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Abbreviations:

As	arsenic
As^{III}	arsenite
As^{V}	arsenate
bAs	blood arsenic
BMI	body mass index
CBS	cystathionine beta synthase
CV	coefficient of variation
Cys	cysteine
CySS	cystine
DMAs(V)	dimethylarsinic acid
E_h	reduction potential
FOX	folate and oxidative stress study
GCL	γ-glutamate-cysteine ligase

GFAA	graphite furnace atomic absorption
GSH	glutathione
GSSG	glutathione disulfide
HEALS	Health Effects of Arsenic Longitudinal Study
ICP-MS	inductively coupled plasma-mass spectrometry
InAs	inorganic arsenic
$MMAs^V$	monomethylarsonic acid
ROS	reactive oxygen species
SAM	S-adenosylmethionine
uAs	urinary arsenic
wAs	water As

ABSTRACT

Background: *In vitro* and rodent studies have shown that arsenic (As) exposure can deplete glutathione (GSH) and induce oxidative stress. GSH is the primary intracellular antioxidant; it donates an electron to reactive oxygen species producing glutathione disulfide (GSSG). Cysteine (Cys) and cystine (CySS) are the predominant thiol/disulfide redox couple found in human plasma. Arsenic, GSH, and Cys are linked in several ways. First, GSH is synthesized via the transulfuration pathway and Cys is the rate limiting substrate. Second, intermediates of the methionine cycle regulate both the transulfuration pathway and As methylation. Third, GSH serves as the electron donor for reduction of arsenate to arsenite. Fourth, As has a high affinity for sulfhydryl groups and therefore binds to GSH and Cys.

Objectives: To test the hypotheses that As exposure is associated with decreases in GSH and Cys and increases in GSSG and CySS, i.e., a more oxidized environment.

Methods: For this cross-sectional study, the Folate and Oxidative Stress Study, we recruited a total of 378 participants from each of 5 water As categories: <10 (n=76), 10-100 (n=104), 101-200 (n=86), 201-300 (n=67), and >300 µg/L (n=45). Concentrations of GSH, GSSG, Cys, and CySS were measured using HPLC.

Results: An interquartile range (IQR) increase in water As was negatively associated with blood GSH (mean change = -25.4 µmol/L; 95% CI: -45.3, -5.31) and plasma CySS (mean change = -3.00 µmol/L; 95% CI: -4.61, -1.40). Similar associations were observed with urine and blood As. There were no significant associations between As exposure and blood GSSG or plasma Cys.

Conclusions: The observed associations are consistent with the hypothesis that As may influence concentrations of GSH and other non-protein sulfhydryls through binding and irreversible loss in bile and/or possibly in urine.

Introduction

Arsenic (As) is a class I human carcinogen that causes several types of cancer (IARC 2004). Chronic As exposure through contaminated drinking water has also been linked to increased mortality (Argos et al. 2010) and both coronary (Chen et al. 2011; States et al. 2009) and respiratory diseases (Parvez et al. 2008; Parvez et al. 2010). Approximately 140 million people worldwide are chronically exposed to As-contaminated drinking water at concentrations greater than 10 µg/L; over 57 million of these exposed individuals live in Bangladesh (World Bank; Kinniburgh and Smedley 2001).

Inorganic As (InAs) undergoes methylation, via nutrient-dependent one-carbon metabolism, first to monomethylarsonic acid ($MMAs^V$), and then to dimethylarsinic acid ($DMAs^V$) (Figure 1). Arsenic metabolites differ in toxicity and individuals vary in their ability to methylate InAs. Epidemiologic studies have shown that a lower capacity to methylate InAs to DMA^V, based on relative amounts of As metabolites in urine, is positively associated with risk for several diseases (Steinmaus et al. 2010). The precise mechanisms through which As causes disease are poorly understood, although oxidative stress, defined as an imbalance between antioxidant defense and the total burden of potentially harmful reactive biochemical species, is often cited as a likely mechanism (Jomova et al. 2011).

Glutathione (GSH), a tripeptide consisting of cysteine (Cys), glutamic acid, and glycine, is a critical component of the body's primary antioxidant defense mechanism (Forman et al. 2009). It is also known to serve as the electron donor for the reduction of arsenate (As^V) to arsenite (As^{III}) (Scott et al. 1993). GSH can detoxify reactive oxygen species (ROS) directly and is also a substrate for glutathione peroxidase, a selenoprotein that enzymatically reduces H_2O_2 and lipid

hydroperoxides (Lu 2009). Detoxification of ROS produces the oxidized metabolite glutathione disulfide (GSSG), which can be reduced back to GSH through the action of glutathione reductase. Another critical function of GSH is to provide a reservoir of Cys in order to maintain overall sulfur amino acid balance (Figure 1). Cells can tolerate only low concentrations of Cys, which is unstable and rapidly oxidizes to cystine (CySS), producing potentially toxic oxygen free radicals (Lu 2009).

A decrease in GSH and increase in GSSG is indicative of chronic oxidative stress, and severe oxidative stress has been shown to deplete GSH (Lu 1999). Furthermore, changes in the reduction potential (E_h) of the GSH/GSSG and Cys/CySS thiol/disulfide pairs may influence redox-sensitive signaling pathways and enzymes (Jones 2002). Concentrations of reduced and oxidized forms of a paired redox couple can be used to estimate the redox state (in mV) with the Nernst equation (Jones et al. 2002). The GSH/GSSG thiol/disulfide redox couple maintains a highly reduced intracellular state that controls cell cycle progression and intracellular antioxidant defenses (Schafer and Buettner 2001). In plasma, Cys and CySS are present at much higher concentrations than GSH and GSSG, and are thus the primary determinant of the extracellular redox state (Jones et al. 2002). The high intracellular (1-10 mM) and lower extracellular (1-10 μM) concentrations of GSH might render extracellular surfaces more vulnerable to oxidative damage (Lu 2009; Smith et al. 1996); this may be partially counteracted by the high extracellular concentrations of the Cys/CySS redox pair.

One-carbon metabolism is the biochemical pathway through which As and numerous other substrates are methylated (Figure 1). A portion of homocysteine generated through one-carbon metabolism is remethylated to form methionine, which is then activated to form the methyl donor, s-adenosyl methionine (SAM). Alternatively, homocysteine can be directed to the

transsulfuration pathway and used in the synthesis of GSH (Figure 1). Under conditions of oxidative stress, shunting of homocysteine to the transulfuration pathway is increased through the upregulation of the GSH biosynthetic enzymes, including γ-glutamate-cysteine ligase (GCL), the rate limiting enzyme of GSH synthesis (Lu 2009). Although most cell types can synthesize GSH, the liver is the major site of synthesis.

There are several mechanisms through which As may influence concentrations of GSH and GSSG. These include 1) inducing the formation of ROS which are then detoxified by GSH, 2) consuming GSH during the reduction of As^{V} to As^{III} (Scott et al. 1993), 3) forming As/GSH complexes which are substrates for the ATP-binding cassette membrane transporters that mediate efflux from cells (Thomas 2009) (Leslie et al. 2004), and 4) inhibiting glutathione reductase, thereby limiting the regeneration of GSH from GSSG (Massey and Williams 1965; Styblo and Thomas 1995; Styblo et al. 1997). *In vitro* and animal studies have shown that As can deplete GSH (Santra et al. 2000). Thus, the depletion of GSH may be one mechanism through which As could lead to oxidative stress. However, given the well-known species differences in As metabolism and susceptibility to As-induced health effects (Drobna et al. 2010), additional data derived from human population studies is needed.

We conducted a cross-sectional study of 378 Bangladeshi adults to test the primary hypothesis that chronic As exposure is associated with reductions in GSH and increases in GSSG. We also wished to test the secondary hypothesis that chronic As exposure is associated with reductions in plasma Cys and increases in CySS.

SUBJECTS AND METHODS

***Participants and procedures*.** Participants in the current study, the Folate and Oxidative Stress (FOX) Study, were recruited between February and July of 2008 in Araihazar, Bangladesh. Potential participants were identified based on well water As (wAs) concentrations, dating from a well survey in the year 2000 (Van Geen et al. 2002), in order to ensure a wide range of As exposures to examine dose-dependent relationships. A new water sample was collected at the time of enrollment for analysis of wAs concentration. Individuals were eligible to participate in FOX if they were: 1) between the ages of 30 and 65, 2) not pregnant and did not plan to become pregnant within 3 months; 3) not taking nutritional supplements; 4) did not have known diabetes, cardiovascular or renal disease, chronic obstructive pulmonary disease, or cancer, and 5) had been drinking from their current well for at least 3 months. Trained recruiters identified eligible participants, explained the nature of the study, obtained informed consent, and scheduled a field clinic visit. All visits were conducted in the laboratory at our field clinic in Araihazar because GSH is unstable, and therefore requires that blood samples be processed immediately. During the field clinic visit, a trained interviewer administered a detailed questionnaire to each participant and a physician collected a venous blood sample. Urine samples were collected in 50-mL acid-washed polypropylene tubes and frozen at –20°C.

The primary aim of this study was to examine the dose-response relationship between As exposure and measures of oxidative stress. We aimed to recruit 75 participants each from the following 5 water As categories: <10, 10-100, 101-200, 201-300, and >300 µg/L. However, the final sample included more participants with lower exposures because many households switched to lower As wells after wells in the region were surveyed for As in 1999-2000 (Chen et

al. 2007). Therefore, the final distribution among well wAs exposure categories was: <10 (n=76), 10-100 (n=104), 101-200 (n=86), 201-300 (n=67), and >300 µg/L (n=45). .

Oral informed consent was obtained by our Bangladeshi field staff physicians, who read an approved assent form to the study participants. This study was approved by the Bangladesh Medical Research Council and the institutional review board of Columbia University Medical Center.

Sample collection and handling. After the initial processing of blood samples in the field clinic, the blood and plasma aliquots were immediately frozen at -80°C. Samples were transported to Dhaka in batches by car on dry ice and again stored in -80°C (blood and plasma) or -20°C (urine) freezers. Samples were then packed on dry ice in coolers and flown to Columbia University.

Water As. Field sample collection and laboratory analyses procedures are described elsewhere in detail (Cheng et al. 2004; Van Geen et al. 2005). Water samples were collected in 20-mL polyethylene scintillation vials. The samples were acidified to 1% with high-purity Optima HCl (Fisher Scientific, Pittsburg, PA, USA) at least 48 hr before analysis (van Geen et al. 2007). Water samples were analyzed by high-resolution inductively coupled plasma mass spectrometry after 1:10 dilution and addition of a Ge spike to correct fluctuations in instrument sensitivity. The detection limit of the method is typically < 0.2 µg/L. A standard with an As concentration of 51 µg/L was run multiple times in each batch. The intra- and inter-assay coefficients of variation (CVs) for this standard were 6.01% and 3.76%, respectively.

Total urinary As. All urine samples were analyzed for total urinary As (uAs) in the Columbia University Trace Metals Core Laboratory by GFAA spectrometry (Nixon et al. 1991) using the

AAnalyst 600 graphite furnace system (PerkinElmer, Shelton, CT, USA). A method based on the Jaffe reaction was used to measure urinary creatinine concentrations (Slot 1965). Method and instrument precision was checked by running 4 different urine samples with known concentrations (to cover the whole linearity range of the standard curve) every day immediately after the instrument calibration with aqueous standards. A urine sample with As concentration in the middle of the linearity range was run after every 10 study samples. The intra- and inter-assay CVs based on this quality-control sample were 3.9% and 5.6%, respectively. For duplicate study samples, the intra- and inter-assay CVs were 3.8% and 5.1%, respectively.

Total blood As. As described previously (Hall et al. 2006), we used a Perkin-Elmer Elan DRC II ICP-MS equipped with an AS 93+ autosampler to analyze whole blood samples for total blood As (bAs) concentration**.** The intra- and inter-assay CVs were 3.2% and 5.7%, respectively, for quality-control samples. For study samples, the intra- and inter-assay CVs were 2.1% and 4.9%, respectively.

Blood GSH and GSSG, plasma Cys and CySS. Whole blood GSH and GSSG and plasma Cys and CySS were assayed essentially as described by Jones et al. (Jones et al. 1998). Blood was collected with a butterfly needle and syringe and then immediately transferred into Eppendorf tubes. For whole blood measurements, the Eppendorf tubes contained 5% perchloric acid (PCA), 0.1 M boric acid, and γ-glutamyl glutamate as an internal standard. For plasma measurements, the tubes contained 0.53 gm L-serine, 25 mg heparin, 50 mg bathophenanthrolene, 300 mg iodoacetic acid, and 10 ml Borate Buffer stock. The Borate Buffer stock consisted of 12.4 gm boric acid, 19 gm sodium tetraborate decahydrate, and 500 ml distilled water. The samples for plasma measurements were centrifuged for 1 min., and 200 µl of supernatant was transferred into Eppendorf tubes containing an equal volume of 10% PCA

and 0.2 M boric acid. For derivatization, plasma samples were centrifuged at 13,000 rpm for 2 minutes, 300 µl of supernatant was transferred to a fresh tube, and the pH was adjusted to 9.0. After incubating for 20 minutes at room temperature, dansyl chloride was added, and samples were incubated at room temperature in the dark for 24 hrs. The derivatized samples were then stored at -80°C until delivered to Columbia University for analysis. Free dansyl chloride was extracted from thawed samples with 500 µl chloroform and then 20 µl of the sample was injected onto the HPLC. Separation was achieved using a Supelcosil LC-NH_2 column (Sigma Cat# 58338). Initial solvent conditions were 60% A (80% methanol, 20% H_2O), 40% B (acetate-buffered methanol pH 4.6) run at 1 ml/min for 10 minutes. A linear gradient to 20% A, 80% B was run over the period from 10 to 50 minutes. From 50 to 52 min, the conditions were returned to 60% A, 40% B. Metabolites were detected using a Waters 474 scanning fluorescence detector, with 335 nm excitation and 515 nm emission (Waters Corp., Milford, MA). Within-assay CVs were all between 0.05 and 0.10 and inter-assay CVs were between 0.11 and 0.18.

Plasma folate. Plasma folate was analyzed by radioproteinbinding assay (SimulTRAC-S, MP Biomedicals, Orangeburg, NY). For folate concentration determination, folic acid as pteroylglutamic acid was used for calibration, and its ^{125}I-labeled analog was used as the tracer. The intra- and inter-assay CVs were 0.06 and 0.14, respectively.

Calculation of the Reduction Potential. The reduction potential of the thiol/disulfide GSH/GSSG and Cys/CySS redox pairs (blood GSH Eh and plasma Cys Eh, respectively) were calculated using the Nernst equation ($E_h = E_o + RT/nF \ln [\text{disulfide}]/[\text{thiol}]^2$ where E_o= standard potential for the redox couple, R= gas constant, T= absolute temperature, n=2 for the number of electrons transferred, and F= Faraday's constant) (Jones et al. 2002). A more positive Eh value indicates a more oxidized redox state.

***Statistical method*s.** We calculated descriptive statistics for characteristics of the study sample, As exposure variables (wAs, uAs, and bAs), and outcome variables (blood GSH and GSSG, plasma Cys and CySS), both for the total sample and by gender. Bivariate associations were examined using scatterplots and Spearman's correlation coefficients. To examine the bivariate associations between dichotomous covariates and As exposure variables or continuous outcome variables we used t-tests or the non-parametric Wilcoxon rank sum test.

We used linear regression models to further examine the associations between As exposure variables, as continuous variables, and the outcome variables, with and without adjustment for potential confounders. Age and sex were included in all covariate-adjusted regression models. Other covariates considered for inclusion in the regression models were variables reported to be associated with the exposures or outcomes based on previous publications and/or variables associated with the exposure and outcome variables in the present study population. These variables included television ownership (as a surrogate for socioeconomic status), cigarette smoking, BMI, urinary creatinine, and plasma folate. We also adjusted for GSH laboratory batch (as a categorical variable) in order to reduce extraneous variation in the outcome variables. We also calculated the change in R^2 between models for each outcome that included covariates only and corresponding models that included both the covariates and As exposure.

To facilitate comparisons among the different measures of exposure (wAs, uAs, and bAs), we report the estimated change in the mean value of blood GSH, blood GSH Eh, plasma CySS, and plasma Cys Eh associated with an interquartile range (IQR) increase in each exposure. For outcome variables that were natural log transformed (blood GSSG and plasma Cys) we report the ratio of estimated geometric means for an IQR change in As exposure.

To examine possible nonlinear relationships, we also created quintiles of As exposure variables and computed covariate-adjusted mean values of the outcome variables for categories of As exposure; plots of quintile specific adjusted mean values were examined to determine if the association was approximately linear.

We ran separate linear regression models to examine the covariate-adjusted associations between As exposure the outcome variables stratified by sex or by folate status. We then used a Wald test to detect differences in the covariate-adjusted associations between As exposure and outcome variables by sex or by folate status. All analyses were performed using SAS (version 9.2; SAS Institute Inc., Cary, NC); all statistical tests were two sided with a significance level of 0.05.

Results

The general characteristics of the study sample are shown in Table 1. The median age was 42 years, approximately half of the study participants were female, and the median BMI was 19.7. Well water As exposure ranged from 0.4 – 700 µg/L; by design, nearly 80% of the participants had wAs concentrations above the WHO standard (10 µg/L) for drinking water. As we have observed previously, males and females differed on the following characteristics: males had more years of formal education (median - males: 4, females: 1, p=0.001), were more likely to report being ever smokers (males: 69%, females: 5.7%, p<0.0001), and were more likely to have a BMI < 18.5 (males: 40.8%, females: 26.4%, p=0.003). Males were also more likely to have plasma folate concentrations < 9 nmol/L (males: 38.8%, females: 20.6%, p<0.0001), which is indicative of marginal folate status, and total homocysteine concentrations above the cutoff for hyperhomocysteinemia (13 µmol/L) (males: 25.5%, females: 7.2%, p=0.0001). There were other gender differences specific to this study sample; the males were older than the females (median -

males: 44.0, females: 40.0, p=0.001) and had higher bAs concentrations (median - males: 12.3, females: 10.3, p=0.009). Blood GSH Eh and plasma Cys Eh also differed significantly by gender; median blood GSH Eh values for females indicated a more oxidized intracellular redox state compared with males (median - males: -203.2, females: -197.4, p<0.0001) while median plasma Cys Eh values for males indicated a more oxidized extracellular redox state compared with females (median - males: -46.1, females: -49.4, p=0.03).

Several covariates were associated with As exposure variables and/or outcome variables. All 3 measures of As exposure were negatively associated with BMI (see Supplemental Material, Table S1). Plasma folate was positively associated with blood GSSG. Age, BMI, and plasma folate were all positively associated with plasma CySS; age was also negatively associated with plasma Cys. There were also differences by sex, smoking, and television ownership (see Supplemental Material, Table S2). Males had higher blood GSH and lower plasma Cys and blood GSH Eh (indicating a less oxidized redox state) than did females. Ever smokers also had significantly higher bAs than never smokers. Contrary to expectation, ever smokers had higher blood GSH concentrations and lower blood GSH Eh than never smokers. However, most smokers were males and after stratification by gender, there were no statistically significant difference in blood GSH and blood GSH Eh by smoking status (data not shown). Participants who owned a television had lower water and uAs concentrations and higher blood GSH, GSSG, and plasma CySS concentrations than participants who did not own a television.

We did not detect any substantial departures from linearity in the models in which we computed covariate-adjusted mean values of the outcome variables for each quintile of As exposure (data not shown). In models using continuous As exposure variables, all three markers of As exposure were negatively associated with blood GSH (estimated changes in mean blood GSH associated

with an IQR increase in exposure from covariate-adjusted models were -25.4 µmol/L; 95% CI: -45.3, -5.31; p = 0.01 for wAs, -54.0 µmol/L; 95% CI: -90.8, -17.2; p = 0.004 for uAs, and -33.4 µmol/L; 95% CI: -62.8, -3.9; p = 0.03 for bAs) and positively, but not always significantly, associated with blood GSH Eh (change in the estimated mean with an IQR increase in exposure from covariate-adjusted models: 1.19; 95% CI: -0.25, 2.62; p = 0.10 for wAs, 2.72; 95% CI: 0.10, 5.35; p=0.04 for uAs, and 1.73; 95% CI: -0.37, 3.83; p = 0.11 for bAs) (Table 2). The change in R^2 values for blood GSH models were 1.3% for wAs, 1.8% for uAs, and 1.1% for bAs; these values can be interpreted as the proportion of the variance in blood GSH that is accounted for by each As exposure variable after controlling for covariates. There were no significant associations between As exposure and blood GSSG concentrations (Table 2). In unadjusted models, water and bAs were significantly negatively associated with plasma Cys. However, associations were positive (though not statistically significant) after adjustment for covariates. All three markers of As exposure were negatively associated with plasma CySS in both covariate-unadjusted and covariate-adjusted models (estimated changes in mean plasma CySS associated with an IQR increase in exposure from covariate-adjusted models were -3.00 µmol/L; 95% CI: -4.61, -1.40; p=0.0002 for wAs, -3.56 µmol/L; 95% CI: -6.47, -0.66; p=0.02 for uAs, and -3.09 µmol/L; 95% CI: -5.40, -0.79; p=0.009 for bAs) and negatively associated with plasma Cys Eh (estimated changes in mean plasma Cys Eh associated with an IQR increase in exposure from covariate-adjusted models were -2.05 µmol/L; 95% CI: -3.89, -0.21; p=0.03 for wAs, -4.04 µmol/L; 95% CI: -7.34, -0.74; p=0.02 for uAs, and -2.01 µmol/L; 95% CI: -4.65, 0.64; p=0.14 for bAs), indicating As exposure is associated with a more reduced extracellular environment. The change in R^2 values for plasma CySS models with adjustment for As exposure (compared with covariates alone) were 2.7% for wAs, 1.2% for uAs, and 1.4% for bAs. Given that males

and females in this study population differed substantially on several characteristics, all regression models were also run stratified by gender; the directions of associations in males and females were the same as in the overall sample but in some cases did not reach statistical significance due to the smaller sample sizes (data not shown). We also examined the possibility that associations might differ by folate status (deficient: < 9 nmol/L, sufficient: ≥ 9 nmol/L) given the influence of folate on As methylation (Gamble et al. 2006). There were no statistically significant differences in the associations between blood As and any of the outcome variables by folate status (see Supplemental Material, Table S3).

Discussion

In this cross-sectional study of Bangladeshi adults chronically exposed to a wide range of As concentrations in drinking water, we observed that three different measures of As exposure (water, urinary, and blood As) were all negatively associated with blood GSH. Contrary to our primary *a priori* hypothesis, As exposure was not positively associated with blood GSSG, the oxidized form of GSH. Also, contrary to our secondary hypotheses, we observed no association between As and plasma Cys, and a negative, rather than positive, association with plasma CySS.

To our knowledge, only one other population-based study has examined the association between As exposure and GSH concentrations. Xu et al (Xu et al. 2008) recruited 208 participants with high wAs exposure (wAs concentration of 90 µg/L or 160 µg/L) and 59 participants with low wAs exposure (20 µg/L) from three villages in Inner Mongolia, China. Participants with high wAs exposure had significantly lower blood total non-protein sulfhydryl concentrations (NPSH, primarily GSH) than those with low wAs exposure. Xu et al did not report findings for blood GSSG.

Our findings and those of Xu et al are generally consistent with rodent studies showing that high-dose As exposure depletes GSH (Maiti and Chatterjee 2001; Santra et al. 2000). Santra et al reported that mice administered As through drinking water (3200 µg/L) ad libitum had elevated hepatic GSH after 2 months and decreased hepatic GSH after 4 months relative to mice administered As-free water. In male Wistar rats, Maiti and Chatterjee found that a single intraperitoneal dose of sodium arsenite (15,860 µg/kg body weight) resulted in a significant reduction in hepatic GSH.

Despite the evidence suggesting that As exposure is associated with reductions in GSH, the question of whether or not chronic exposure, at doses relevant to human populations, results in oxidative stress per se remains unclear. Some *in vitro* and animal model studies have reported that As exposure led to an increase in the production of ROS and a decline in GSH (Flora et al. 2012; Thompson and Franklin 2010; Zhang et al. 2011). However, these studies tend to use very high doses of As – doses that are often not relevant to actual human exposures. For example, Flora et al administered an As dose of 50 ppm in drinking water (~50,000 µg/L) to mice (Flora et al. 2012). We would not expect very high doses to produce the same effects as the doses to which our study participants were exposed (median 114 µg/L). In addition, there are well known species differences in both As metabolism and susceptibility to As-induced health effects (Drobna et al. 2010).

The negative associations between As exposure and blood GSH and plasma CySS, while statistically significant, accounted for a small proportion of the estimated variance in blood GSH (1.1–1.8%) and plasma CySS (1.2–2.7%). Our findings, as well as the inconsistent findings from other population-based studies examining associations between As exposure and blood markers of oxidative damage (Burgess et al. 2007; Engstrom et al. 2010a; Engstrom et al. 2010b; Pi et al.

2002), raise the possibility that, despite reductions in GSH, As may not induce substantial, sustained oxidative stress that is measurable in blood using these biomarkers at environmentally relevant As exposures. We can't determine from this study what the functional effects of the observed reduction in blood GSH might be; however, alterations in GSH have been linked to the development and/or progression of numerous diseases (Ballatori et al. 2009).

Research by Waalkes and colleagues suggests that human cells can adapt to low-level As exposure through alterations in the one-carbon metabolic pathway (Coppin et al. 2008). Exposure of human prostate epithelial RWPE-1 cells (which do not methylate As) to 5 uM sodium arsenite for up to 16 weeks resulted in reductions in SAM and increases in s-adenosylhomocysteine and homocysteine. In addition, As exposure increased the expression of cystathionine β-synthase (CBS), the enzyme that catalyzes the first step in GSH production via the transulfuration pathway, and increased cellular reduced GSH by 5-fold (Coppin et al. 2008). Collectively, these findings suggest that in RWPE-1 cells low-level As exposure increases shunting of homocysteine to the transulfuration pathway to increase GSH production, resulting in decreased remethylation of homocysteine to SAM. Exposure to As also resulted in increased expression of the ATP-binding cassette protein C1 (ABBC1) and increased efflux of As from the RWPE-1cells (Coppin et al. 2008). Given that *ABBC1* encodes a protein involved in the efflux of a *tri*glutathione-arsenical complex (Leslie et al. 2004), these findings suggest that As may exert a strong influence on GSH concentrations through this mechanism.

We observed a negative association between As exposure and blood GSH but no association with blood GSSG, a finding that is most consistent with the hypothesis that As may reduce GSH through binding and irreversible loss in bile and/or possibly in urine (Kala et al. 2004). This finding also suggests that other proposed As-mediated mechanisms [specifically, GSH depletion

during the detoxification of reactive oxygen species; GSH depletion during the reduction of As^{V} to As^{III} (Scott et al. 1993); or As-mediated glutathione reductase inhibition resulting in reduced regeneration of GSH from GSSG (Styblo et al. 1997)] did not *strongly* influence GSH status in our chronically exposed Bangladeshi study population. Associations between the IQR increases in wAs and bAs and the outcomes were generally quite similar (Table 2). For blood GSH, blood GSH Eh, plasma CySS, and plasma Cys Eh, the magnitudes of the associations were somewhat stronger for an IQR increase in uAs compared with IQR increases in wAs and bAs.

A secondary hypothesis of the present study was that As would be negatively associated with Cys because Cys is required for GSH synthesis. However, our findings showed a positive but not statistically significant association with plasma Cys and a negative association with CySS. The lack of a statistically significant association with Cys may potentially be explained by the fact that free Cys is extremely unstable extracellularly and rapidly oxidizes to CySS (Lu 2009). In fact, one of the functions of GSH is to serve as a storage form of Cys through the γ-glutamyl cycle (Meister and Anderson 1983). Given that Cys is constantly in flux, the measurement of plasma Cys may reflect one snapshot of Cys concentrations rather than the overall Cys status of an individual. In a study designed to examine the long-term stability of blood glutathione and cyst(e)ine (i.e., cysteine + cystine) measurements, Richie et al (Richie et al. 1996) reported low interindividual variability in cyst(e)ine, which suggests that measures of Cys and/or CySS in whole blood or plasma may not be as useful as a biomarker of long-term cyst(e)ine status.

There are a number of factors that influence GSH concentrations (including age, smoking, physical activity, and diet) and an individual's response to As might depend on their background level of oxidative stress. A mathematical model of one-carbon metabolism developed by Reed and colleagues (Reed et al. 2008) predicts that changes in GSH and Cys with oxidative stress

depend on the severity of the oxidative stress. For example, with moderate overall oxidative stress, blood and cytosolic GSH and cytosolic Cys increase while blood Cys decreases. This initial increase in GSH results from the stimulation of enzymes involved in GSH synthesis. With severe oxidative stress, values of all four metabolites decrease sharply (Reed et al. 2008). In our statistical analysis, we did not find evidence of nonlinear relationships between As exposure and the outcome variables (data not shown). In addition, we controlled for several potential confounding factors. However, we were unable to adjust for physical activity, a variable previously shown to be associated with GSH (Rundle et al. 2005), and uncontrolled confounding by this or other unmeasured factors is possible.

In summary, we observed that As exposure was negatively associated with blood GSH and plasma CySS in a population chronically exposed to a wide range of As concentrations in drinking water. These findings, and the lack of association between As exposure and blood GSSG or plasma Cys, suggest that As may exert its greatest influence on GSH through binding and irreversible loss in bile and/or possibly in urine. Our findings do not lend strong support to the hypothesis that As induces levels of oxidative stress that are measurable in blood using these biomarkers and at these environmentally relevant exposures. However, our findings cannot rule out the possibility of As-induced oxidative stress in tissue or cellular compartments that we were unable to measure in healthy human volunteers.

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Table 1. Characteristics of the study sample, for all participants and by gender [a]

Characteristic	Total Sample (n=378)	Females (n=194)	Males (n=184)
Age (yrs)	42.0 (13.0)	40.0 (13.0)	44.0 (14.0)
Education (yrs)	3.0 (5.0)	1.0 (5.0)	4.0 (6.0)
BMI (kg/m^2)	19.7 (5.0)	20.7 (5.0)	19.0 (4.2)
BMI < 18.5 (%)	33.4	26.4	40.8
Ever smoker (%)	36.5	5.7	69.0
Ever betel nut use (%)	42.6	41.2	44.0
TV ownership (%)	58.2	57.2	59.2
Water As (µg/L)	114.0 (190.3)	113.1 (186.9)	114.1 (215.2)
Water As > 10 µg/L (%)	79.9	80.4	79.4
Water As > 50 µg/L (%)	68.3	68.6	67.9
Urinary As (µg/L)	122.5 (223.0)	119.0 (217.0)	126.0 (218.0)
Urinary creatinine (mg/dL)	40.3 (57.0)	36.2 (49.8)	44.0 (57.1)
Urinary As / g creatinine	329.5 (454.0)	360.0 (465.0)	307.5(392.0)
Blood As (µg/L)	10.9 (13.3)	10.3 (12.4)	12.3 (14.3)
Plasma folate (nmol/L)	11.1 (6.5)	12.5 (6.9)	9.7 (5.1)
Folate deficient - <9 nmol/L (%)	29.4	20.6	38.8
Homocysteine (µmol/L)	9.0 (4.3)	7.7 (3.5)	10.7 (5.1)
Hyperhomocysteinemia - >13 µmol/L (%)	16.1	7.2	25.5
Blood GSH (µmol/L)	494.6 (227.3)	436.6 (212.0)	529.7 (209.7)
Blood GSSG (µmol/L)	34.3 (23.4)	34.5 (20.6)	33.9 (25.8)
Blood GSH Eh	-199.9 (14.3)	-197.4 (12.4)	-203.2 (12.3)
Plasma Cys (µmol/L)	3.2 (2.8)	3.5 (2.5)	2.9 (2.9)
Plasma CySS (µmol/L)	55.5 (19.0)	53.6 (19.7)	56.2 (19.0)
Plasma Cys Eh	-48.5 (22.6)	-49.4 (19.3)	-46.1 (24.0)

[a] Values are medians (interquartile range) unless otherwise noted

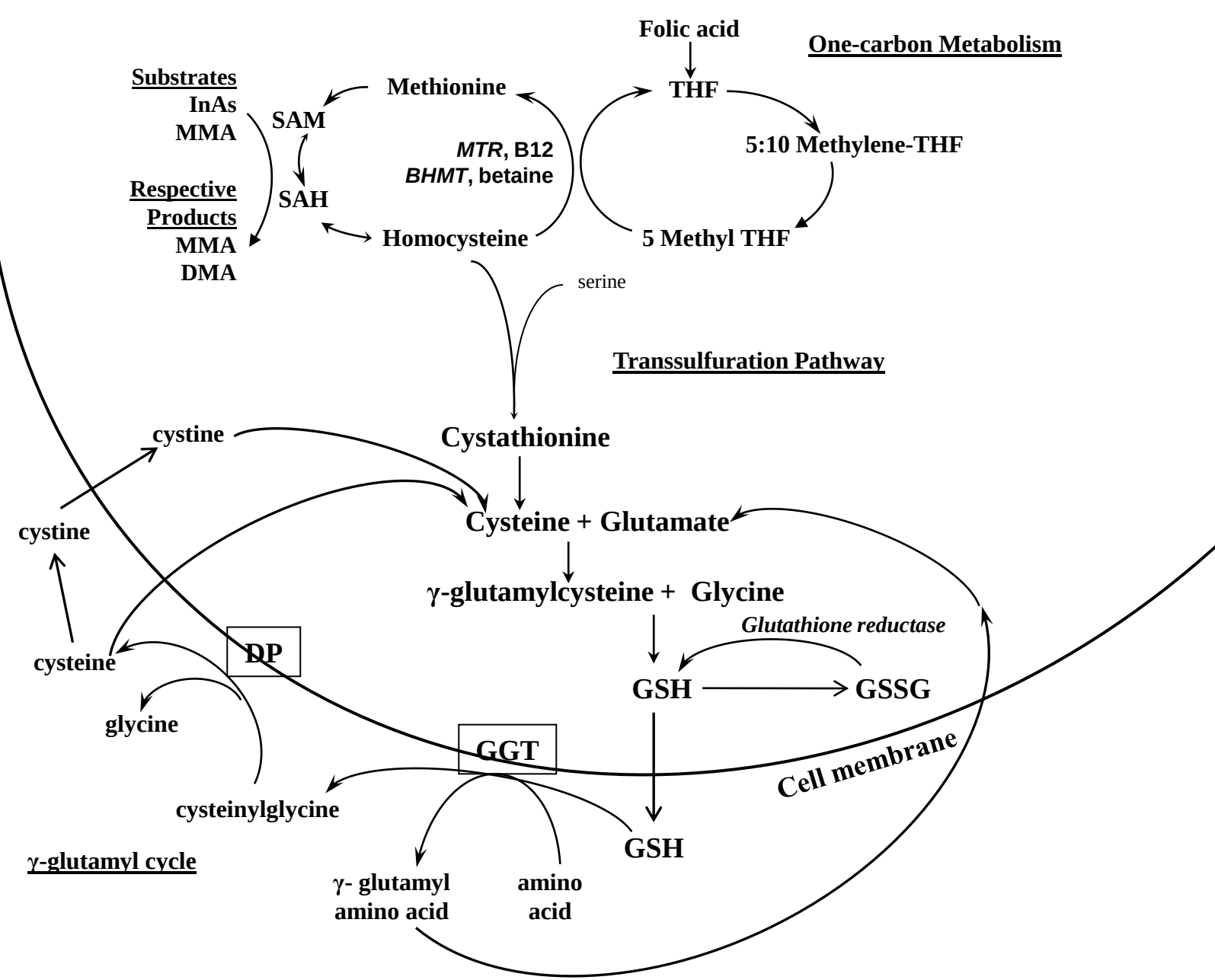
Table 2. Covariate-unadjusted and adjusted effect size estimates for associations between measures of As exposure and GSH, GSSG, Cys, and CySS

	Water As (µg/L)		Urinary As (µg/L)		Blood As (µg/L)	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[a]
Blood GSH (µmol/L)						
Mean change (95% CI)[c]	-33.8 (-49.3, -18.0)	-25.4 (-45,3, -5.31)	-59.6 (-84.0, -35.3)	-54.0 (-90.8, -17.2)	-46.6 (-72.3, -21.1)	-33.4 (-62.8, -3.9)
p-value	<0.0001	0.01	<0.0001	0.004	0.0004	0.03
R^2	4.6	23.5	5.8	24.8	3.3	23.3
ΔR^2		1.3		1.8		1.1
Blood GSSG (µmol/L)						
Mean ratio (95% CI)[d]	1.00 (0.95, 1.04)	0.98 (0.95, 1.02)	1.07 (0.99, 1.14)	0.95 (0.88, 1.05)	0.99 (0.92, 1.06)	0.98 (0.91, 1.04)
p-value	0.88	0.42	0.07	0.31	0.63	0.43
R^2	0.0	47.8	0.8	48.0	0.06	47.8
ΔR^2		0.1		0.2		0.1
Blood GSH Eh (mV)[e]						
Mean change (95% CI)[c]	1.92 (0.74, 3.11)	1.19 (-0.25, 2.62)	4.51 (2.67, 6.35)	2.72 (0.10, 5.35)	2.64 (0.70, 4.58)	1.73 (-0.37, 3.83)
p-value	0.002	0.10	<0.0001	0.04	0.008	0.11
R^2	2.6	31.1	5.8	32.2	1.9	31.1
ΔR^2		0.5		0.8		0.5
Plasma Cys (µmol/L)						
Mean ratio (95% CI)[d]	0.93 (0.88, 0.98)	1.06 (0.98, 1.14)	0.98 (0.89, 1.09)	1.12 (0.99, 1.28)	0.90 (0.81, 0.99)	1.05 (0.95, 1.16)
p-value	0.01	0.14	0.73	0.06	0.03	0.34
R^2	1.7	39.4	0.0	40.6	1.3	39.2
ΔR^2		0.3		0.6		0.1
Plasma CySS (µmol/L)						
Mean change (95% CI)[c]	-2.94 (-4.19, -1.69)	-3.00 (-4.61, -1.40)	-1.78 (-3.80, 0.25)	-3.56 (-6.47, -0.66)	-4.11 (-6.17, -2.06)	-3.09 (-5.40, -0.79)
p-value	<0.0001	0.0002	0.08	0.02	0.0001	0.009
R^2	5.3	29.8	0.8	28.8	4.0	28.5
ΔR^2		2.7		1.2		1.4
Plasma Cys Eh (mV)[f]						
Mean change (95% CI)[c]	1.38 (-0.21,2.98)	-2.05 (-3.89, -0.21)	-0.007 (-2.51, 2.49)	-4.04 (-7.34, -0.74)	1.90 (-0.67, 4.48)	-2.01 (-4.65, 0.64)
p-value	0.09	0.03	0.99	0.02	0.15	0.14
R^2	0.8	38.3	0.0	39.1	0.6	37.8
ΔR^2		0.9		1.0		0.4

a – adjusted for log transformed age, sex, television ownership(yes/no), smoking (ever/never), log transformed body mass index and plasma folate, and GSH/Cys laboratory batch, b – additionally adjusted for log urinary creatinine, c –represents the mean change in the outcome for a change in the exposure from the 25^{th} to the 75^{th} percentile, d – represents the ratio of the geometric mean in the outcome for a change in the exposure from the 25^{th} to the 75^{th} percentile, e - reduction potential of the GSH/GSSG redox pair, f – reduction potential of the Cys/CySS redox pair

Figure Legend

Figure 1. One-carbon Metabolism and Glutathione Synthesis and Metabolism. Folic acid is reduced to tetrahydrofolate (THF) and subsequently converted to 5-methyl THF. In a reaction catalyzed by methionine synthetase (MTR) the methyl group of 5-methyl-THF can be transferred to homocysteine, generating methionine. Methionine is activated to form s-adenosylmethionine (SAM), the universal methyl donor. The by-product of methylation reactions, s-adenosylhomocysteine (SAH), is hydrolyzed to homocysteine. Homocysteine is either used to regenerate methionine, or is directed to the transsulfuration pathway. Glutathione (GSH) is the end product of the transsulfuration pathway. GSH can serve as a continuous source of cysteine, which is extremely unstable, via the γ-glutamyl cycle (adapted from Lu 2009). GSH is exported from the cell and the enzyme gamma-glutamyltransferase (GGT) transfers the γ-glutamyl moiety of GSH to an amino acid, often cystine, producing cysteinylglycine and γ-glutamyl amino acid. The γ-glutamyl amino acid can be transported back into the cell and ultimately metabolized to glutamate. Cysteinylglycine is converted to cysteine and glycine by dipeptidase (DP). Cysteine is unstable extracellularly and can oxidize to cystine; both cysteine and cystine can be imported back into the cell for GSH production.


Folic acid
One-carbon Metabolism
Substrates
InAs
MMA
Respective
Products
MMA
DMA
SAM
SAH
Methionine
MTR, B12
BHMT, betaine
THF
5:10 Methylene-THF
5 Methyl THF
Homocysteine
serine
Transsulfuration Pathway
Cystathionine
cystine
cystine
Cysteine + Glutamate
γ-glutamylcysteine + Glycine
Glutathione reductase
cysteine
DP
GSH
GSSG
glycine
GGT
Cell membrane
cysteinylglycine
GSH
γ-glutamyl cycle
γ- glutamyl
amino acid
amino
acid