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Influence of GSTT1 Genetic Polymorphisms on Arsenic Metabolism

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SUMMARY

A repeated measures study was conducted in Pabna, Bangladesh to investigate factors that influence biomarkers of arsenic exposure. Drinking water arsenic concentrations were measured by inductively-coupled plasma mass spectrometry (ICP-MS) and urinary arsenic species [arsenite (As_3), arsenate (As_5), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)] were detected using High Performance Liquid Chromatography (HPLC) and Hydride Generated Atomic Absorption Spectrometry (HGAAS). Linear mixed effects models with random intercepts were used to evaluate the effects of arsenic contaminated drinking water, genetic polymorphisms in glutathione-S-transferase (GSTT1 and GSTM1) on total urinary arsenic, primary methylation index [MMA/(As_3+As_5)], secondary methylation index (DMA/MMA), and total methylation index [(MMA+DMA)/(As_3+As_5)]. Drinking water arsenic concentrations were positively associated with total urinary arsenic concentrations and total methylation index. A significant gene-environment interaction was observed between urinary arsenic exposure in drinking water GSTT1 but not GSTM1 where GSTT1 *null* individuals had a slightly higher excretion rate of arsenic compared to GSTT1 *wildtypes* after adjusting for other factors. Additionally, individuals with GSTT1 *null* genotypes had a higher primary methylation index and lower secondary methylation index compared to GSTT1 *wildtype* after adjusting for other factors. This data suggests that GSTT1 contributes to the observed variability in arsenic metabolism. Since individuals with a higher primary methylation index and lower secondary methylation index are more susceptible to arsenic related disease, these results suggest that GSTT1 *null* individuals may be more susceptible to arsenic-related toxicity. No significant associations were observed between GSTM1 and any of the arsenic methylation indices.

Keywords: Arsenic, Methylation, Urinary arsenic, GSTT1, Gene environment interaction, Bangladesh, Environmental health.

1. INTRODUCTION

Approximately 330 million people living in the Ganges Delta are at risk of ingesting arsenic-contaminated water as the result of a public health initiative which switched the population's drinking water from surface to groundwater Kumar (2003). Beginning in the 1970s, international aid agencies and public health authorities, in conjunction with the Indian and Bangladeshi governments, installed millions of shallow tubewells to combat waterborne diseases. In the late 1980's, individuals with arsenic-related diseases were diagnosed in India but it wasn't until the 1990s that the extent of the arsenic crisis in Bangladesh was fully understood.

Currently, it is estimated that 95% of the drinking water in Bangladesh is provided by shallow tubewells. A national survey conducted by the Dhaka Community Hospital found that 73% of the tubewells sampled

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exceeded the World Health Organization's (WHO) provisional arsenic drinking water limit of 10 μg/L and 59% exceeded the Bangladesh drinking water standard of 50 μg/L. These findings led the WHO to call this the largest mass poisoning of a population in history (SoEs and DCH 2000). Chronic exposure to arsenic is associated with numerous adverse health effects including non-cancerous skin lesions Paul *et al.* (2000), neuropathy (Lagerkvist and Zetterlund 1994, Feldman *et al.* 1979) vascular diseases (Chiou *et al*. 2005, Yu *et al*. 2002) and an increased risk of skin (Haque *et al*. 2003, Beane *et al*. 2004) bladder (Smith *et al*. 1992) and lung cancers (Peters *et al.* 1986, Smith *et al*. 1998).

Once ingested, inorganic arsenic (As_{in}; arsenate and arsenite) is metabolized through a series of reduction and oxidative methylation reactions forming monomethyl arsonic acid (MMA) and dimethyl arsinic acid (DMA) where glutathione acts as the reducing agent (Gebel 1999). In humans, approximately 60-70% of the ingested dose is excreted in the urine within 3-days but the methylation process is not complete and urine contains, on an average, 10-30% As_{in}, 10-20% MMA, and 60-70% DMA (Pomroy *et al*. 1980, Calderon *et al*. 1999, Hopenhayn-Rich 1993). Data from experimental studies show that the toxicity of each of these arsenic species differ depending on their oxidation and methylation state where trivalent species are more toxic than their pentavalent analogs and MMA is more toxic than DMA (Styblo *et al.* 2000). Due to the incomplete arsenic metabolism in humans, data from epidemiological studies have determined that methylation of inorganic arsenic may be a bioactivation pathway rather than a detoxification pathway (Kitchin 2001). Subsequently, researchers frequently use methylation indices to characterize an arsenic methylation capacity. Data from epidemiological studies show that arsenic methylation capacity is associated with an increased risk of disease (Styblo *et al*. 2002 Yu *et al*. 2003). For instance, data from a cohort in Mexico where people were ingesting arsenic contaminated-drinking water showed that individuals with arsenic-induced skin lesions had a higher proportion of MMA/As_{in} and lower proportions of DMA/MMA compared to individuals without arsenic-induced skin lesions (Del Razo *et al*. 1997). In Taiwan, a region with historically high levels of arsenic contaminated drinking water, several case-control studies found that individuals with arsenic-induced skin lesions, skin cancer, bladder cancer, and peripheral vascular disease had a higher ratio of MMA/DMA and a decreased ability to metabolize As_{in} to DMA compared to matched controls with similar arsenic exposures (Yu *et al*. 2002, Chen *et al*. 2003, Tseng *et al*. 1986). Thus, it seems that individuals who excrete higher proportions of MMA are more susceptible to arsenic-induced disease. However, these studies were all cross sectional and therefore it is not possible to determine whether arsenic methylation capacity is a function of disease.

There is considerable inter-individual variation in urinary arsenic metabolism (Hopenhayn-Rich *et al.* 1993, Vahter 1999, Calderon *et al*. 1999). Genetic differences are widely cited as contributing to this variability. Specifically, genetic polymorphisms in the glutathione s-transferase (GST) superfamily of enzymes that catalyze the conjugation of reduced glutathione (GSH) to hydrophobic and electrophilic compounds, are believed to influence the biotransformation of arsenic (Tanaka-Kagawa *et al*. 2003, Chiou *et al.* 1997, Thomas *et al*. 2004, Buchet *et al*. 1981). Data from many studies have shown that glutathione-*s*-transferase omega (GSTO1) which is identical to monomethyl arsenate reductase and GSTO2 which encodes a protein that shares 64% amino acid identity with GSTO1 and play an important role in arsenic biotransformation (Whitbread *et al*. 2003, Radabough and Aposhion 2000, Zakharyan and Aposhian 1999, Zakharyan *et al*. 2001, Schmuck *et al*. 2005). Additionally, it has been shown that arsenic (+3) methyltransferase (As3MT) also contributes to differences in arsenic metabolism, specifically higher levels of methylated arsenic compounds (Rodrigues *et al.* 2012, Wood *et al*. 2006).

The main objective of this study was to determine whether single nucleotide polymorphisms (SNPs) in two glutathione s-transferase genes (GSTM1 and GSTT1) were associated with altered arsenic metabolism. We specifically chose these SNPs that are loss-of-function variants which affect enzymatic functions and are highly prevalent in South East Asians (Girisha *et al*. 2004, Polimanti *et al*. 2013) We used data from a repeated measures study that was conducted in an arsenic-endemic region of Bangladesh. This cohort was comprised of individuals who showed no visible symptoms of arsenic toxicity in order to evaluate the effects of genetic polymorphisms on biomarkers of arsenic exposure in a healthy population. We hypothesized that individuals with the deletion SNP

would excrete higher proportions of MMA indicating that they had impaired arsenic metabolism.

2. METHODS

2.1 Study Design and Participant Selection

The study was approved by the IRB's of the Harvard School of Public Health and the Dhaka Community Hospital. Informed consent was obtained from all adult participants prior to participation and parental consent was obtained for all participants less than 18 years of age.

The study was described through a series of community meetings held in Pabna, Bangladesh. Pabna is the administrative capital of the Pabna district located north-western Bangladesh. The city has a population of approximately 138,000. Pabna was chosen for the following reasons: a range of high and low arsenic concentrations in tubewells based on prior geological assessments; Dhaka Community Hospital has a well-established clinic network in the area, and Pabna is representative of the socio-economic status (SES) of much of non-urban Bangladesh. The community meetings asked for volunteers to be in the study if they were long-term residents of Pabna, obtained their drinking water from tubewells, and received primary health care from the Pabna Community Clinic, an affiliate of Dhaka Community Hospital (DCH), and multiple family members within a household were willing to participate. This process recruited 50 households (N = 248) into the study.

During the initial visit in September 2001, a behavioral and demographic questionnaire was administered and a blood sample collected. Researchers then visited participants at their homes every 3 months for four years to collect urine, toenail, and water samples. This analysis used data collected from January 2002-March 2003, representing 4 sampling collection periods. When samples were collected on multiple days, only data from the first day was included in this analysis.

Of the 50 households (N = 248) who were recruited into this study, two households (n = 13) moved out of the study area prior to January 2002. Five individuals from different households did not provide urine samples during the time period included in this analysis. In addition, the data collected from 4 participants who had diabetes and 29 children under the age of 15 were excluded. Subsequently, 197 participants representing 48 households were included in this analysis.

2.2 Water Sample Collection and Analysis

Each quarter, drinking water samples were collected from the tubewell each family identified as their primary source of drinking water. Tubewells were purged for several minutes before collecting 50 ml of water in an acid-washed polypropylene tube (BD Falcon, BD Bioscience, Bedford, MA). Samples were preserved with Reagent Grade HNO_3 (Merck, Germany) to a pH < 2 and kept at room temperature until analysis.

Total inorganic arsenic was quantified by inductively coupled plasma-mass spectrometry using US EPA method 200.8 (Environmental Laboratory Services, North Syracuse, New York). Analysis was validated using PlasmaCAL multi-element QC standard # 1 solution (SCP Science, Canada). The average percent recovery for As_{in} was 96.0 ± 2.9%. The limit of detection (LOD) for this method is 1 mg As/L. Thirty-nine percent of the water samples were below the LOD and assigned a value of 0.5 mg As/L.

2.3 Urine Sample Collection and Analysis

Participants were provided with sterile urine collection containers (VWR International) and instructed to collect a first void urine sample. Each morning, members of the field team would pick up the samples and transfer them to 15-ml polyethylene tubes (BD Falcon, BD Bioscience, Bedford, MA) which were frozen at -20ºC. Samples were shipped on dry ice to Taipei Medical University for analysis.

Frozen urine samples were thawed at room temperature, dispersed by ultrasonic wave, and filtered through a Sep-Pak C18 column to remove protein (Mallinckrodt Baker In., NJ). An aliquot of 200 ml was used to determine arsenic species. Urinary arsenic species, arsenite (As_3), arsenate (As_5), monomethyl arsonic acid (MMA) and dimethyl arsinic acid (DMA) were separated by High Performance Liquid Chromatography (Waters 501; Waters Associates, Milford, MA, USA) using a Nucleosil 10u SB 100A column (Phenomenex, Torrance, CA, USA). Individual species were then detected using Hydride Generated

Atomic Absorption Spectrometry (Perkin-Elmer Flow Injection Analysis System 400-AA 100, USA) as described by Hsueh *et al.* (1998). This analytical approach eliminates interference from arsenobetanine and arsenocholine, non-toxic organic arsenic species found in seafood. This method was evaluated using freeze-dried urine (SRM 2670-2 (Elevated) National Institutes of Standards and Technology, Gaithersburg, MD) containing 480 ± 100 μgAs/L. A value of 507 ± 17 μgAs/L (N = 4) was achieved.

The average limit of detection, determined by 115 method blanks run on separate days, for As_3, As_5, MMA, and DMA were 0.04 ug/L, 0.06 μg/L, 0.05 ug/L, and 0.06 ug/L respectively. Quality control procedures included spiked samples where a known amount of As_3, As_5, MMA, and DMA standard reagent was added to one sample within each batch. The average percent recovery for 348 spiked samples for As_3, As_5, MMA, and DMA was 98.9 ± 6.5%, 100 ± 6.5%, 99.9 ± 6.4%, and 100.1 ± 6.5% respectively. Replicates of standard solutions were also analyzed during each laboratory day and all were ± 5% for each arsenic specie. Specifically, the percent difference for As_3, As_5, MMA, and DMA were -1.0 ± 3.5%, 0 ± 3.9%, -0.3 ± 3.4%, and -1.3 ± 3.4%.

Urinary creatinine was measured using the kinetic Jaffe Method. Under alkaline conditions, creatinine binds with picric acid (2,4,6-trinitrophenol) forming an orange-red complex, which was measured at 505 nm using an Hitachi 7170S autoanalyzer (Tokyo, Japan).

2.4 Genotyping

Multiplex polymerase chain reaction amplifications were performed from genomic DNA extracted from whole blood following the Puregene Protocol (Gentra Systems, Minnesota). Genotyping of *GSTM1* and *GSTT1* followed the protocol described by Liu *et al.* (2001). Homozygosity for the *GSTM1* and *GSTT1* null allele was indicated by the presence of an internal control product in concurrence with the absence of a 230 and 480 base pair fragment, respectively. Genotyping procedures were validated by randomly selecting 5% of the samples and subjecting them to repeat analysis. Two researchers independently reviewed all genotyping results. The concordance rate for all duplicate samples was 100%. All SNPs passed the Hardy-Weinberg equilibrium chi-squared test with p-value > 0.05.

2.5 Statistical Analysis

While arsenic metabolites are stable in frozen urine, it is possible that As_3 will convert to As_5 after 6 months (Chen *et al*. 2002). Subsequently, As_3 and As_5 were summed to define As_{in}. Total urinary arsenic (TUA μg/L) was defined as the sum of the four arsenic metabolites As_3, As_5, MMA and DMA. Literature commonly reports total urinary arsenic per gram creatinine (TUA μg/g) and therefore both outcomes were included in our analysis. Arsenic metabolism was evaluated using the ratios of metabolites. Primary, secondary and total methylation index were defined as MMA/As_{in}, DMA/MMA, and $(MMA+DMA)/As_{in}$, respectively. Descriptive statistics were generated for each outcome.

Tubewell arsenic concentrations, total urinary arsenic concentrations, primary, secondary, and total methylation were all positively skewed and subsequently transformed to their common logarithms. Linear mixed effects regression models were used to evaluate the relationship between urinary arsenic outcomes and tubewell arsenic concentrations. A random intercept was included to account for autocorrelation among repeated measures on a single participant. All statistical goals were achieved by estimating parameters in the following model: $Y_i = X_i\beta + Z_i b_i + \varepsilon_i$ where Y_i denotes any of the four urinary arsenic indices for the i^{th} individual; X_i and Z_i are the fixed covariate matrixes, β is a vector of corresponding population slope parameters, and b_i is a vector of random intercepts (i^{th} individual) which allowed each participant to have an intercept that deviated from the population mean, and ε represents the unknown random error. Different covariance structures for the random effects matrix were evaluated using restricted maximum likelihood (REML) and ultimately the default "variance components" (VC) was selected because it had the lowest Akaike Information Criterion (AIC) score. Many covariates were evaluated in each model including genetic polymorphisms in *GSTT1* and *GSTM1*, environmental tobacco smoke, chewing betel nuts, gender, age, urinary creatinine, duration of tubewell use, season, diabetes, body mass index (BMI), and BMI^2. Covariates that were not significant ($\alpha < 0.05$) were removed to achieve the most parsimonious model. The residuals from all models were evaluated and heavy-tailed distributions were observed. Thus, empirical standard error which computes the estimated variance-

covariance matrix of the fixed-effects parameters by using the asymptotically consistent estimator, commonly known as the "sandwich estimator" was reported. Two outliers were identified in the analyses, and all models were analyzed both with and without these outliers. Removing the outliers did not change the results in any meaningful way. Subsequently, all models include all observations. SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for all calculations.

Table 1: Population characteristics of the 197 adults included in this study. Standard deviations are calculated by assuming that all observations are independent. by assuming that all observations are independent.

	Mean(SD)	Median	Range
Male	42.9%		
GSTT1 (null)	18.4%		
GSTM1 (null)	35.1%		
Age	33.0 ± 13.7	30	15 - 77
Body Mass Index	20.5 ± 3.5	20	13 - 30
Tubewell Arsenic (μg/L)	79.0 ± 122.8	9.7	< 1 - 752
Total Urinary Arsenic (μg/L)	45.0 ± 59.6	28.4	0.66 - 845.9
Total Urinary Arsenic (μg/g creatinine)	104.4 ± 138.1	66.81	8.97- 2128.33
Urinary As_{in}(μg/L)	6.35 ± 11.02	3.49	< 0.10 - 161.96
Urinary MMA (μg/L)	5.05 ± 9.21	2.55	< 0.05 - 126.36
Urinary DMA (μg/L)	34.58 ± 42.39	21.53	< 0.06 - 557.61
% As_{in}	13.42 ± 8.36	12.24	0 - 91.12
% MMA	9.38 ± 6.63	9.07	0 - 38.91
% DMA	77.21 ± 10.52	78.15	8.02 - 100
Primary methylation (MMA/As_{in})	0.95 ± 1.39	0.72	–0.28 - 25.16
Secondary methylation (DMA/MMA)	23.60 ± 228.79	7.75	–1132.76 - 5617.89
Total methylation ($MMA+DMA/As_{in}$)	35.93 ± 42.62	22.95	0.06 - 558.39

3. RESULTS

In total, 197 individuals from 48 households provided 739 urine samples that were analyzed for arsenic. Of those samples, 13 were missing creatinine measurements and were not included in the subsequent analysis. Furthermore, 9 samples had creatinine levels below 5 mg/dL and 1 sample had more than 300 mg/dLcreatinine and were excluded (Barbanel *et al*. 2002). Thus 187, 173, 176, and 181 urine samples were included from quarter 1-4, respectively. Missing samples were considered to be random. Of the samples with observations, 0.14% had a DMA concentration below the LOD, while 14% and 10.3% of the samples had As_{in} or MMA concentrations below the LOD. The reported urinary arsenic concentrations were used in all analysis instead of assigning half the LOD.

Participant characteristics are described in Table 1. The average adult participant was female, 33 years of age, and had a normal body weight. Median tubewell arsenic concentrations were 9.7 μg/L (range: < 1 – 752). For those participants whose tubewell had non-detectable arsenic concentrations, median total urinary arsenic concentrations were 18.1 μg/L (range: 0.66 – 135.4) and 48.5 μg/g creatinine (range: 8.9 – 346.9), whereas for participants with tubewell having detectable arsenic levels median total urinary arsenic

Table 2: A multivariate linear regression model including all factors that significantly influenced total urinary arsenic concentrations in 197 adults.

	Log_{10} Total Urinary Arsenic (μg/L)			
Effect	Estimate	SE	95% CL	p-value
Intercept	0.05	0.05	(-0.05, 0.16)	0.30
Log_{10}Tubewell As (μg/L)	0.10	0.01	(0.08, 0.13)	< 0.001
Monsoon	0.09	0.02	(0.05, 0.12)	< 0.001
Summer	0.12	0.02	(0.09,0.16)	< 0.001
Winter	0.000			
Log_{10}Creatinine (mg/dL)	0.73	0.03	(0.68, 0.78)	< 0.001
Duration of well use (yr)	0.01	0.003	(0.004, 0.01)	< 0.001

concentrations were 40.0 μg/L (range: 2.8 – 845.9) and 78.8 μg/g (range: 14.2 – 2128.4).

Linear mixed effect models found total urinary arsenic (TUA) concentrations were significantly associated with the concentration of arsenic in drinking water, season in which the urine sample was collected, urinary creatinine levels, and duration of tubewell use (Table 2). GSTT1 was modestly associated with increased TUA (β = 0.08, SE = 0.04, p-value = 0.06) in adjusted models whereas GSTM1 did not appear to be associated with TUA (β = -0.04, SE = 0.03, p-value = 0.21). Normalizing total urinary arsenic (TUA/g) for creatinine concentrations or including creatinine as a covariate in linear regression models generated essentially the same slope for tubewell arsenic. Subsequently, we have only included model results that adjusted for creatinine as an independent variable and not TUA/g. Adjusting for all other covariates, every 10-fold increase in tubewell As resulted in a 1.26-fold increase in TUA for a participant of median age. In addition, urine samples collected in the monsoon season (June– September) and summer season (March – June) had more TUA compared to those collected in winter (October – February). This could be a reflection of increased water consumption due to hotter weather experienced in these seasons or a change in dietary sources that led to increased ingestion of arsenic-contaminated foods. Gene-environment interactions were evaluated by including an interaction term in the models. We observed that individuals who possess the null genotype had a slightly higher excretion rate for arsenic given any drinking water exposure compared to individuals with a wildtype GSTT1 genotype after adjusting for season, urinary creatinine, duration of tubewell use (β = 0.09, SE = 0.03, p-value = 0.004). However, no significant gene-environment interaction was detected for GSTM1 in similarly adjusted models (β = 0.05, SE = 0.03, p-value = 0.08).

Primary methylation, which could approximate first-pass metabolism of inorganic arsenic, was increased in participants who possessed the GSTT1 genotype (Table 3). Other factors that were also associated with primary methylation included sex, age, and season. Specifically, males and increasing age were associated with increased primary methylation. Interestingly, the season in which the urine samples were collected also influenced primary methylation with samples collected in the monsoon season have lower primary methylation. While it is unknown why arsenic methylation would change with season, it is possible that this is a function in dietary changes during this period. The same factors were also observed to influence secondary methylation (DMA/MMA) although in an opposite directions (Table 3). Thus, those factors that were associated with increased primary methylation but decreased secondary methylation are related to impaired arsenic methylation capacity as indicated by an increased proportion of MMA being excreted in urine. No association was observed between GSTM1 and either primary or secondary methylation indices.

The factors that were associated with total methylation are presented in Table 4. Unlike the other

Table 3: Adjusted effects of parameters for $\log_{10}$ primary methylation (MMA/As_{in})and log10 secondary methylation (DMA/MMA) where winter is the comparison group.

Parameter	$\log_{10}$ Primary Methylation					$\log_{10}$ Secondary Methylation				
	Estimate	SE	95% LCL	95% UCL	p-value	Estimate	SE	95% LCL	95% UCL	p-value
Intercept	–0.214	0.030	–0.264	–0.144	< 0.0001	1.070	0.030	1.010	1.130	< 0.0001
GSTT1 (null)	0.126	0.040	0.034	0.215	0.0100	–0.090	0.040	–0.180	–0.170	0.0300
Gender (male)	0.119	0.040	0.050	0.190	0.0002	–0.240	0.040	–0.180	–0.010	< 0.0001
Monsoon	–0.082	0.040	–0.156	–0.007	0.0300	0.070	0.030	0.010	0.140	0.0300
Summer	0.015	0.040	–0.055	0.086	0.6700	0.001	0.030	–0.070	0.070	0.9700
Age (years)	0.004	0.001	0.002	0.007	0.0002	–0.003	0.001	–0.003	0.002	0.8000

Table 4: Factors associated with $\log_{10}$ total methylation index (MMA+DMA/As_{in}) where winter is the reference season. All estimates are adjusted for the other parameters in the table.

Log_{10} Total Methylation					
Parameter	Estimate	SE	95% LCL	95% UCL	p-value
Intercept	–0.830	0.441	–1.699	0.039	0.0600
Gender (male)	–0.100	0.031	–0.161	–0.038	0.0020
Log_{10}Tubewell As (μg/L)	0.100	0.014	0.072	0.128	< 0.0001
Log_{10}Creatinine (mg/dL)	0.725	0.037	0.652	0.798	< 0.0001
Monsoon	0.086	0.018	0.050	0.122	< 0.0001
Summer	0.127	0.019	0.089	0.165	< 0.0001
Age (years)	0.003	0.001	0.000	0.005	0.0400
BMI	0.091	0.041	0.011	0.171	0.0300
BMI-squared	–0.002	0.001	–0.004	0.000	0.0200

indices, total methylation index was observed to increase with tubewell arsenic concentration, creatinine, season, age and body mass index. Total methylation was lower in males compared to females. Whereas, arsenic concentration in drinking water, urinary creatinine, season, age and bmi-squared were positive associated with total methylation. The association between drinking water arsenic concentrations and total methylation was surprising and may indicate that total methylation increases with exposure, a finding that warrants further investigation. No associations were observed between GSTM1 and total methylation. Nor were any gene-environment interactions observed between genotype and drinking water arsenic on total methylation.

4. DISCUSSION

By evaluating the association between drinking water exposures and urinary arsenic metabolites, we were able to identify parameters including genetic factors that influenced arsenic methylation capacity. This information is useful because it helps to explain inter-individual variations in biomarker response. Notably, individuals who were GSTT1 null or male excreted a higher proportion of MMA in their urine. A number of studies have reported that individuals who excrete a higher proportion of MMA or have a higher primary methylation ratio have an increased risk of arsenic related disease.

As expected, there was a positive relationship between drinking water arsenic concentration and total urinary arsenic. Although this relationship was not as strong as what has been reported in the United States where Calderon *et al.* (1999) reported log-linear slopes ranging from 0.626 to 0.659. However, our estimates were similar to what others have reported in Bangladesh. Watanabe (2001) observed that the slope of the log-linear relationship between tubewell arsenic and TUA µg/g creatinine ranged from 0.294 – 0.356 in males and 0.320 – 0.328 in females, which equates to a 1.97 – 2.27-fold increase in TUA µg/g creatinine for every 10-fold increase in tubewell arsenic exposure. Our analysis found a 1.26-fold increase in TUA µg/g for every 10-fold increase in tubewell arsenic exposure, although our models adjusted for additional covariates which could explain the small differences in slopes. Additionally, we observed a similar gender effect as those observed by both Calderon and Watanabe where males excreted less total urinary arsenic compared to females. Furthermore, primary methylation and secondary methylation was influenced by gender suggesting that females have a greater arsenic methylation capacity than males. It is also interesting to note that arsenic exposure, as measured in personal drinking water samples, was not associated with primary or secondary methylation capacity suggesting that arsenic exposure was not contributing to a change in the proportion of MMA. However, exposure was associated with total methylation capacity (MMA + DMA); a relationship that has not been observed previously and warrants further investigation.

In humans, inorganic arsenic is methylated via one-carbon metabolism, a biochemical pathway dependent upon folate, vitamin B_{12} (cobalamin) and vitamin B_6 for the recruitment of methyl groups. Animal studies have suggested that diets deficient in folate or protein led to decreased production of DMA and diminished total urinary arsenic excretion which ultimately result in an increased retention of arsenic in tissues (Vahter and Marafante 1987, Spiegelstein *et al.* 2003). A cross-sectional study in arsenic-exposed individuals in Bangladesh found that the percent of

methylated arsenic metabolites was influenced by folate, specifically the percentage of DMA in urine was positively associated with plasma folate whereas the percentage of As_{in} and MMA in urine was negatively associated with plasma folate concentrations (Gamble *et al*. 2005). Another study conducted in the United States measured the concentration of micro-nutrients in plasma serum and ratios of urinary arsenic metabolites observed that low intake of dietary protein, iron, zinc, and niacin decreased the production of DMA and increased the concentration of MMA in arsenic-exposed individuals (Steinmaus *et al*. 2005).

Dietary intakes of selenium and vitamin E also appear to influence arsenic metabolism. For instance, in a historically arsenic-contaminated region of Taiwan, a positive relationship was found between total urinary arsenic, selenium concentrations in both urine and serum, and α-tocopherol (Hsueh *et al*. 2003). Considering that arsenic metabolism could influence an individual's susceptibility to arsenic-induced disease by altering the ratio of urinary arsenic metabolites further research is warranted on investigating the influence of diet on arsenic metabolism.

Rather than considering total urinary arsenic as a biomarker of exposure, it might be more relevant to consider urinary arsenic as a biomarker of internal dose and the individual arsenic metabolites as biomarkers of biologically relevant dose on the exposure to disease continuum. In this regard, genetic polymorphisms in GSTT1 could influence arsenic induced disease by altering the dose of active agent. Participants with GSTT1 *null* genotypes had a significantly higher primary methylation index and a significantly lower secondary methylation index. In addition, at any given tubewell arsenic concentrations GSTT1 *null* genotypes excreted more total urinary arsenic compared to GSTT1 *wildtype* genotypes.

Thus it appeared that glutathione-s-transferase theta but not glutathione-s-transferase mu influenced the relative concentration of methylated urinary arsenic metabolites. This observation is supported by a study from Taiwan that found participants with GSTT1 *null* genotypes had an elevated percentage of DMA in urine (Chiou *et al*. 1997). Furthermore, researchers have reported that GSTT1 *null* individuals have an increased risk of developing bladder cancer and arsenic-induced skin disease (Chen *et al*. 2004, McCarty 2005). Therefore, it appears that GSTT1 is involved in arsenic metabolism and can influence the internal dose of methylated arsenic although more research is needed before its role can be fully elucidated.

There are several potential limitations in this study. It is possible that there was misclassification of exposure in this study since drinking water samples were collected at the same time as urine samples and the half-life of arsenic in urine is three days. It is also possible that some participants utilize more than one source of drinking water during the day if they travelled away from their home and by measuring only the drinking water at the participant's home, we have missed additional sources of exposure. Also, we did not adjust for the amount of water consumed by each individual which could influence the concentration of urinary arsenic metabolites. Nor did we account for any other potential sources of ingested arsenic, such as diet which can be an additional source of arsenic exposure. Other limitations include only evaluating two GST genotypes.

In conclusion, several factors were identified that influenced arsenic excretion and arsenic methylation capacity in a healthy population. Future studies are needed to determine if individuals who have the *GSTT1* deletion polymorphisms have a greater susceptibility to arsenic-related disease.

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