Association Between Variants in Arsenic (+3 Oxidation State) Methyltranserase (*AS3MT*) and Urinary Metabolites of Inorganic Arsenic: Role of Exposure Level

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ABSTRACT

Variants in *AS3MT*, the gene encoding arsenic (+3 oxidation state) methyltransferase, have been shown to influence patterns of inorganic arsenic (iAs) metabolism. Several studies have suggested that capacity to metabolize iAs may vary depending on levels of iAs exposure. However, it is not known whether the influence of variants in *AS3MT* on iAs metabolism also vary by level of exposure. We investigated, in a population of Mexican adults exposed to drinking water As, whether associations between 7 candidate variants in *AS3MT* and urinary iAs metabolites were consistent with prior studies, and whether these associations varied depending on the level of exposure. Overall, associations between urinary iAs metabolites and *AS3MT* variants were consistent with the literature. Referent genotypes, defined as the genotype

previously associated with a higher percentage of urinary dimethylated As (DMAs%), were associated with significant increases in the DMAs% and ratio of DMAs to monomethylated As (MAs), and significant reductions in MAs% and iAs%. For 3 variants, associations between genotypes and iAs metabolism were significantly stronger among subjects exposed to water As >50 versus ≤50 ppb (water As X genotype interaction $P < .05$). In contrast, for 1 variant (rs17881215), associations were significantly stronger at exposures ≤50 ppb. Results suggest that iAs exposure may influence the extent to which several AS3MT variants affect iAs metabolism. The variants most strongly associated with iAs metabolism—and perhaps with susceptibility to iAs-associated disease—may vary in settings with exposure level.

Chronic exposure to inorganic arsenic (iAs) has been associated with increased risk of several types of cancer, with a substantial literature suggesting iAs exposure may also be associated with other health outcomes, including cardiovascular diseases and diabetes (Iarc Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012; Maull et al., 2012; Moon et al., 2012; Sung et al., 2015). There are multiple sources of exposure to iAs, including contaminated drinking water, food, soil, and air, as well as occupational settings (Iarc Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Contaminated drinking water is a common source of high exposure, and is a widespread public health problem, estimated to affect around 140 million people worldwide (Iarc Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012; Smith et al., 2000). There is growing evidence that, along with levels of exposure to iAs, inter-individual variation in the capacity to metabolize iAs is an important determinant of toxicity, and thus of health risks related to this exposure (Pierce et al., 2013; Sun et al., 2007). Using measures of proportions of iAs metabolites in urine, which have been postulated to reflect capacity to metabolize iAs, numerous studies suggest that individual variation in patterns of iAs metabolism may influence susceptibility to adverse health outcomes among subjects exposed to iAs in drinking water (Chen et al., 2013; Mendez et al., 2015), or may be directly associated with health risks (Del Razo et al., 2011; Huang et al., 2007; Kuo et al., 2015; Lindberg et al., 2008; Sun et al., 2007). Although the indicators of iAs metabolism most strongly related to risk have varied, associations between measures of iAs metabolism and health risks have been reported in settings with widely varying levels of iAs exposure (Chen et al., 2005, 2013; Del Razo et al., 2011; Engstrom et al., 2015; Lindberg et al., 2008; Mendez et al., 2015).

In humans, the primary pathway for metabolism of iAs involves sequential methylation to form monomethylated As (MAs) and dimethylated As (DMAs) metabolites, which are excreted in the urine (Thomas et al., 2007). Higher percentages of total urinary As represented by DMAs (DMAs%), and lower percentages of MAs or the unmethylated iAs (MAs% and iAs%, respectively) in urine have been hypothesized to be indicators of higher capacity to metabolize iAs (Thomas et al., 2007; Tseng, 2007; Vahter, 2002). The ratios of MAs to iAs (MAs/iAs) and of DMAs to MAs (DMAs/MAs) in urine have also been widely used as indicators of capacity for the first and second methylation steps. However, the measures of iAs metabolism most predictive of increased health risks remain to be established, given the conflicting associations reported in recent studies (Chen et al., 2013; Del Razo et al., 2011; Mendez et al., 2015; Nizam et al., 2013; Thomas et al., 2007).

Arsenic (+3 oxidation state) methyltrasferase (AS3MT) is a key enzyme in the pathway for the methylation of iAs, and variants in the AS3MT have been shown to be associated with inter-individual differences in iAs metabolism (Antonelli et al., 2014; Fu et al., 2014; Gribble et al., 2015; Pierce et al., 2012; Schlebusch et al., 2015; Wood et al., 2006). Previous studies have linked polymorphic sites in this gene to significant differences in urinary measures of iAs metabolism in various populations (Antonelli et al., 2014; Fu et al., 2014; Gao et al., 2015; Gribble et al., 2015; Pierce et al., 2012; Wood et al., 2006). It has been suggested that iAs exposure level may modify iAs metabolism, as reflected by changes in urinary As methylation profiles, with a shift in the proportions of urinary metabolites among persons exposed to levels approximately >50 versus ≤50 ppb (Kile et al., 2009; Lindberg et al., 2008; Pierce et al., 2013; Schlebusch et al., 2015). We have previously reported based on laboratory experiments that levels and proportions of the methylated products, including DMAs/MAs ratio, differ between recombinant variants of human AS3MT and depend on the substrate concentration (Ding et al., 2012). However, to our knowledge, no population study has formally explored to what extent associations between AS3MT variants and measures of iAs metabolism may vary depending on levels of iAs exposure. Such heterogeneity, if present, could lead to inconsistencies across populations with varying iAs exposure in the extent to which genetic variants either relate to measures of iAs metabolism, or modify health risks associated with environmental iAs exposure.

The aims of this study were to examine the consistency of previously established associations between multiple AS3MT variants and the profiles of urinary iAs metabolites in a population with substantial variability in exposure, and to assess evidence of heterogeneity in the magnitude of these associations depending on the extent to which subjects are exposed to iAs in drinking water.

METHODS

Study population

Participants were originally recruited for a cross-sectional study on the association of iAs exposure with prevalence of diabetes mellitus in Chihuahua, Mexico, which has been described previously (Mendez et al., 2015). Briefly, in the parent study, a total of 1160 adults were recruited with a minimum of 5-year uninterrupted residency in the area. Pregnant women, subjects with urinary tract infection, and individuals with potential occupational exposure to iAs (eg, those working with pesticides or in mines or smelters) were excluded since these conditions affect the urinary profiles of iAs metabolites(Cocker et al., 2006; Colin-Torres et al., 2014; Gardner et al., 2012; Li et al., 2016; Loh et al., 2016). Participants provided samples of household drinking water and spot urine samples in which As metabolites were measured, and

interviewer-administered questionnaires were used to collect a wide array of information on factors including health status (including diagnosed diabetes), use of medications, smoking, use of alcohol. Physical exams included an oral glucose tolerance test (OGTT) for detecting undiagnosed diabetes, as well as measures of weight, height, body mass index (BMI), waist and hip circumferences, blood pressure, and skin lesions associated with iAs exposure. For use in sensitivity analyses exploring effects of diabetes, subjects were classified as having diabetes based on fasting plasma glucose ≥ 126 mg/dl or 2-h post OGTT glucose (2 HPG ≥ 200 mg/dl), or on self-reported diagnosis or use of diabetes medication (Kerner *et al.*, 2014; World Health Organization, 2006). All subjects provided signed informed consent, and the study was approved by the Institutional Review Boards of UNC-Chapel Hill and the Centro de Investigación y de Estudios Avanzados of the Instituto Politécnico Nacional in Mexico City.

Measurements of water and urinary As

The analyses of As in water and urines were described in detail in the parent study (Mendez *et al.*, 2015). Drinking water was collected in subjects' homes, and the concentration of iAs in these water samples was measured by hydride generation (HG)-cryotrapping (CT)-atomic absorption spectrometry (AAS) (Hernandez-Zavala *et al.*, 2008). Spot urine samples were collected during morning medical exams as described previously (Currier *et al.*, 2014). Concentrations of iAs, MAs and DMAs in spot urine were measured by HG-CT-AAS. Certified standard reference materials (SRMs) from the inter-laboratory comparison program in Quebec and the SRM 2669 (Arsenic Species in Frozen Human Urine) from National Institute of Standards and Technology were used as quality controls.

The limit of detection (LOD) for iAs in water as well As species in urine was 0.01 µg As/l. Concentrations of water iAs and urinary As species which were below LOD (1.9% for water iAs, 1.6% for urinary iAs) were imputed at LOD/2. Total speciated As in urine (tAs) was calculated as sum of the iAs, MAs and DMAs. iAs metabolism was characterized using measures recommended in the literature (Thomas *et al.*, 2001; Vahter and Concha, 2001), namely percentages of each iAs metabolite (DMAs%, MAs%, iAs%), as well as the ratios of MAs/iAs (also known as the primary methylation index) and DMAs/MAs (the secondary methylation index) in urine.

Genotyping

DNA was isolated from venous blood collected in the OGTT using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. We reviewed the available literature to look for candidate *AS3MT* variants associated with urinary As profiles. Nine *AS3MT* variants linked to differences in iAs metabolism or susceptibility to iAs toxicity were identified for analysis in this study based on literature published through April 2011 (Agusa *et al.*, 2009; Chung *et al.*, 2009a,b; Engstrom *et al.*, 2011; Hernandez-Zavala *et al.*, 2008; Hwang *et al.*, 2010; Lindberg *et al.*, 2007; Sampayo-Reyes *et al.*, 2010; Schlawicke Engstrom *et al.*, 2007; Thomas *et al.*, 2001; Valenzuela *et al.*, 2009; Wood *et al.*, 2006). These *AS3MT* variants included 8 single nucleotide polymorphisms (SNPs), rs35232887 (Arg173Trp), rs34556438 (Thr306lle), rs11191439 (Met287Thr), rs17881215 (G4965C), rs3740393 (G12390C), rs3740390 (C14215T), rs11191453 (T35587C), rs10748835 (G35991A), and 3 variable number of tandem repeats (VNTR) variants, AB, A2B, and A3B. The *AS3MT* variants were analyzed either in the Mammalian Genotyping Core

(UNC, Chapel Hill, North Carolina, USA) or in our laboratory (VNTR and rs17181215), using predesigned or custom TaqMan assays (Applied Biosystems, Carlsbad, CA, USA). The ABI Dual 384-Well GeneAmp PCR System 9700 and ABI PRISM 7900HT Sequence Detection System from Applied Biosystems was used for genotyping and the ABI SDS software for data analysis. VNTR variants and rs17881215 were identified by sequencing a PCR-amplified promoter region.

Due to funding constraints, samples for a random subset of approximately half the subjects in the full sample were sent to the Mammalian Genotyping Core ($N = 543$). A random sample was also analyzed for rs17881215 and VNTR in our laboratory. Due to genotyping failure, the analysis sample available for each variant measured in the core facility varied in size from $N = 500$ (for rs10748835) to $N = 506$ (for rs3740393), with $N = 715$ available for rs17881215 and VNTR. 772 subjects had data on at least 1 candidate genetic marker along with measures of water As, iAs metabolism, age and gender. In addition to our primary analyses in the maximum sample with available data for each variant, we analyzed associations in a sample limited to subjects with data available for all candidate variants ($N = 483$) to confirm that effects were not affected by varied sample size; results were not meaningfully different (Supplementary Appendix Table 1).

Statistical analysis

We estimated associations between candidate variants in *AS3MT* and each measure of iAs metabolism described above. In all analyses, we defined the variant previously reported to be associated with a higher DMAs% as the referent genotype in order to facilitate comparisons with existing literature. Genotype frequencies were estimated and tested for departure from Hardy-Weinberg equilibrium by calculating pairwise r^2 coefficients between variants.

To determine whether candidate *AS3MT* variants were related to biomarker estimates of overall iAs exposure versus to measures of iAs metabolism, we first compared median (25th and 75th percentile) tAs, as well as each indicator of iAs metabolism, across allelic variants. Medians were used rather than means given the highly non-normal distribution of tAs (Shapiro-Wilk $P < .01$). The non-parametric Kruskall-Wallis test was used to identify statistically significant differences in each As measure across genotypes for each polymorphism. For all analyses, $P < .10$ was used *a priori* to define marginal significance and $P < .05$ to define significance, given the moderate sample size. We further evaluated associations between *AS3MT* genotypes and indicators of metabolism using multiple linear regression models adjusting for age and gender, which are known to influence iAs metabolism (Lindberg *et al.*, 2007, 2008; Loffredo *et al.*, 2003; Tseng, 2009). Coefficients from these models estimated the mean differences in each measure of iAs metabolism among different genotypes of each variant, adjusting for age and sex.

Next, we explored whether associations between *AS3MT* variants and iAs metabolism appeared to vary depending on levels of exposure. To identify *AS3MT* variants for which associations with measures of iAs metabolism differed significantly with changes in the concentration of drinking water As, we tested the significance of polymorphism × water As interactions in age- and sex-adjusted linear regression models using global F tests. Based on previous literature, interactions were tested defining high versus low water As exposure with a cutoff of 50 ppb, close to the sample median of 48.64 ppb. Age- and sex-adjusted models

were also run stratified by higher versus lower water As to compare the magnitude of associations between AS3MT variants and measures of iAs metabolism among individuals more versus less exposed, regardless of the significance of interactions.

In addition to sensitivity analyses to evaluate the influence of varying sample size described above, we compared the associations between urinary As profiles and AS3MT variants before and after adjusting for or excluding persons with diabetes; results did not differ meaningfully (Supplementary Appendix Table 2). Additionally, because numerous epidemiological studies have shown that BMI may influence the metabolism of iAs (Gomez-Rubio et al., 2011; Grashow et al., 2014; Gribble et al., 2013; Su et al., 2012), we evaluated the impact of adjusting for BMI; again, results did not differ meaningfully (data not shown). Adjusting for self-reported parental race-/ethnicity (Hispanic 93.5%, Amerindian/indigenous 2.9%, white 3.3%, and other 0.4%) did not affect our findings (not shown). Similarly, additionally adjusting for either total water As or urinary tAs had no meaningful effect (data not shown). Statistical analysis was performed using STATA version 13 (Stata Corporation, College Station, Texas) except Global F test for the polymorphism × water As interactions, which was performed with PROC GLM in SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

RESULTS

Characteristics of the Population and AS3MT Genotype Frequencies

After excluding subjects with missing data on urinary As species, age, or gender (N = 42), there were 772 individuals (520 females, 252 males) with available information on at least 1 candidate variant. Exposure to water As varied substantially, with a median (25–75th percentile) of 48.6 (37.1–74.1) ppb and range of

0.01–419.77 ppb. Of the 9 initial candidate AS3MT variants, 2 SNPs (rs35232887 and rs34556438) had a very low frequency (N < 5) for the non-wildtype genotypes and were not used for further analysis. Genotype frequencies varied substantially for all other variants (Table 1). Three pairs of SNPs—rs11191439 and rs17881215, rs3740393 and rs3740390, rs3740390 and rs11191453—were in linkage disequilibrium with $r^2 > 0.8$. The referent genotype—which we defined as the genotype associated with a higher DMAs% in previously published studies—corresponded to the wildtype (based on global genotype frequency reports from National Center for Biotechnology Information) for only 2 SNPs (rs11191439 and rs17881215, highly correlated with each other) (Table 1). For rs11191453, since there was no observation in our sample with the homozygous variant (CC) previously associated with the highest DMAs%, we used the heterozygous variant (TC) as the referent. For VNTR, as only N = 1 subject had the true referent genotype A3B, we defined A2B, which was also associated with a higher DMAs% (Fu et al., 2014; Wood et al., 2006), as the referent. A3B was excluded from further analyses due to low frequency (N = 1).

Urinary Measures of Arsenic Metabolism Varied Substantially With Variants

Table 1 also shows the median (25th and 75th percentile) of each measure of iAs metabolism—DMAs%, MAs%, iAs%, DMAs/MAs, and MAs/iAs—overall and stratified by genotypes of each AS3MT variant. Values for the percentages and ratios of urinary metabolites were consistent with the full study population (Mendez et al., 2015). The median (25th and 75th percentiles) for tAs, DMAs%, MAs%, iAs%, DMAs/MAs, and MAs/iAs were 62.9 µg/l (34.2, and 108.4 µg/l), 76.9% (71.3%, 81.4%), 13.9% (10.9%, 17.4%), 8.8% (6.3%, 12.2%), 5.5 (4.2, 7.4), and 1.6 (1.2, 2.1),

TABLE 1. Urinary As Profiles of Arsenic Metabolites Overall and by AS3MT Variants (Median and 25 and 75th Percentiles)

	N	%	tAs		DMAs%		MAs%		Unmethylated iAs%		DMAs/MAs Ratio		MAs/iAs Ratio	
			p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)
All subjects	772		62.9	(34.2, 108.4)	76.9	(71.3. 81.4)	13.9	(10.9,17.4)	8.8	(6.3, 12.2)	5.5	(4.2, 7.4)	1.6	(1.2, 2.1)
By AS3MT variant:														
rs11191439[a] [d]TT	412	82	59.5	(31.3, 105.5)	**77.3****	(71.7, 81.8)	**13.5****	(10.8, 16.7)	8.9	(6.1, 12.4)	**5.7****	(4.4, 7.6)	1.5	(1.1, 2.1)
TC	86	17	52.5	(30.4, 99.7)	74.9	(66.5, 79.0)	15.8	(13.0, 20.0)	9.8	(6.3, 14.5)	4.7	(3.3, 6.0)	1.8	(1.2, 2.5)
CC	3	1	54.8	(26.6, 151.0)	65.6	(59.2, 73.8)	19.7	(13.9, 27.8)	13.0	(12.2, 14.7)	3.3	(2.1, 5.3)	1.3	(1.1, 2.1)
rs17881215[a] [d]GG	579	81	61.4	(33.1, 107.4)	**77.6****	(72.8, 82.5)	**13.4****	(10.7, 16.6)	8.6	(6.1, 11.6)	**5.8****	(4.4, 7.6)	1.6	(1.2, 2.1)
GC	124	17	67.5	(38.0, 112.8)	74.9	(69.0, 80.5)	15.1	(12.8, 19.2)	8.8	(6.7, 13.9)	4.9	(3.6, 6.3)	1.6	(1.2, 2.3)
CC	12	2	45.2	(34.3, 82.4)	73.9	(65.3, 78.7)	19.8	(12.6, 21.6)	11.2	(5.0, 13.1)	3.7	(3.0, 6.2)	1.8	(1.3, 3.0)
rs3740393[b] GG	280	55	59.0	(30.9, 97.4)	**75.3****	(69.3, 79.6)	**14.7****	(11.7, 18.9)	**9.9****	(7.1, 13.1)	**5.1****	(3.8, 6.8)	1.6	(1.2, 2.1)
GC	200	40	57.2	(30.6, 107.6)	78.6	(72.9, 83.3)	13.0	(10.8, 16.0)	8.3	(5.4, 12.0)	5.8	(4.6, 7.6)	1.6	(1.1, 2.3)
[d]CC	26	5	72.6	(46.5, 125.0)	80.7	(74.0, 84.5)	12.0	(9.4, 15.0)	9.2	(4.3, 12.0)	6.7	(5.3, 9.0)	1.2	(1.0, 1.8)
rs3740390[a,b,c] CC	293	58	59.9	(32.4, 99.5)	**75.5****	(69.4, 79.7)	**14.5****	(11.8, 18.8)	**10.0****	(7.1, 13.3)	**5.3****	(3.8, 6.8)	1.6	(1.1, 2.1)
CT	192	38	56.6	(30.1, 105.6)	78.7	(73.5, 83.4)	12.8	(10.5, 15.9)	8.1	(5.4, 11.9)	6.2	(4.7, 7.8)	1.6	(1.1, 2.3)
[d]TT	20	4	71.3	(36.9, 119.2)	80.3	(70.2, 86.0)	12.6	(9.0, 15.3)	7.9	(4.4, 14.8)	6.3	(4.7, 9.5)	1.2	(0.9, 1.7)
rs11191453[c] TT	290	58	59.7	(32.4, 99.5)	**75.3****	(69.2, 79.7)	**14.6****	(11.8, 18.9)	**10.0****	(7.1, 13.4)	**5.2****	(3.7, 6.8)	1.6	(1.2, 2.1)
[d]TC	212	42	57.9	(30.1, 107.6)	78.8	(73.4, 83.5)	12.8	(10.3, 15.9)	8.1	(5.3, 12.0)	6.2	(4.7, 8.0)	1.6	(1.1, 2.3)
rs10748835 GG	151	30	60.5	(33.1, 99.5)	**76.0***	(70.1, 80.2)	14.5	(12.0,18.1)	9.5	(7.1, 12.9)	**5.4***	(3.9, 6.6)	1.5	(1.1, 2.1)
GA	251	50	57.2	(29.4, 104.3)	76.7	(70.3, 81.3)	13.7	(10.8, 17.4)	9.0	(6.1, 12.9)	5.5	(4.1, 7.6)	1.6	(1.1, 2.1)
[d]AA	98	20	64.8	(32.0, 112.6)	78.5	(73.8, 83.5)	13.7	(10.3, 15.9)	8.3	(5.1, 11.7)	5.7	(4.6, 7.7)	1.6	(1.1, 2.2)
VNTR AB	587	82	61.5	(33.2, 112.0)	**76.9***	(70.9, 81.5)	13.9	(11.0, 17.4)	8.7	(6.2, 12.3)	5.5	(4.1, 7.3)	1.6	(1.2, 2.1)
[d]A2B	127	18	60.5	(34.2, 92.6)	77.9	(73.8, 82.1)	13.6	(10.6, 16.1)	8.3	(6.3, 10.5)	5.6	(4.6, 7.9)	1.6	(1.2, 2.2)

[a,b,c]The pairs of variants shown were in linkage disequilibrium with $r^2 > 0.8$.
[d]Identifies the referent genotype, defined as the genotype associated in previous literature with a higher DMAs%.
*P < .10,
**P < 0.05 for Kruskall wallis test for differences in urinary As metabolites among different genotypes of the AS3MT variants. Medians bolded when the Kruskall Wallis test reached significance (P < .10).

respectively. Water As was significantly positively correlated with urinary tAs (Spearman's $R = 0.23$, $P < 0.01$).

In descriptive analysis, no significant difference in tAs was observed across genotypes for any of the 7 candidate variants ($P > 0.10$; Table 1). Descriptive analyses also showed that consistent with prior literature, the referent genotype was associated with increases in the DMAs% for all 7 candidate markers. These differences were significant ($P < .05$) for 5 variants, and marginally significant for 2 (VNTR and rs10748835, $P < .10$). The 5 variants associated with significant increases in the DMAs% were also associated with significant reductions in the MAs% ($P < 0.05$); VNTR and rs10748835 were associated with small but non-significant reductions in this measure. Similarly, the DMAs/MAs ratio was significantly higher among participants with the referent genotype for 6 variants, the exception being VNTR. There was a reduction in iAs% observed for the referent genotypes for 6 of the candidate markers examined, although differences were at least of marginally significance for only 3 variants. The exception to this pattern, rs3740393, showed lower iAs% for the heterozygous (GC) instead of referent homozygous (CC) genotype. In contrast to the patterns observed for all other measures of iAs metabolism, there were no clear pattern and no significant differences in the MAs/iAs ratio across genotypes in any of the candidate variants.

Adjusted mean differences in urinary measures of iAs metabolism associated with having polymorphisms other than the referent genotypes are shown in Table 2, based on multivariable linear models adjusted for age and gender. Results were similar to descriptive analyses. Having non-referent genotypes was associated with significant ($P < .05$) decreases in the DMAs% for at least one genotype in all 7 variants, significant increases in the MAs% for 5 variants, and marginally significant increases ($P < .10$) in the MAs% for rs10748835 and VNTR. Excluding some genotypes with very small cell sizes ($N < 5$), the 5 SNPs significantly associated with these measures of iAs metabolism (rs3740390 and 2 correlated SNPs rs3740393 and rs11191453; rs17881215 and the correlated SNP rs11191439) were associated with the largest adjusted mean differences in both DMAs% (from 3.3 ± 0.7 to $4.6 \pm 2.2\%$) and MAs% (from 1.8 ± 0.5 to $3.9 \pm 1.4\%$), with smaller differences associated with polymorphisms in rs10748835 and VNTR.

Significant differences in measures of iAs metabolism associated with genetic polymorphisms were most notable for the DMAs/MAs ratio. When compared with the referent genotype, all other genotypes in each variant were associated with at least marginally significant ($P < .10$) differences in DMAs/MAs with only 2 exceptions: the homozygous variant of rs11191439, for which the cell size was small ($N = 3$), and polymorphisms in VNTR, for which the association was null. The magnitude of association with DMAs/MAs was strongest—a >2-unit change—for variants in rs3740393 and the correlated SNP rs3740390, with the weakest associations—of about half this magnitude—for rs10748835. Like the descriptive analyses, few variants ($N = 3$) were significantly associated with the iAs%, and none with the MAs/iAs ratio, after multivariable adjustment.

Associations Between AS3MT Variants and Urinary Measures of iAs Metabolism Among Subjects With Higher Versus Lower Exposure to Drinking Water As

There were interactions between elevated (>50 ppb) exposure to water As and several genetic variants, suggesting that the degree to which genotype in these variants influence patterns of iAs metabolism may vary with increasing exposure. Interactions were significant primarily for the DMAs/MAs ratio ($P < .05$ for 3 variants: the correlated SNPS rs3740390 and rs3740393, as well as rs10748835; Figure 1C, Supplementary Appendix Table 3). There were also marginal significant ($P < .10$) interactions between exposure level and one variant (rs17881215) for the DMAs% and MAs%.

As shown in Figure 1C, the decline in the DMAs/MAs ratio associated with having genotypes other than the referent was considerably as well as significantly larger among more versus less highly exposed individuals for 3 SNPs: rs3740390, the correlated SNP rs3740393, and rs10748835 (see also Supplementary Appendix Table 4). In the sample as a whole, the magnitude of associations with the DMA/MAs ratio was strongest for the first 2 SNPs. However, except for the null relationship with VNTR, this last SNP—rs10748835—was the variant most weakly associated with the DMAs/MAs in the overall sample. Though

TABLE 2. Adjusted Associations Between A S3MT Variants and Urinary Profiles of Arsenic Metabolites[a]

Polymorphism		N	Dimethylated Arsenic % (DMAs%)		MAs%		Unmethylated iAs%		DMAs/MAs Ratio		MAs/iAs Ratio	
			Coefficient (SE)	P	Coefficient (SE)	P	Coefficient (SE)	P	Coefficient (SE)	P	Coefficient (SE)	P
rs11191439[b] (*ref TT, N = 412*)	TC	86	**−3.5 (1.0)**	.00**	**2.3 (0.6)**	.00**	**1.2 (0.6)**	.04**	**−1.2 (0.4)**	.00**	−1.4 (1.2)	.23
	CC	3	**−9.1 (4.7)**	.05*	**6.5 (3.1)**	.04**	2.7 (2.9)	.36	−2.6 (1.8)	.15	−1.6 (5.8)	.79
rs17881215[b] (*ref GG, N = 579*)	GC	124	**−3.3 (0.8)**	.00**	**1.9 (0.5)**	.00**	**1.4 (0.5)**	.00**	**−1.1 (0.3)**	.00**	−0.2 (1.5)	.92
	CC	12	**−4.6 (2.2)**	.04**	**3.9 (1.4)**	.01**	0.7 (1.4)	.63	**−1.6 (0.9)**	.06*	−1.5 (4.3)	.72
rs3740393[c] (*ref CC, N = 26*)	GC	200	−1.4 (1.7)	.41	1.8 (1.1)	.10	−0.4 (1.0)	.68	**−2.1 (0.6)**	.00**	1.4 (2.1)	.50
	GG	280	**−4.2 (1.7)**	.01**	**3.3 (1.1)**	.00**	0.9 (1.0)	.36	**−3.1 (0.6)**	.00**	−0.5 (2.0)	.80
rs3740390[c,d] (*ref TT, N = 20*)	CT	192	−0.6 (1.9)	.73	1.4 (1.3)	.27	−0.7 (1.2)	.53	**−2.3 (0.7)**	.00**	0.2 (2.3)	.93
	CC	293	**−3.8 (1.9)**	.05**	**3.0 (1.2)**	.02**	0.8 (1.2)	.50	**−3.4 (0.7)**	.00**	−0.5 (2.3)	.82
rs11191453[d] (*ref TC, N = 212*)	TT	290	**−3.3 (0.7)**	.00**	**1.8 (0.5)**	.00**	**1.5 (0.5)**	.00**	**−1.3 (0.3)**	.00**	−0.7 (0.9)	.43
rs10748835 (*ref AA, N = 98*)	GA	251	−1.5 (1.0)	.12	0.7 (0.6)	.27	0.8 (0.6)	.19	**−0.6 (0.4)**	.10*	0.7 (1.2)	.59
	GG	151	**−2.2 (1.1)**	.04**	**1.3 (.7)**	.07*	0.9 (0.7)	.16	**−1.1 (0.4)**	.01**	0.1 (1.3)	.95
VNTR (*ref A2B, N = 127*)	AB	587	**−1.6 (0.8)**	.04**	**0.9 (0.5)**	.09*	0.7 (0.5)	.14	−0.2 (0.3)	.45	0.2 (1.4)	.88

[a]Linear regression model adjusted for age and gender; referent genotype defined as the genotype associated in previous literature with a higher DMAs%.

[b,c,d]The pairs of variants shown were in linkage disequilibrium with $r^2 > 0.80$.

*$P < .10$,

**$P < .05$ for coefficients of associations between *AS3MT* variants and urinary As profiles, results come from multiple linear regression model adjusted for age and gender. Coefficients (SE) were bolded when they reached significance ($P < .10$).
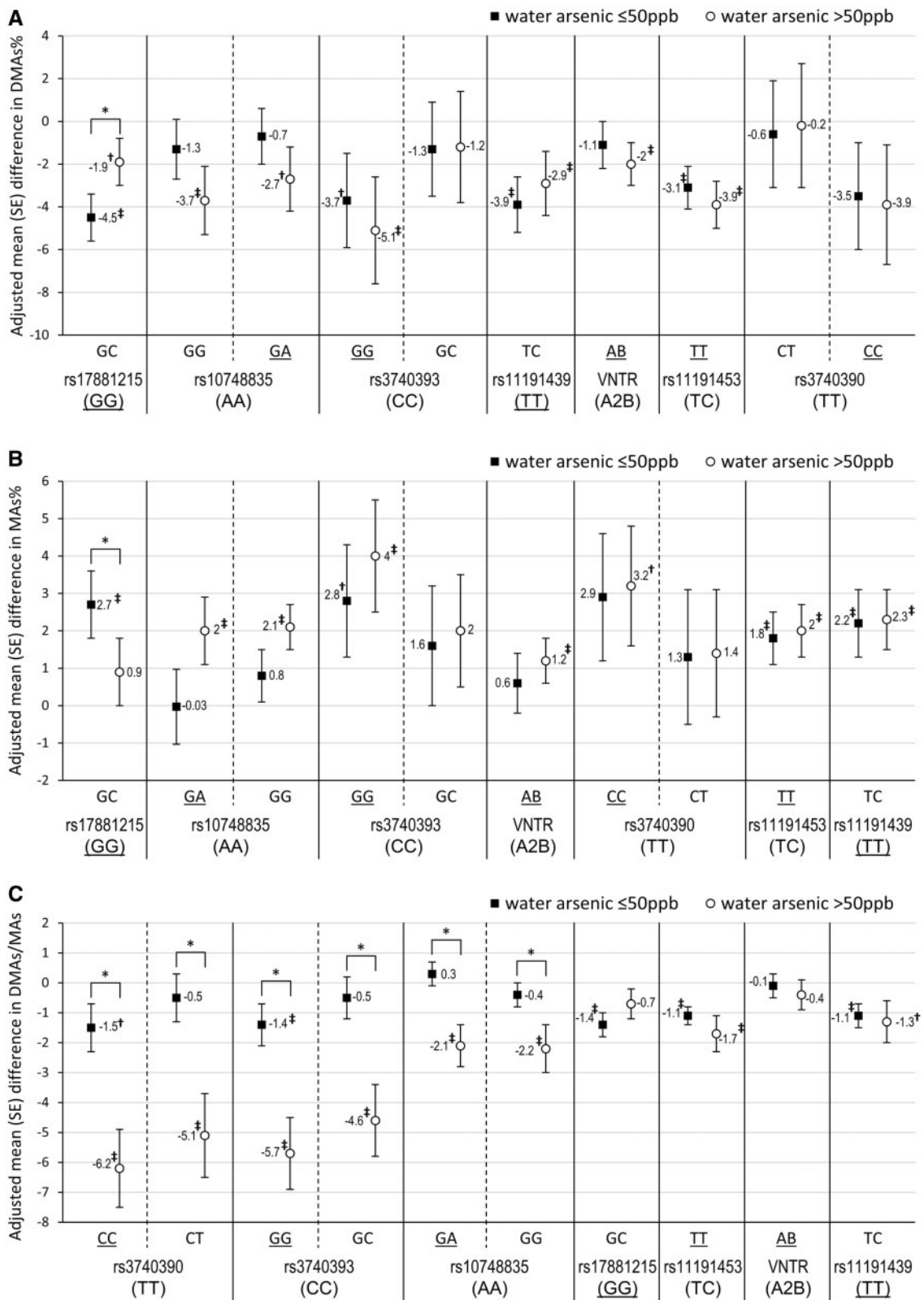
FIG. 1. Associations between AS3MT variants and urinary arsenic profiles among subjects with higher versus lower water arsenic (As). †P < .10 ‡P < .05 for differences in urinary As profiles associated with having non-referent AS3MT variants genotypes compared with referent genotypes, where referent genotypes were defined as those associated with a higher DMAs% in previous studies. Wild type genotypes, defined based on global genotype frequency reports from the National Center for Biotechnology Information, were underlined. *Indicates interaction (P < .10) for variant X categorical water iAs (>50 vs ≤ 50 ppb). Results come from multiple linear regression models adjusted for age and gender.
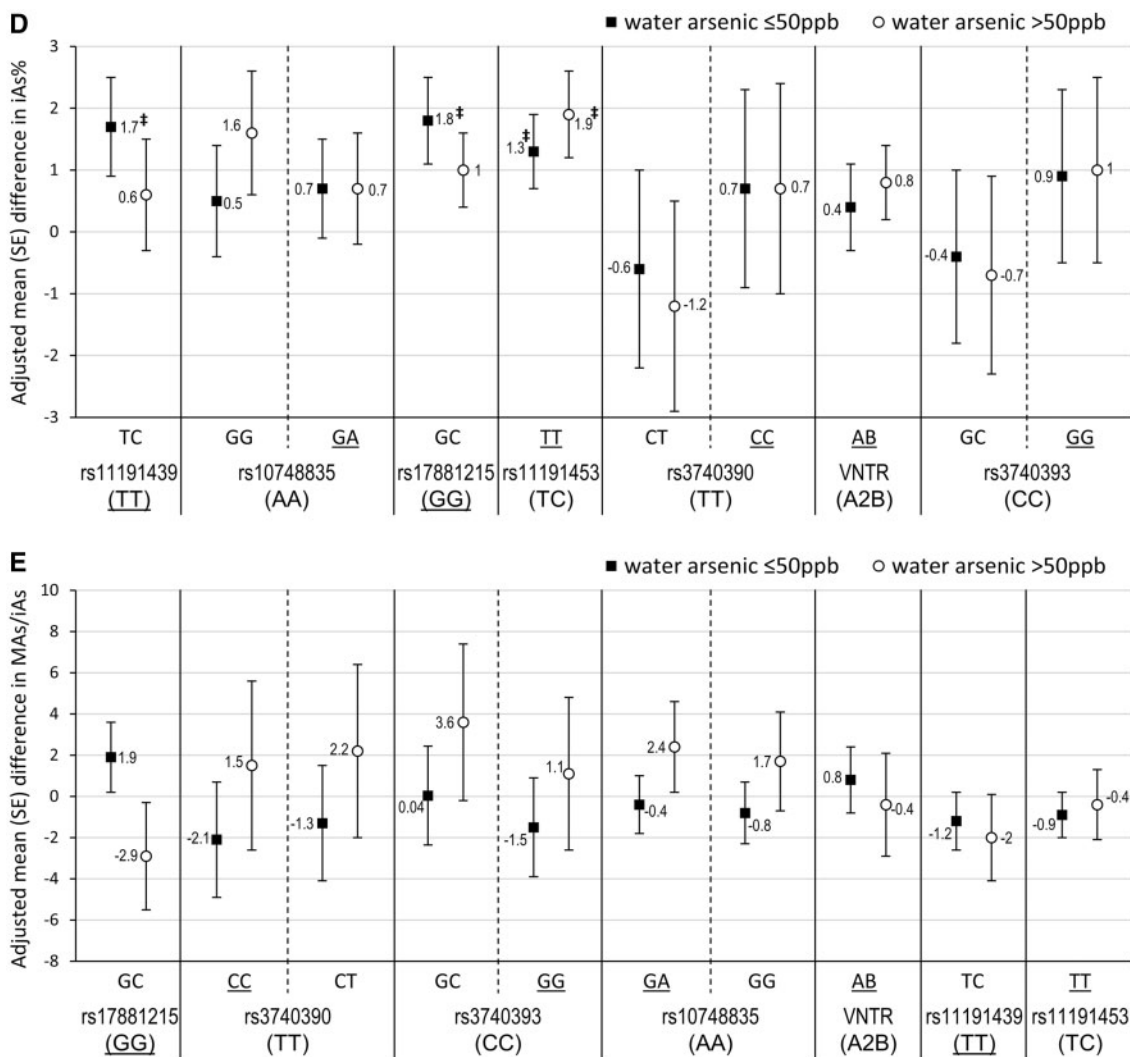
FIG. 1. Continued

interactions did not reach significance, the magnitude of associations with polymorphisms in rs10748835 and both the DMAs% and MAs% were more than 2 times larger among highly versus more moderately exposed individuals (Figure 1A and B, Supplementary Appendix Table 4).

In contrast to the stronger associations seen at higher levels of exposure for these 3 variants, for polymorphisms in rs17881215 the magnitude of association with both the DMAs% and MAs% was marginally significantly weaker at higher exposure (P < .10; Figure 1A and B, Supplementary Appendix Tables 3 and 4). There was, however, no difference in the magnitude of association between polymorphisms in this SNP and the DMAs/MAs ratio at high versus low levels of exposure.

In models predicting variation in the iAs% and the MAs/iAs ratio, no significant effect modification by water As was observed. However, for both outcomes, the magnitude of associations appeared to vary depending on the level of water As exposure for polymorphisms that included both rs10748835 and rs17881215, for which interactions reached significance for other measures (Supplementary Appendix Table 4, Figure 1D and E).

In sensitivity analyses exploring the effects of additionally adjusting for diabetes (Supplementary Appendix Table 2), or for

BMI, urinary tAs and water As (not shown), there were no meaningful differences in results. False discovery rate adjustment (Benjamini et al., 2001) of models for each measure of metabolism led to loss of significance for associations with a number of variants and iAs%, and for the association between variants in rs3740390 and MAs% among subjects exposed to low levels of drinking water arsenic. Interactions for models predicting the DMA/MAs ratio remained significant.

DISCUSSION

Inter-individual variation in urinary measures of iAs metabolism has been associated with the risk of adverse health outcomes associated with iAs exposure (Kuo et al., 2015; Mendez et al., 2015; Pierce et al., 2013; Sun et al., 2007), indicating that factors influencing metabolism may affect susceptibility to disease. There is growing evidence—including a recent review—that a number of candidate variants in AS3MT affect iAs metabolism (Antonelli et al., 2014; Fu et al., 2014; Gribble et al., 2015; Pierce et al., 2012; Schlebusch et al., 2015; Wood et al., 2006). At present, evidence on the consistency with which these variants relate to markers of metabolism across populations is limited

and mixed, perhaps in part due to the small sample size and modest power in most previous studies ($N < 300$) (Drobna et al., 2013; Fu et al., 2014). Moreover, although it has been suggested that the extent to which some variants influence iAs metabolism might be stronger among subjects more highly exposed to iAs (Pierce et al., 2013), to our knowledge, studies have yet to formally explore such heterogeneity. In this study, we aimed to confirm relationships between 7 candidate AS3MT variants and urinary markers of iAs metabolism (Antonelli et al., 2014; Beebe-Dimmer et al., 2012; Drobna et al., 2013; Engstrom et al., 2011, 2015; Hernandez et al., 2014; Hernandez-Zavala et al., 2008; Hwang et al., 2010; Lindberg et al., 2007; Sampayo-Reyes et al., 2010; Valenzuela et al., 2009; Wood et al., 2006), and examine the extent to which these associations may vary depending on the level of exposure to drinking water As.

In this study, genotypes associated with a higher DMAs% in previous studies were consistently associated with significantly higher DMAs%, DMAs/MAs and lower MAs%; almost all associations were at least marginally significant at $P < .10$. Reference genotypes also tended to be associated with a lower iAs%, though these relationships were largely not significant. However, associations with the ratio of MAs to iAs, which has been used as an indicator of the efficiency of the first methylation step, were consistently non-significant, and varied considerably in terms of direction and magnitude.

We found significant interactions between 4 of the 7 candidate AS3MT variants and concentrations of As in drinking water. Three SNPs (rs3740393, rs3740390, and rs10748835) had somewhat stronger associations with indicators of iAs metabolism among individuals exposed to higher versus lower levels of As in drinking water, with significant differences in the magnitude of association for the DMAs/MAs ratio. In contrast, one SNP (rs17881215) was more strongly associated with 2 markers of iAs metabolism—the DMAs% and MAs%—among participants with lower rather than with higher exposure. This suggests that the genetic variants most influential for aspects of iAs metabolism may vary across populations, depending on prevailing levels of exposure. This finding also suggests the possibility that the variants most influential for modifying health risks may differ in more highly exposed populations than in settings with low exposure. Though earlier studies, to the best of our knowledge, have not formally examined whether the degree of exposure to iAs may modify the influence of AS3MT variants on iAs metabolism, prior literature has suggested that patterns of metabolism may vary by level of exposure (Ahsan et al., 2007; Kile et al., 2009; Lindberg et al., 2008; Styblo et al., 1999; Tseng, 2009). Several studies have suggested that at exposures exceeding 50 ppb, there may be an increase in the MAs% in urine and decrease in DMAs%, perhaps due to saturated capacity for the secondary methylation step or the inhibition of AS3MT activity by high levels of iAs (Kile et al., 2009; Lindberg et al., 2008). At these higher exposures, genetic variants may be either more, or less, influential on iAs metabolism. However, in our sample, we did not observe meaningful differences in urinary As profiles between groups exposed to higher (≥ 50 ppb) versus lower concentrations of water As in our study. The median (25th and 75th percentile) for more versus less exposed groups were 77.2% (71.4%, 81.8%) versus 76.5% (70.9%, 81.2%), 14.0% (11.1%, 17.4%) versus 13.6% (10.9%. 17.4%), and 5.4 (4.2, 7.3) versus 5.6 (4.2, 7.4) for DMAs%, MAs%, and DMAs/MAs accordingly (Kruskall-Wallis $P > .10$ for all pairwise comparisons).

Interactions between AS3MT variants and levels of exposure (ie, water iAs) did not depend on the magnitude of the association between those variants and urinary iAs profiles in the overall population. For example, in the population as a whole, polymorphisms in rs10748835 were much more weakly associated with urinary iAs metabolites than those in rs3740393. However, there were significant differences in the magnitude of association with the DMAs/MAs ratio among participants with high versus low exposure for both variants.

In our analyses, the referent genotype, defined as the one previously associated with a higher DMAs%—and postulated to be associated as well with a higher DMAs/MAs, lower MAs%, and lower iAs%—was not always the major (ie, most frequent) genotype in our sample. Indeed, the postulated beneficial referent genotype was the major genotype only for rs11191439 and rs17881215, the SNPs with the strongest magnitude of association with methylation indicators in our sample (see Table 2).

It is unclear what the implications of the AS3MT variant-iAs metabolism associations are for health of iAs-exposed individuals. Based on previous literature, the relevance of a high versus low DMAs%, MAs%, or DMAs/MAs ratio for health risks is uncertain, and may depend on the level of exposure. Several studies in high exposure settings have found a high MAs% to be associated with increased risk of cancer and other health outcomes, including cardiovascular diseases and diabetes (Chen et al., 2005; Chung et al., 2009a,b; Lindberg et al., 2008). However, numerous studies in settings with more moderate exposure have reported a higher DMAs% to be associated with increased risk of diabetes and other cardiometabolic outcomes (Chen et al., 2013; Del Razo et al., 2011; Mendez et al., 2015; Nizam et al., 2013). The conflicting results for the associations between urinary iAs methylation profiles and health outcomes may be due to differences in the distribution of trivalent and pentavalent methylated metabolites (MAsIII vs MAsV; DMAsIII vs DMAsV) which exhibit different toxicities in laboratory models (Chung et al., 2009; Del Razo et al., 2011; Kligerman and Tennant, 2007; Nizam et al., 2013; Petrick et al., 2000; Schwerdtle et al., 2003; Styblo et al., 2000; Tseng, 2007). However, differentiating between the trivalent and pentavalent As species in urine is technically challenging and is rarely implemented in population studies (Valenzuela et al., 2005). Therefore, analyses performed in most population studies, including this study, are typically limited to measurements of total iAs, MAs, and DMAs, and the percentages and ratios of these metabolites in urine.

Though a considerable literature indicates that inter-individual variation in the capacity to metabolize iAs is associated with diverse health risks, including cancer, diabetes, cardiovascular disease, and skin lesions (Chen et al., 2013; Gribble et al., 2014; Huang et al., 2007; Karagas et al., 1998; Kuo et al., 2015; Lesseur et al., 2012; Mendez et al., 2015; Pierce et al., 2013; Tseng, 2007), less is known about the health effects of variants in AS3MT that influence metabolism. Previous studies of candidate SNPs in AS3MT, which strongly influences arsenic metabolism, have also reported significant associations between arsenic-related health outcomes and the variants in the SNPs we found to most strongly relate to lower DMAs% and higher MAs%, rs11191439 and rs17881215. In a separate Mexican population with moderately high levels of drinking water arsenic, C versus T alleles in rs11191439 [M287T] were associated with significantly higher levels of trivalent DMAs in urine and increases in 2-h and fasting glucose (Drobna et al., 2013), as well as with increased odds of skin lesions (Valenzuela et al., 2009). Variants in this SNP have also been associated with increases in cytogenetic damage in subjects occupationally exposed to arsenic (Hernandez et al., 2014). However, in a U.S. population with low exposure (only 2.5% of the sample had >50 ppb drinking water As), associations between rs11191439 variants and bladder

cancer were null (Karagas *et al.*, 1998; Lesseur *et al.*, 2012). Similarly, C versus G alleles in rs17881215 [G4965C] were associated with increases in trivalent DMAs and higher fasting and 2-h glucose (Drobna *et al.*, 2013). For rs3740393 (G vs C), rs3740390 (C vs T), and/or rs10748835 (G vs A)—the 3 variants for which we found significant interactions with exposure in our analysis of methylation patterns—there were null associations with the outcomes under study: glucose levels (Drobna *et al.*, 2013), skin lesions (Valenzuela *et al.*, 2009), or bladder cancer (Lesseur *et al.*, 2012). These studies did not examine interactions between SNPs and arsenic exposure. A recent study in Bangladesh (Pierce *et al.*, 2013) found evidence of such an interaction. Variants in rs9527 (*P* < .05), the *AS3MT* SNP that was associated with DMAs% and MAs% in urine, were also associated with increased odds of skin lesions, but only among individuals in the highest tertile of water arsenic exposure (>87 ppb). Though mechanisms remain to be fully elucidated, a study in a U.S. population reported differential methylation of the *AS3MT* promoter depending on arsenic exposure (Gribble *et al.*, 2014).

This study was conducted in the Chihuahua area of Mexico, with moderately elevated levels of iAs in drinking water (median concentration of 48.6 ppb). Many previous studies have been conducted in areas with substantially higher exposure (Maull *et al.*, 2012) (eg, water iAs exposure level for studies done in Bangladesh is likely to be between 100 and 200 ppb (Ahsan *et al.*, 2007; Chen *et al.*, 2013; Farzan *et al.*, 2015; Lindberg et al., 2008)). Thus there is uncertainty regarding the health effects of more moderate levels of iAs exposure, and the influence of genetic polymorphisms on iAs metabolism and toxicity, in areas with moderate exposure, which are more typical population exposures around the world. The range of exposure in this Chihuahua cohort—with nearly 50% of subjects at levels below 50 ppb—enabled us to analyze interactions between *AS3MT* variants in the moderate range of water As exposure at which some studies have suggested iAs metabolism may shift (Kile *et al.*, 2009; Lindberg et al., 2008).

A limitation of this study is that, although the sample size was larger than in many previous studies (Drobna *et al.*, 2013; Engstrom *et al.*, 2011; Fu *et al.*, 2014; Lindberg *et al.*, 2007; Valenzuela *et al.*, 2009), we had small cell sizes for genotypes of several candidate *AS3MT* variants, particularly in analyses stratified by water As. The small cell sizes may have reduced our power to detect interactions between level of exposure and methylation profiles for those variants.

CONCLUSION

In this study, 7 *AS3MT* variants which may play a role in iAs metabolism were examined based on results of previous studies. The patterns of association between markers of iAs metabolism and these variants were highly consistent with those reported in previous studies, confirming that these variants are in part responsible for the inter-individual differences in urinary profiles of iAs metabolites. We found that specific genotypes in 5 SNPs, rs17881215, rs3740393, rs3740390, rs11191439, and rs11191453, were associated with significantly higher DMAs% and lower MAs% in urine. Polymorphisms in these SNPs, along with rs10748835, were also associated with the DMAs/MAs ratio.

Our results also suggested that the role of several of these *AS3MT* variants in iAs metabolism may differ among populations with different levels of iAs exposure. Three SNPs, rs3740393, rs3740390, and rs10748835, appeared to have significantly more potent effects, based on associations with larger decreases in the DMAs/MAs ratio, among subjects highly exposed to As in drinking water (>50 ppb). In contrast, rs17881215 had significantly more potent effects among subjects with lower water As levels. Since measures of iAs metabolism have been associated with risk of adverse health outcomes, these findings suggest that variants in *AS3MT* may influence susceptibility to health effects of iAs exposure, and that the role of these variants may depend on the level of iAs exposure. However, given that toxicity of iAs metabolites varies by oxidation status (Del Razo *et al.*, 2011; Kligerman and Tennant, 2007; Nizam *et al.*, 2013; Petrick *et al.*, 2000; Schwerdtle *et al.*, 2003; Styblo *et al.*, 2000; Tseng, 2007), further research focusing on whether and how these variants relate to the distribution of trivalent and pentavalent metabolites is needed to better clarify the influence of *AS3MT* polymorphism on iAs methylation profiles, and on health outcomes.

SUPPLEMENTARY DATA

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

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