Associations between Methylated Metabolites of Arsenic and Selenium in Urine of Pregnant Bangladeshi Women and Interactions between the Main Genes Involved

Helena Skröder,1 Karin Engström,1,2 Doris Kuhehnelt,3 Maria Kippler,1 Kevin Francesconi,3 Barbro Nermell,1 Fahmida Tofail,4 Karin Broberg,1 and Marie Vahter1

1Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
2Division of Occupational and Environmental Medicine, Department of Laboratory Medicine, Lund University, Lund, Sweden
3Institute of Chemistry, NAWI Graz, University of Graz, Graz, Austria
4International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), Dhaka, Bangladesh

BACKGROUND: It has been proposed that interactions between selenium and arsenic in the body may affect their kinetics and toxicity. However, it is unknown how the elements influence each other in humans.

OBJECTIVES: We aimed to investigate potential interactions in the methylation of selenium and arsenic.

METHODS: Urinary selenium (U-Se) and arsenic (U-As) were measured using inductively coupled plasma mass spectrometry (ICPMS) in samples collected from pregnant women (n = 226) in rural Bangladesh at gestational weeks (GW) 8, 14, 19, and 30. Urinary concentrations of trimethyl selenium ion (TMSe) were measured by HPLC–vapor generation–ICPMS, as were inorganic arsenic (iAs), methylarsonic acid (MMA), and dimethylarsonic acid (DMA). Methylation efficiency was assessed based on relative amounts (%) of arsenic and selenium metabolites in urine. Genotyping for the main arsenite and selenium methyltransferases, AS3MT and INMT, was performed using TaqMan probes or Sequenom.

RESULTS: Multivariable-adjusted linear regression analyses indicated that %TMSe (at GW8) was positively associated with %MMA (β = 1.3, 95% CI: 0.5, 2.0) and U-As, and inversely associated with %DMA and U-Se in producers of TMSe (INMT rs6970396 AG + AA, n = 74), who had a wide range of urinary TMSe (12–42%). Also, %TMSe decreased in parallel to %MMA during pregnancy, especially in the first trimester (~0.58 %TMSe per gestational week). We found a gene–gene interaction for %MMA (p-interaction = 0.076 for haplotype 1). In analysis stratified by INMT genotype, the association between %MMA and both AS3MT haplotypes 1 and 3 was stronger in women with the INMT GG (TMSe nonproducers, 5th–95th percentile: 0.2–2%TMSe) vs. AG + AA genotype.

CONCLUSIONS: Our findings for Bangladeshi women suggest a positive association between urinary %MMA and %TMSe. Genes involved in the methylation of selenium and arsenic may interact on associations with urinary %MMA. https://doi.org/10.1289/EHP1912

Introduction

An interaction between arsenic and selenium was first observed in 1938, when feeding of arsenic was found to prevent selenium poisoning in rats (Moxon 1938). Since then, interactions in effects and metabolism of arsenic and selenium have been extensively studied (Levander 1977; Pilsner et al. 2011; Sun et al. 2014; Zwolak and Zaporowska 2012), in particular whether there is any protective effect of selenium on arsenic toxicity. Mechanisms proposed to explain antagonistic effects of selenium on arsenic toxicity include the formation of a complex that increases excretion of both elements through bile (Gailer 2009) and the antioxidative effects of selenium (Sun et al. 2014). A complex of arsenic and selenium in the form of seleno-but(S-glutathionyl) arsinium ion ([GS]2 AsSe) has been identified in the bile of rabbits and rats (Gailer et al. 2002; George et al. 2016); however, to our knowledge, the presence of this complex has not been demonstrated in humans. Because the main excretory route for both arsenic (Vahter 2002) and selenium (Anan et al. 2009) in humans is through urine and not bile, the mechanism for any interaction of toxicological importance between the two elements in humans needs further investigation. Both elements are methylated in the body through the one-carbon metabolism (Jackson et al. 2013; Vahter 2002) in order to facilitate excretion via urine (Suzuki et al. 2005), suggesting a possible pathway for a potential interaction.

In humans, inorganic arsenic (iAs), a potent toxicant and carcinogen that may be present in drinking water and certain foods, is methylated to methylarsonic acid (MMA) and dimethylarsonic acid (DMA), which are excreted in the urine (Vahter 2002). It is well established that the metabolism of arsenic—as measured by the relative distribution of iAs, MMA, and DMA in urine—varies markedly between individuals and populations (Vahter 2002). This can be explained partly by polymorphisms in the arsenic metabolizing genes. Recent genome-wide association studies of Bangladeshi and Argentinean adults identified polymorphisms in the S-adenosylmethionine-dependent arsenite methyltransferase (AS3MT) gene on chromosome 10 as the strongest genetic predictors of variation in arsenic metabolism (Pierce et al. 2012; Schlebusch et al. 2015), a finding further supported in an American Indian population (Balakrishnan et al. 2017). In a series of studies performed in Matlab, in rural Bangladesh, our group has also identified age, gender, body weight, and smoking as predictors of arsenic methylation efficiency (Gardner et al. 2011b; Lindberg et al. 2008, 2010). In addition, we previously reported that the efficiency of arsenic methylation appeared to increase over the course of pregnancy (as indicated by an increase in %DMA and a decrease in %MMA in urine) among women participating in a population-based randomized food and micronutrient supplementation trial (MINIMat: Maternal and Infant Nutritional Interventions in Matlab; Persson et al. 2012). There appeared to be no evidence of modification by AS3MT genotypes on the pregnancy-related changes in arsenic methylation efficiency (Gardner et al. 2012). It is unknown whether these factors also influence the methylation of selenium, and if the pregnancy-related increase in arsenic methylation is accompanied by any change in the methylation of selenium.

The metabolism of the essential element selenium involves an initial reduction to selenide, followed by incorporation into...
multiple selenoproteins, mainly in the form of selenocysteine (Kryukov et al. 2003; Papp et al. 2010; Rayman 2012; Suzuki and Ogra 2002). Different methylated selenium species, especially various selenosugars and the trimethylselenonium ion (TMSe), are the main forms of selenium excreted in urine (Anan et al. 2009), with marked interindividual variations (Kuehnelt et al. 2006). We recently reported such a marked interindividual variation in TMSe excretion among MINIMat women in the Matlab area in Bangladesh, with the relative amount of urinary TMSe being 24 ± 12% (0.76 ± 0.61 μg/L) in about one-third of the women and 1.9 ± 1.1% (0.046 ± 0.038 μg/L) in two-thirds (Kuehnelt et al. 2015). A genome-wide association study performed in the same women identified single nucleotide polymorphisms (SNPs) in the 3'-adenosylmethionine–dependent indolethylamine N-methyltransferase (INMT) gene on chromosome 7 as the strongest predictors of %TMSe in urine.

Previous studies in vitro and in mice have shown that selenium may inhibit arsenic methylation (Kenyon et al. 1997; Styblo et al. 1996; Styblo and Thomas 2001), and we recently found an inverse association between blood selenium and methylation of iAs to DMA (%DMA in urine), and a positive association between total urinary selenium and %DMA, among 488 9-y-old Bangladeshi children who had been born to mothers participating in the MINIMat trial (Skröder Löveborn et al. 2016). Positive associations between total urinary selenium and arsenic methylation have also been reported for study populations of pregnant women in Chile (Christian et al. 2006) and adults in Taiwan (Hsueh et al. 2003). The aim of the present study was to elucidate potential interactions in the methylation of selenium and arsenic in order to further understand the mechanisms involved in reported toxicological interactions.

Materials and Methods

Study Population

The present analysis is based on a subset of pregnant women originally recruited to the MINIMat supplementation trial in pregnancy (Persson et al. 2012). The trial recruited a population-based sample of pregnant women in early pregnancy living in the rural Matlab region in Bangladesh during November 2001 to October 2003. Women were assigned to one of three micronutrient supplementation interventions beginning in gestational week (GW) 14: 30 mg iron plus 400 μg folic acid; 60 mg iron plus 400 μg folic acid; or 30 mg iron and 400 μg folic acid plus 13 additional micronutrients, including 65 μg of selenium in the form of sodium selenite. The present analysis originates from a subset of 500 women who were randomly selected from women recruited during the first year of the trial for additional evaluation of their exposure to arsenic (Valter et al. 2006) and essential elements across pregnancy (Figure 1).

From this group of women, 408 provided blood samples at GW14 and 394 had sufficient high-quality DNA for genotyping. Also, 271 of the women had provided complete series of urine samples analyzed for arsenic species and selenium.

Figure 1. Flow chart of the pregnant Bangladeshi women included in the present study (adapted from Kuehnelt et al. 2015), originating from 500 randomly selected women recruited to the MINIMat trial during the first year of the trial. Note: GW, gestational week.
samples at approximately GW8 (time of pregnancy testing), GW14, GW19, and GW30, and these samples had previously been analyzed for arsenic metabolites (Gardner et al. 2011b). For 228 of these women, there were sufficient archival urine samples to measure urinary TMsSe concentrations. The present evaluation included 226 of the women (Figure 1), after excluding 1 woman missing the date of last menstrual period and 1 with unreasonable arsenic metabolite fractions (no MMA and 94% iAs, which may imply that urine had been diluted with water). These 226 women also had blood selenium and arsenic concentrations measured at GW14 (erythrocyte fraction; Ery-Se and Ery-As; Kippler et al. 2009).

The project was approved by the ethical committees at the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b), and the Regional Ethical Review Board Stockholm, Sweden. The MINIMat trial obtained informed written consent from the pregnant women prior to recruitment (Persson et al. 2012; Rahman et al. 2011). This consent included both the clinical trial and investigations of arsenic and other toxicants. The procedures were in accordance with the Helsinki Declaration.

**Determination of Selenium and Arsenic in Erythrocytes and Urine**

Blood samples were collected in lithium heparin tubes at the four health care facilities in Matlab. Plasma and erythrocytes were separated by centrifugation and frozen. Erythrocyte samples were transported frozen to Karolinska Institutet, Sweden.

The concentrations of Ery-As and Ery-Se were measured at Karolinska Institutet during 2007 by inductively coupled plasma mass spectrometry (ICPMS; Agilent 7700x, Agilent Technologies, Tokyo, Japan) with the collision/reaction cell system in hydrogen mode for selenium and in helium mode for arsenic. Sample collection, preparation [high temperature/pressure acid digestion by Milestone ultraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany)], ICPMS analyses, and quality control have been described previously in detail (Kippler et al. 2009). No samples were below the limit of detection (LOD; 0.70 and 11 L, respectively; Kuehnelt et al. 2015). Selenium in erythrocytes is mainly bound to hemoglobin (Hb) and is therefore expressed as µg Se/g Hb to adjust for differences in hematocrit (Skröder et al. 2015; Stefanowicz et al. 2013). Hemoglobin concentrations were measured in venous blood using a HemoCue photometer (HemoCue AB, Angelholm, Sweden).

Spot urine samples were collected in plastic urine collection cups at GW8, 14, 19, and 30, either at home or at the health care facilities. The urine was transferred to 24-mL plastic bottles, frozen, and transported frozen to Karolinska Institutet. The concentrations of total U-Se were determined during 2012 using ICPMS (Agilent 7700x; Agilent Technologies, Tokyo, Japan) with the collision/reaction cell system in hydrogen mode. The urine samples were diluted 1:10 in 1% nitric acid (prepared from 65% suprapur; Merck, Darmstadt, Germany). Standard solutions for the external calibrations of selenium (Merk VI, Darmstadt, Germany; CPI International, Amsterdam, Netherlands; Ultra Scientific Analytical Solutions, North Kingstown, RI) and internal standards (Ge; CPI International) were prepared fresh in 1% nitric acid before every run. The LOD for U-Se was 0.04 µg/L, and no sample was below this level. Quality control of the ICPMS analyses included commercial reference materials for urine (Seronorm™ Trace Elements Urine 101164 and 101165, and National Institute of Standards and Technology (NIST) Standard Reference Material® 2670a H Toxic Elements in Freeze-Dried Urine), and obtained values (14, 70, and 204 µg/L, respectively) were in general in good agreement with reference values (13.9, 70.1, and 229.5 µg/L, respectively; Kuehnelt et al. 2015).

Urinary concentrations of TMsSe were measured during 2014 at the University of Graz by high-performance liquid chromatography (HPLC) with on-line vapor generation and detection by ICPMS with an Agilent 1,100 HPLC system (Agilent, Waldbronn, Germany) connected to an Agilent 7500ce ICPMS equipped with an Agilent Hydride Generation Accessory and an octopole reaction cell, operated in hydrogen mode (Kuehnelt et al. 2015). The concentrations of TMsSe were calculated by external calibration with aqueous solutions of TMsSe (0.025 to 10 µg Se/L). Interday variability was within 5%, assessed by repeated analysis of a urine sample containing 0.18 µg Se/L from TMsSe. The LOD for TMsSe was 0.01 µg/L, and 9% of all urine samples had concentrations below this value. These values were replaced with LOD divided by the square root of 2. Other U-Se species, mainly selenosugars, were not investigated in this study because these compounds, unlike TMsSe (Zhao et al. 2010; Zheng et al. 2002), have limited stability (Juresa et al. 2006) and the urine samples had been stored frozen at −20°C to −40°C for about 13 y, with occasional thawing.

The urinary concentrations of iAs, MMA, DMA were measured at Karolinska Institutet by HPLC on-line with hydride generation and ICPMS, as described previously (Gardner et al. 2011a, 2011b). We used a Hamilton PRP-X100 anion-exchange column (4.6 mm × 250 mm) for separation of As(III), DMA, MMA, and As(V). For the mobile phase, we used a 20-mM ammonium dihydrogen phosphate buffer with pH 5.8. We did not measure MMA(III) because this is likely to be oxidized to MMA (V) (Francesconi and Kuehnelt 2004). The sum metabolite concentration (U-As) was used as a measure of exposure to iAs, whereas the fractions of the different metabolites in relation to total metabolite concentration (%) were used as measures of arsenic methylation efficiency (Vahter 2002). Quality control included comparison with other analytical methods (Lindberg et al. 2007) and analyses of reference material CRM no. 18 (National Institute for Environmental Studies, Ibaraki, Japan) with a certified DMA concentration of 36 ± 9 µg/L. The obtained concentration of DMA was 41 ± 3.4 µg/L (mean ± SD; n = 18; Gardner et al. 2011b).

All urinary concentrations were adjusted for specific gravity (SG) using the formula C_adjusted=C_measured × (SG_average−1)/ (SG_sample−1) (Nermell et al. 2008). The average specific gravity across pregnancy for the women in the present study was 1.010.

**Genotyping of INMT and AS3MT**

Genotyping was performed as previously described (Engström et al. 2011; Kuehnelt et al. 2015). In brief, genomic DNA was isolated from peripheral blood using the QIAGEN DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping for INMT SNPs (rs6970396, rs1061644, both in the 3’ untranslated region of INMT, and rs4270015, a phenylalanine-cysteine exchange) was performed by real-time PCR on a ABI 7,900-HT instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan probes (Assay ID C_29402264_10; Applied Biosystems), followed by allelic discrimination (Kuehnelt et al. 2015). Genotyping for eight AS3MT SNPs (listed below) was performed using Sequenom™ (San Diego, CA, USA) technology by Swegene’s DNA Facility at Malmö University Hospital, Malmö, Sweden (Engström et al. 2011). All of the AS3MT SNPs are noncoding except for rs11191439, which is a methionine to threonine substitution. Linkage disequilibrium (LD) analysis was performed using Haploview (Barrett et al. 2005), and AS3MT haplotypes were inferred by PHASE software (Stephens and Donnelly 2003). Deviations from Hardy-Weinberg equilibrium were tested using chi-square (χ-square) analysis. None of the SNPs in the
The three INMT SNPs were in complete linkage disequilibrium (LD) in the study population (Kuehnelt et al. 2015), and for the present analysis we report data for rs6970396 only. In addition, we evaluated four AS3MT haplotypes identified in a previous study of 361 MINIMat participants (overlap with present study n = 226; Engström et al. 2011), with the following sequences (listed 5’ to 3’ for rs7085104, rs3740400, rs3740393, rs3740390, rs1191439, rs1191453, rs101478835, and rs1046778, respectively): haplotype 1 (AAGGTGTT), haplotype 2 (GCCATCAC), haplotype 3 (ACGGTTAC), and haplotype 4 (GCCGCTAT). Increasing copies of haplotype 1 (women had either no or, one, or two copies) were associated with higher %MMA, whereas the opposite was found for haplotype 3 (also no, one, or two copies). Women with one copy of haplotype 4 had higher %iAs and lower %DMA, compared with women with no copies (no woman had two copies of haplotype 4). In contrast, for haplotype 2, women with one or two copies had lower %iAs and higher %DMA compared with women with no copies (Engström et al. 2011).

Statistical Analyses

Statistical analyses were performed using Stata (version 12; Statacorp). Analyses were performed for all women combined and stratified according to INMT rs6970396 genotypes when appropriate. We classified women with AG or AA genotypes as TMSe “producers” and those with the GG genotype as “nonproducers” based on our previous study showing that 99% of the same MINIMat women with urine %TMSe ≥4 had INMT rs6970396 AG or AA (Kuehnelt et al. 2015).

For assessment of differences in characteristics between producers and nonproducers, and when assessing associations between continuous and categorical variables, we used Wilcoxon signed-rank test, Kruskal-Wallis, and Fisher’s exact test, whereas associations between continuous variables were assessed using Spearman correlation coefficients (rS). p-Values of <0.05 were considered statistically significant. We also used box plots to visually compare distributions of %TMSe and %MMA according to INMT genotype and copies of AS3MT haplotypes. All evaluations of potential associations between methylated selenium and arsenic metabolites were performed on data in early pregnancy (GW8), as arsenic methylation efficiency has been shown to change during pregnancy (Gardner et al. 2011b), which we also found for TMSe (see below). Also, GW8 was prior to the micronutrient supplementation, which started in GW14.

We used multivariable linear regression analyses to identify predictors of GW8 %TMSe in the study population. Because the nonproducers had very small variation in urinary %TMSe, influencing factors were not evaluated in these women. All models included GW8 U-As, GW8 U-Se, plus either %iAs, %MMA, or %DMA (also GW8) in separate models. The models did not include factors that were not statistically significant predictors of %TMSe in a previous analysis of the same MINIMat participants, that is, body mass index (BMI), age, socioeconomic status (SES), or Ery-Se (Kuehnelt et al. 2015). To determine whether the associations were confounded by U-As, we repeated these analyses after excluding U-As. To evaluate to what extent the presence of the two distinct groups of %TMSe (producers and nonproducers) confounded the associations, we also repeated the analyses with all women (not only producers of TMSe), including also the INMT genotype (GG or AG + AA).

In addition, we performed statistical testing of interactions between INMT genotypes [AG + AA (producers), or GG (nonproducers)] and AS3MT haplotype copy number (no, one, or two copies for haplotypes 1–3, no or one for haplotype 4) by including haplotype copy number in the models and adding a product interaction term to each respective model (significant interactions at p <0.10). Finally, we modeled %TMSe and INMT genotype stratified on copy numbers of all the AS3MT haplotypes (adjusting for U-As, %MMA, and U-Se).

We also performed linear regression models of GW8 %MMA, %DMA, and %iAs, respectively (as dependent variables) with GW8 U-Se, U-As, %TMSe, and AS3MT haplotype copy numbers as predictors. We included the haplotypes (1–4) in separate models, and for each metabolite we included the haplotype associated with differences in that particular metabolite according to a previous study, including some of the same MINIMat women as those in the present study (Engström et al. 2011). Thus, when assessing %iAs and %DMA, we included copy number of haplotypes 2 and 4 (separate models), and when assessing %MMA, we included haplotypes 1 and 3. We performed statistical tests of interactions between INMT genotypes (AG + AA, or GG) and AS3MT haplotype copy number by including INMT genotype in the model, as well as a product interaction term, and stratified the analyses on producers and nonproducers of TMSe.

We assessed how the %TMSe changed across pregnancy with linear mixed effects models using maximum likelihood estimation, with %TMSe as the dependent variable and gestational week as the predictor (adjusting for %MMA, U-As, and U-Se). Also this analysis was restricted to producers (INMT AA + AG) because there was essentially no variation in %TMSe among the nonproducers. The scatter plot of %TMSe over time among producers suggested a nonlinear trend, which was why we included a spline knot at GW13. This was the knot used in a previous MINIMat study assessing changes in arsenic methylation efficiency over pregnancy (Gardner et al. 2011b), and visual examination of the plot for %TMSe suggested that this was the GW after which the decrease flattened out. We compared the slopes before and after GW13 using the Wald test. In addition, we used scatter plots to compare %TMSe and %MMA according to the GW, as well as U-Se across pregnancy. We also used mixed effects linear regression to assess the average change in U-Se across pregnancy among producers and nonproducers separately (adjusting for %MMA, U-As, and %TMSe).

Finally, we compared in silico the amino acid sequences in INMT (UniProt accession number O95050) and AS3MT (Q9BBK9) using EMBOSS needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/index.html). The identity (%) is calculated by multiplying the number of perfect matches in the sequence pair by 100 and dividing by the length of the aligned region, including gaps. Similarity (%) also considers the degree of similarity of amino acids to one another.

Results

General Characteristics

The main characteristics of the women are summarized in Table 1, together with concentrations of total selenium and arsenic in blood and urine, and methylated selenium (TMSe) and arsenic (MMA and DMSA) metabolites in urine. The women were 14–44 y of age, 33% had a BMI <18.5 kg/m², and <5% had a BMI >25 kg/m². In total, 67% of the women were carriers of INMT rs6970396 GG (associated with low %TMSe), 28% carried AG, and the remaining about 4% carried AA. Because few women had AA, all evaluations have been done on AG + AA combined (producers of TMSe), compared with GG (nonproducers). The most common AS3MT haplotype was haplotype 1 (77% with either one or two copies; associated with higher %MMA), and the least common was haplotype 4 (11% with one copy, no woman with two copies). For both haplotype 2 and 3, there were about 30% with one or two
Table 1. Characteristics of the studied women (n = 226) classified as producers (carriers of INMT rs6970396 genotype AG or AA) or nonproducers of TMSe (INMT GG).

| Characteristic | TMSe producers (n = 74) | Nonproducers (n = 152) | p-Valuea |
|----------------|------------------------|------------------------|----------|
| Age (y)        | 27 ± 6.3               | 27 ± 6.2               | 0.49     |
| Height (cm)    | 150 ± 5.3              | 150 ± 4.2              | 0.67     |
| Weight at GW8 (kg) | 45 ± 6.2          | 45 ± 6.8               | 0.85     |
| Parity (n births) | 1.6 ± 1.3             | 1.6 ± 1.5              | 0.55     |
| Hemoglobin GW14 (g/L) | 117 ± 13             | 116 ± 11               | 0.98     |
| Ery-As GW14 (µg/kg) | 11 ± 13            | 9.1 ± 8.0              | 0.78     |
| Ery-Se GW14 (µg/g Hb) | 0.54 ± 0.10       | 0.56 ± 0.36            | 0.59     |
| U-As GW8 (µg/L)  | 7.4 ± 5.5              | 7.3 ± 3.6              | <0.001   |
| TMSe GW8 (µg/L)  | 1.9 ± 1.1              | 0.055 ± 1.2            | <0.001   |
| TMSe GW8 (%)    | 24 ± 12                | 0.08 ± 0.5             | <0.001   |
| iAs GW8 (µg/L)  | 139 ± 176              | 11 ± 12               | <0.001   |
| iAs GW8 (%)     | 24 ± 34                | 21 ± 13               | 0.45     |
| MMA GW8 (µg/L)  | 15 ± 6.0               | 15 ± 9.5              | 0.54     |
| TMSe (µg/L)     | 11 ± 3.5               | 11 ± 4.4              | 0.77     |
| DMA GW8 (µg/L)  | 97 ± 115               | 97 ± 99               | 0.43     |
| DMA GW8 (%)     | 74 ± 7.7               | 74 ± 10               | 0.98     |
| TMSe (%)        | 18/33/23               | 34/88/30              |          |
| TMSe-1 (%)      | 56/16/2                | 107/40/5              |          |
| TMSe-2 (%)      | 51/21/2                | 99/50/3               |          |
| TMSe-4 (%)      | 69/5                   | 132/20                |          |

Note: DMA, dimethylarsinic acid; Ery-As, erythrocyte arsenic; Ery-Se, erythrocyte selenium; GW, gestational week; iAs, inorganic arsenic; MMA, methylarsonic acid; TMSe, trimethylselenonium ion; U-As, urinary arsenic; U-Se, total urinary selenium.

aWilcoxon’s signed rank test for differences between TMSe producers and nonproducers.

bInversely with %DMA (p = 0.001) and %MMA, with stronger correlations among TMSe producers than in the nonproducers who had very small variations in TMSe (Table 2). The correlations based on all women were also weaker than for the producers, indicating that the clustering of both TMSe (micrograms per liter) and %TMSe in producers (high levels) and nonproducers (low levels) markedly influenced the correlations (Table 2). Therefore, the association between %TMSe and the arsenic metabolites has been evaluated in producers.

When comparing women with different number of copies of the AS3MT haplotypes, we found no significant difference in urinary %TMSe (all women, Kruskal-Wallis p = 0.29, 0.68, 0.22, and 0.73, respectively). However, the association between the copy number of AS3MT haplotype 1–4 and %MMA seemed to differ between the INMT genotypes (Figure 2, left panel). In contrast, the association between INMT genotype and %TMSe looked similar regardless of AS3MT haplotype copy number (Figure 2, right panel).
Multiple Linear Regression Analyses

In the producers (INMT AG + AA), the associations with %TMSe were positive for %MMA ($\beta = 1.3$, 95% confidence interval (CI): 0.56, 2.0] and U-As ($\beta = 0.026$, 95% CI: 0.012, 0.040), and inverse with U-Se ($\beta = -0.53$, 95% CI: -0.92, -0.15; Table 3, Model 1). Excluding U-As from the model increased the estimate for %MMA from 1.3 to 2.0 (95% CI: 1.3, 2.7; Table 3, Model 2), suggesting that U-As partly explained the association between %TMSe and %MMA. In support, the associations between %TMSe and %DMA and %iAs also increased by excluding U-As. Including all women (see Table S2) resulted in markedly weaker associations (%MMA: $\beta = 0.25$, 95% CI: 0.027, 0.47; U-As: $\beta = 0.016$, 95% CI: 0.0099, 0.022; U-Se: $\beta = -0.29$, 95% CI: -0.48, -0.098), supporting confounding from clustering of TMSe in two distinct groups.

Figure 2. Box plots for %MMA (left panel) and %TMSe (right panel; n = 226) at gestational week 8 by copy number for AS3MT haplotypes 1, 2, 3, and 4 (0, 1, or 2 copies for haplotypes 1–3; no or one copies for haplotype 4) and INMT rs6970396 genotype (GG or AG + AA). The bottom and top of each box represents the 75th and 25th percentile (interquartile range), respectively, and the line inside the box represents the median. Whiskers represent the smallest and largest values within the interquartile range*1.5. Note: MMA, methylarsonic acid; TMSe, trimethylselenonium ion.
To further evaluate factors that predict %TMSe and %MMA, we included a product interaction term for INMT genotype (GG or AG + AA) and AS3MT haplotype copy number (no, one, or two for haplotypes 1–3; no or one for haplotype 4) in models of %TMSe and %MMA, respectively, that also included U-As, U-Se, and either %TMSe (for models of %MMA) or %MMA (for models of %TMSe). When assessing %TMSe as the dependent variable, we found no significant interactions between INMT and the AS3MT haplotypes (haplotype 1–4; \( p = 0.13, 0.60, 0.69, \) and 0.27, respectively). When stratifying the models by AS3MT haplotype copy number (Table 4; see also Tables S3–S5), we found that %TMSe was higher among carriers of AG + AA, compared with those with GG, in all strata.

When assessing %MMA as the dependent variable, we found a significant interaction for haplotype 1 (\( p = 0.076 \)), but not for haplotypes 2–4 (\( p = 0.23, 0.17, \) and 0.33, respectively). For %MMA, we modeled associations with haplotypes 1 and 3 (separate models) stratified by INMT (i.e., GG vs. AG + AA), whereas for %iAs and %DMA, we included haplotypes 2 and 4 (separate models). This showed that the estimates (prediction of %MMA) for each increase in haplotype copy number were much larger among the nonproducers compared with the producers (\( p \)-values close to 0), and followed the expected trend (i.e., stronger estimates for two copies compared with one, in the direction expected from data by Engström et al. 2011) only among the nonproducers. We found no significant interaction between INMT genotype and AS3MT haplotype copy number when assessing %iAs and %DMA, although the stratified analyses indicated similar patterns as those for %MMA, that is, stronger associations in the expected direction for %iAs and %DMA with AS3MT haplotype copy number (haplotype 2 and 4, chosen due to stronger associations with %iAs and %DMA) among nonproducers of TMSe (see Tables S6–S7).

**TMSe, U-Se, and Arsenic Metabolites across Pregnancy**

The mixed effects linear spline regression showed that %TMSe decreased across pregnancy among the producers (Figure 3), and that the main part of the decrease occurred in early pregnancy. On average, the %TMSe decreased by \(-0.58\) percentage points per gestational week (95% CI: \(-0.98, -0.17\)) up to GW13. After GW13, the decrease was much weaker (\( \beta = -0.035, 95\% \) CI: \(-0.15, 0.080\); \( p \) for difference between estimates = 0.022). Still, %TMSe was much higher in late pregnancy in producers (median 18% TMSe) than in the nonproducers, in whom the small amounts of TMSe remained fairly constant across pregnancy (median %TMSe at GW8, 14, 19, and 30: 0.50, 0.60, 0.62, and 0.70, respectively). The decrease in %TMSe among the producers seemed to follow that of %MMA (Figure 3).

As for U-Se, the mixed effects linear regression analyses adjusted for %TMSe, %MMA, and U-As showed a slight increase in U-Se across pregnancy among both producers (per gestational week: \( \beta = 0.011, 95\% \) CI: \(-0.053, 0.074\)) and nonproducers (\( \beta = 0.085; 95\% \) CI: 0.049, 0.12; Figure 4).

**Bioinformatic Analyses**

When comparing the amino acid sequences of INMT and AS3MT, we found small similarity (identity 12.9% and similarity 21.9%). There were limited similarities in the 5'-adenosylmethionine binding domains: five of seven active site positions binding with 5'-adenosyl-homocysteine in INMT were identical in AS3MT (http://www.rcsb.org/pdb/explore/explore.do?structureId=2a14). There were no clear similarities between the selenium and arsenic binding domains; only two of the four conserved cysteine residues in AS3MT, required for arsenic methylation (Ajees and Rosen 2015) were identical in INMT (Figure 5).

**Discussion**

This study shows a positive association between early pregnancy %TMSe and %MMA in urine, in spite of the fact that their methylation is catalyzed by different methyltransferases with little sequence similarities; TMSe by INMT (Kuehnelt et al. 2015) and iAs and MMA largely by AS3MT (Engström et al. 2011;...
Schlebusch et al. 2015; Pierce et al. 2012). The association between %TMSe and %MMA (GW8) was stronger among producers of TMSe (A-allele carriers of \textit{INMT} rs6970396), who had more variation in %TMSe (5th–95th percentile: 12–42%) than nonproducers (5th–95th percentile: 0.21–2.0%). The total variation in GW8 %MMA was 4–19% (all women), with a weak impact of the \textit{AS3MT} genotype (model $R^2$ = 0.26–0.30, Table 5) compared with that of the \textit{INMT} genotype for %TMSe (model $R^2$ = 0.78; see Table S2). To note, the %DMA, the endproduct of human methylation of inorganic arsenic, decreased with increasing %TMSe. Another similarity between %MMA and %TMSe was the parallel decrease in early gestation, whereas %DMA increased with increasing gestational week, as shown previously (Gardner et al. 2011b). Because U-Se increased across pregnancy, it remains to be elucidated which other selenium metabolites are induced by pregnancy and excreted in urine.

A new finding is the significant interaction between \textit{INMT} and \textit{AS3MT} genotypes for %MMA in urine. We found limited sequence similarities between \textit{INMT} and \textit{AS3MT}, indicating inability of the proteins to methylate each other’s substrates. Instead, the associations between \textit{AS3MT} haplotype 1 and 3 and %MMA (and between haplotype 2 and 4 and %iAs and %DMA, as indicated in Tables S6–S7) were much more pronounced in nonproducers of TMSe (GG carriers of \textit{INMT} rs6970396) than in producers. This gene–gene interaction is supported by our recent evaluation of urinary TMSe in women in the Andean part of Argentina who were previously found to have an unusual \textit{INMT}–\textit{AS3MT} gene interaction is supported by our recent evaluation of urinary TMSe in women in the Andean part of Argentina who were previously found to have an unusual \textit{AS3MT} genotype, resulting in an efficient methylation of iAs to DMA (and lower %MMA in urine) compared with most other populations studied (Vahter et al. 1995, Engström et al. 2011; Schlebusch et al. 2015). We found that these women have an exceptionally high frequency of the \textit{INMT} rs6970396 G-allele (98.5%; Kuehnelt et al. 2015), and essentially all of the studied individuals lacked TMSe in the urine. The mechanism for this interaction is not known. It can be speculated that formation of TMSe influences the regulation of \textit{AS3MT} expression, but future studies in this area are warranted.

Also gene–environment interactions might have contributed to our results. Very high arsenic exposure (85 mg/L in drinking water) of pregnant mice was shown to reduce the expression of \textit{INMT} in the liver of male fetuses (Liu et al. 2007). However, in the present study with much lower arsenic exposure levels, we found a positive association between U-As and urinary TMSe.

Walton et al. (2003) showed that selenite inhibited As(III) methylation via inhibition of \textit{AS3MT} in rat and human hepatocytes in vitro (Walton et al. 2003). Song et al. (2010) suggested, based on their observations in a cell-free assay, that selenite can modify the structure and activity of AS3MT through the formation of adducts (RS–Se–SR) with cysteine residues in AS3MT, subsequently inhibiting arsenite methylation. However, it is hard to...
understand how adduct formation of selenium would be occurring only in producers of TMSe. Whether arsenic also interacts with INMT, or selenite inhibits AS3MT in humans in vivo, should be the focus of additional research.

Both %MMA and %TMSe in urine increased with increasing arsenic exposure (Table 3), and U-As predicted part of the association between %TMSe and both %MMA (positive) and %DMA (inverse). Still, these latter associations were significant when adjusted for U-As. This similarity between %MMA and %TMSe might be related to the one-carbon metabolism, through which both selenium and arsenic are methylated, using S-adenosylmethionine as the methyl donor (Jackson et al. 2013; Marafante et al. 1985), prior to excretion in urine. The urinary %MMA is known to increase with increasing arsenic exposure, possibly due to inhibition of the further methylation of MMA to DMA (Vahter 2002). Thus, it is possible that arsenic also inhibits the biotransformation of selenium to other metabolites, for example, the more complex selenosugars (also methylated), the main urinary form of selenium (Kobayashi et al. 2002), leading to an increase in the percentage of TMSe.

Similarly, U-Se was positively associated with %MMA in the linear regression analyses, especially in the producers. Indeed, selenium has been shown to decrease the methylation of iAs to DMA in animals and in vitro (Kenyon et al. 1997; Styblo et al. 1996; Styblo and Thomas 2001; Walton et al. 2003), and we recently found a positive association between Ery-Se and both %iAs and %MMA (and a corresponding inverse association with %DMA) in the urine of 9-year-old Bangladeshi children (n = 488; Skröder Löveborn et al. 2016). Thus, it is possible that the positive association between %MMA and %TMSe is also partly due to inhibition of the methylation of MMA to DMA by selenium, but this needs to be evaluated experimentally. To note, the complex between arsenic and selenium, [(GS)2AsSe] that has been detected in bile from rabbits and rats (Gailer et al. 2002) and also in vitro by mixing arsenite and selenite (Manley et al. 2006) has never been identified in humans; however, to our knowledge, no one has attempted to identify it. Since the main excretory route for arsenic in humans is through urine (Vahter 2002), it seems unlikely that an arsenic–selenium complex excreted through bile would have any significant impact on human arsenic retention.

It is now well documented that appreciable amounts of TMSe is present in the urine of only some people, that is, those with INMT rs6970396 AG or AA (Kuehnelt et al. 2015; Lajin et al. 2016b), and not only at elevated selenium intake as once believed (EFSA 2014). One-third of the women in the present study excreted TMSe in urine (6–73% of total urinary Se), in spite of fairly low selenium status. About 60% of a subsample of women enrolled in the MINIMat trial had plasma selenium of <60 μg/L (n = 89, of whom 55 were included in the present study; Li et al. 2008), indicating deficiency (Fairweather-Tait et al. 2011). Furthermore, the above mentioned Andean women, who did not produce much TMSe, appeared to have adequate selenium status with an average blood selenium concentration of 180 μg/kg and an average urinary concentration of 28 μg/L (n = 54 and 83, respectively; Kuehnelt et al. 2015). The main predictor for TMSe in the studied Bangladeshi women was polymorphisms in INMT (Kuehnelt et al. 2015; see also Table S2), and the production appeared to be independent of general nutrition, assessed as BMI, SES, as well as selenium status (Ery-Se; Kuehnelt et al. 2015). Interestingly, the %TMSe in urine decreased with increasing U-Se, although the production of TMSe (μg/L) appeared to increase. This would indicate that the excretion of other metabolites (probably selenosugars) increases at higher rates than TMSe with increasing selenium intake. This was recently found in healthy adults (Lajin et al. 2016a) as well as in cancer patients (Kuehnelt et al. 2007), where administration of selenite or selenomethionine lead to lower proportions of TMSe in relation to the selenosugars (i.e., lower %TMSe) in urine, although the concentrations of TMSe increased. The factors that might influence the proportions of TMSe and selenosugars in urine need to be further elucidated.

Strengths of the present study include the fairly large fraction of TMSe producers, multiple sampling times during pregnancy, and the concentrations of TMSe in the urine being analyzed by HPLC/VG/ICPMS, which allows selective determination of the TMSe with an LOD of 0.01 μg Se/L. Also, the exposure to arsenic through drinking water and rice varied greatly, with concentrations in drinking water ranging from 0.02 to 882 μg/L, and in rice from 56 to 316 μg/kg dry weight (Kippler et al. 2016). A limitation is the lack of data regarding concentrations of different selenosugars in urine—the urinary samples had been frozen at −20°C to −40°C since 2002–2004, and thus the selenosugars had probably degraded. Also, we could not evaluate associations between TMSe and plasma selenium, or interactions between plasma selenium and arsenic metabolism because very few women had plasma selenium measured. Finally, this study included only pregnant women. Because the arsenic methylation has been shown to be more efficient in women (Lindberg et al. 2008), it would be of interest to investigate whether this is also the case for methylation of selenium and to broaden this research to men and nonpregnant women with a wider age range.

To conclude, we observed a strong positive association between %TMSe and %MMA in urine and several common predictors such as urinary concentrations of arsenic and selenium, gestational week, and genetics. In particular, we found an interaction between AS3MT and INMT for the production of the highly

Table 5. Linear regression analysis of associations between %MMA and AS3MT haplotypes 1 and 3 (adjusted for U-Se, U-As, and %TMSe) among Bangladeshi women in gestational week 8 (n = 226), stratified by TMSe producers (INMT rs6970396 genotype AG + AA) and nonproducers (rs6970396 GG).

| Haplotype 1 | Haplotype 3 |
|-------------|-------------|
| Predictors  | TMSe producers | TMSe nonproducers | TMSe producers | TMSe nonproducers |
|             | [β (95% CI); p-Value] | [β (95% CI); p-Value] | [β (95% CI); p-Value] | [β (95% CI); p-Value] |
| Total U-Se (μg/L) | 0.15 (0.030, 0.27) 0.015 0.073 (−0.10, 0.25) 0.41 | 0.16 (0.042, 0.28) 0.009 0.042 (−0.13, 0.21) 0.63 |
| U-As (μg/L) | 0.0060 (0.001, 0.011) 0.011 0.0122 (0.008, 0.017) <0.0001 | 0.0600 (0.015, 0.010) 0.010 0.0133 (0.008, 0.017) <0.0001 |
| %TMSe | 0.12 (0.052, 0.19) 0.001 0.56 (0.26, 1.5) 0.006 | 0.12 (0.052, 0.19) <0.0001 0.77 (0.17, 1.4) 0.011 |
| INMT haplotype copies | Reference | Reference | Reference | Reference |
| 0 | 0.67 (−0.98, 2.3) 0.42 1.8 (0.33, 3.3) 0.018 | Reference |
| 1 | 0.0012 (−1.18, 1.8) >0.99 2.0 (0.16, 3.9) 0.033 | Reference |
| 2 | −0.87 (−4.8, 3.0) 0.66 −4.4 (−8.7, −0.11) 0.045 | 0.076 |

Note: MMA, monomethylarsonic acid; TMSe, trimethylselenonium ion; U-As, urinary arsenic; U-Se, total urinary selenium.

*p-Value derived from including a product interaction term for INMT genotypes (AG + AA, or GG) and AS3MT haplotype copy number (0, 1, or 2 copies for haplotypes 1–3; 0 or 1 for haplotype 4).
toxic MMA metabolite, with stronger associations between AS3MT haplotypes 1 and 3 and %MMA in nonproducers of TMSe (INMT genotype AG + AA). More research is needed to understand the underlying mechanisms and pathways. It also remains to be elucidated whether the variations in selenium metabolism may influence the selenium status in terms of plasma selenium, expression and function of selenoproteins, and health effects of selenium intake (both low and high) as previously shown for arsenic through, for example, higher susceptibility to toxicity in individuals with higher percentages of MMA (de la Rosa et al. 2017).

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