (deCastro *et al.*, 2014; Kordas *et al.*, 2016). This may also explain why the highest %DMA appeared during the pre-monsoon period, with higher food security (Hillbruner and Egan, 2008). The highest U-As appeared during the monsoon period, possibly due to higher water arsenic concentrations (Bhattacharya *et al.*, 2011) and/or increased use of nearby, shallow wells.

The strengths of our study include the relatively large study sample ($n=488$ mother-child pairs) and the longitudinal design. Also, biomarkers of arsenic and selenium status were analyzed by ICPMS, and we had the possibility to compare different media for each element. Limitations include lack of plasma selenium to assess selenium kinetics, no data regarding other nutrients known to affect the methylation of arsenic, such as homocysteine and vitamin B6, involved in the 1-carbon metabolism, and lack of AS3MT genotype. Also, functional markers of selenium status such as glutathione peroxidases or thioredoxin reductases could have provided information regarding their impact on the arsenic metabolism through the provision of reducing equivalents necessary for the reduction of iAs and its metabolites (Burk and Levander, 2006).

To conclude, children had higher arsenic methylation efficiency than adults, and there was no difference between boys and girls. The main influencing factors were selenium and folate status. The strong influence of selenium is in contrast to that observed in adults, and the mechanisms of the interaction need to be further elucidated. Minor influencing factors were genetic predisposition, SES, and ongoing exposure to arsenic.

The results emphasize the need for considering the dietary intake of different arsenic species when evaluating the arsenic metabolite pattern in urine.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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