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Blood glutathione redox status and global methylation of peripheral blood mononuclear cell DNA in Bangladeshi adults

Megan M. Niedzwiecki,[1] Megan N. Hall,[2] Xinhua Liu,[3] Julie Oka,[1] Kristin N. Harper,[1] Vesna Slavkovich,[1] Vesna Ilievski,[1] Diane Levy,[3] Alexander van Geen,[4] Jacob L. Mey,[4] Shafiul Alam,[5] Abu B. Siddique,[5] Faruque Parvez,[1] Joseph H. Graziano[1] and Mary V. Gamble[1,*]

[1]Department of Environmental Health Sciences; Mailman School of Public Health; Columbia University; New York, NY USA; [2]Department of Epidemiology; Mailman School of Public Health; Columbia University; New York, NY USA; [3]Department of Biostatistics; Mailman School of Public Health; Columbia University; New York, NY USA; [4]Lamont-Doherty Earth Observatory of Columbia University; Palisades, NY USA; [5]Columbia University Arsenic Project in Bangladesh; Dhaka, Bangladesh

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Abbreviations: 8-oxodG, urinary 8-oxo-2'-deoxyguanosine; As, arsenic; BMI, body mass index; CBS, cystathionine-β-synthase; uCr, urinary creatinine; CV, coefficient of variation; Cys, cysteine; DPM, disintegrations per minute; DTT, dithiothreitol; E_h, reduction potential of redox couple, in millivolts; FOX, folate and oxidative stress study; GFAA, graphite furnace atomic absorption; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; Hcys, homocysteine; ICP-MS, inductively coupled mass spectrometry; LINE-1, long interspersed nuclear element-1; MAT, methionine adenosyltransferase; MTHFR, methylenetetrahydrofolate reductase; PBL, peripheral blood leukocyte; PBMC, peripheral blood mononuclear cell; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; wAs, water As

Oxidative stress and DNA methylation are metabolically linked through the relationship between one-carbon metabolism and the transsulfuration pathway, but possible modulating effects of oxidative stress on DNA methylation have not been extensively studied in humans. Enzymes involved in DNA methylation, including DNA methyltransferases and histone deacetylases, may show altered activity under oxidized cellular conditions. Additionally, in vitro studies suggest that glutathione (GSH) depletion leads to global DNA hypomethylation, possibly through the depletion of *S*-adenosylmethionine (SAM). We tested the hypothesis that a more oxidized blood GSH redox status is associated with decreased global peripheral blood mononuclear cell (PBMC) DNA methylation in a sample of Bangladeshi adults. Global PBMC DNA methylation and whole blood GSH, glutathione disulfide (GSSG), and SAM concentrations were measured in 320 adults. DNA methylation was measured by using the [^{3}H]-methyl incorporation assay; values are inversely related to global DNA methylation. Whole blood GSH redox status (E_h) was calculated using the Nernst equation. We found that a more oxidized blood GSH E_h was associated with decreased global DNA methylation (B ± SE, 271 ± 103, p = 0.009). Blood SAM and blood GSH were associated with global DNA methylation, but these relationships did not achieve statistical significance. Our findings support the hypothesis that a more oxidized blood GSH redox status is associated with decreased global methylation of PBMC DNA. Furthermore, blood SAM does not appear to mediate this association. Future research should explore mechanisms through which cellular redox might influence global DNA methylation.

Introduction

Methylation of cytosines in CpG dinucleotides is an epigenetic mechanism involved in the regulation of gene expression and cellular differentiation.[1,2] The methyl donor for this reaction is *S*-adenosylmethionine (SAM); SAM synthesis is regulated by folate-dependent one-carbon metabolism, as described in **Figure 1**. Decreased levels of global DNA methylation are associated with increased chromatin accessibility and gene transcriptional activity,[3] as well as genomic instability.[4] Consequently, global DNA hypomethylation, along with site-specific DNA hypermethylation of tumor suppressor genes, is commonly found in tumor tissue and transformed cells[5] and is believed to play a role in carcinogenesis.[6]

Oxidative stress is a risk factor also commonly implicated in carcinogenesis.[7] The body's primary antioxidant is glutathione (GSH), a thiol-containing tripeptide (γ-glutamyl-cysteinyl-glycine) that readily donates an electron to reactive oxygen species (ROS) via glutathione peroxidase (GPx) and quickly reacts with another free radical GSH molecule to form glutathione

*Correspondence to: Mary V. Gamble; Email: mvg7@columbia.edu
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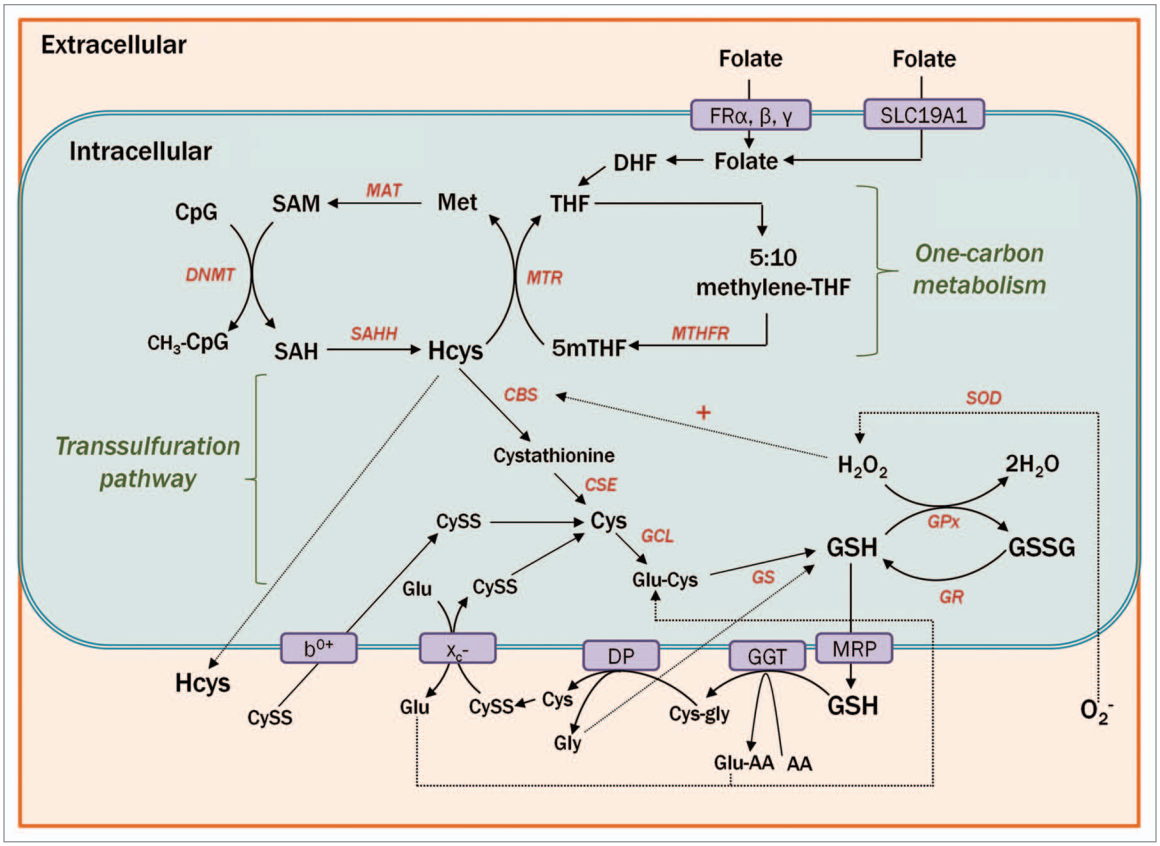

Figure 1. Overview of one-carbon metabolism, transsulfuration pathway, and glutathione influx/efflux. Folate is delivered into the cell via a receptor-mediated [folate receptor alpha (FRα), beta (FRβ) or gamma (FRγ)] or carrier-mediated [solute carrier family 19, member 1 (SLC19A1), also known as RFC1] transport mechanism and is subsequently reduced to dihydrofolate (DHF) and tetrahydrofolate (THF), which then enters the one-carbon metabolic pathway. THF picks up a methyl group from serine and is converted to 5-methyl THF (5mTHF). The methyl group of 5mTHF can be transferred to Hcys via methionine synthetase (MTR), generating methionine (Met) and THF. Met is activated to form *S*-adenosylmethionine (SAM), which serves as the methyl donor for the methylation of CpG dinucleotides and many other substrates, yielding the methylated product and *S*-adenosylhomocysteine (SAH), which is hydrolyzed to regenerate Hcys. Hcys can be used to regenerate Met or be directed through the transsulfuration pathway via cystathionine-β-synthase (CBS). Glutathione (GSH), a product of the transsulfuration pathway, is a tripeptide comprised of glutamate (Glu), cysteine (Cys) and glycine (Gly). As the primary endogenous antioxidant, GSH donates an electron to reactive oxygen species (e.g., H_2O_2) with the enzyme glutathione peroxidase (GPx) and quickly reacts with another free radical GSH to form glutathione disulfide (GSSG). GSH can be regenerated from GSSG in a reaction catalyzed by glutathione reductase (GR). Under conditions of oxidative stress, CBS activity is upregulated to direct Hcys flux through the transsulfuration pathway for GSH production. Excess intracellular Hcys can also be exported extracellularly. GSH can be exported out of the cell and catabolized via the cell membrane enzyme γ-glutamyltransferase (GGT). GGT transfers the γ-glutamyl group to an amino acid, producing cysteinylglycine (Cys-Gly), which can be broken down to Cys and Gly via dipeptidase (DP). Cys is unstable extracellularly and rapidly oxidizes to cystine (CySS). The x_c^- antiporter can import CySS using a transmembrane Glu gradient, and the b^{0+} system can directly import CySS, which can be converted back to Cys to maintain the intracellular Cys pool.

disulfide (GSSG).[8] Reduced levels of GSH, increased levels of GSSG and a decrease of the ratio of GSH to GSSG are indicative of a more oxidized intracellular environment.[9] Since the GSH-GSSG couple is the most abundant intracellular redox pair, their absolute concentrations can be used in the Nernst equation to estimate the intracellular redox state, E_h (in mV).[10] Approximately 50% of the cysteine (Cys) used in the production of GSH is derived from the conversion of homocysteine (Hcys) to cystathionine in the first step of the transsulfuration pathway (**Fig. 1**).[11]

Increased oxidative stress and aberrant DNA methylation often co-occur in carcinogenesis.[12] Although DNA methylation and oxidative stress are metabolically linked through the relationship between one-carbon metabolism and the transsulfuration pathway, possible modulating effects of oxidative stress on DNA methylation have not been extensively studied in humans. Two mechanisms have been proposed in the literature.

First, a more oxidized cellular redox state may lead to a decrease in genomic DNA methylation through redox regulation of related enzymes. A BLAST analysis of gene sequences containing possible redox-sensitive cysteine residues identified the SAM-dependent methyltransferases as potentially redox-sensitive.[13] Additionally, the activity of methionine adenosyltransferase (MAT), which catalyzes the enzymatic addition of methionine to adenosine for the synthesis of SAM, is decreased in environments with a more oxidized GSH/GSSG ratio.[14]

Second, it has been hypothesized by others that GSH depletion under conditions of chronic oxidative stress may lead to

Table 1. Descriptive characteristics for study sample (n = 320)

Baseline variables	Mean ± SD (range) or N(%)
Age (years)	43.2 ± 8.3 (30–63)
Male	159 (49.7)
BMI (kg/m^2)	20.3 ± 3.5 (13.8–35.3)
Underweight (BMI < 18.5 kg/m^2)	112 (35.1)
Ever cigarette smoking	123 (38.4)
Ever betel nut use	142 (44.4)
Television ownership	185 (57.8)
Water As (μg/L)	144.9 ± 122.5 (0.4–699.9)
Water As > 50 μg/L	226 (70.9)
Plasma folate (nmol/L)[a]	12.6 ± 7.0 (2.4–60.6)
Folate deficiency (folate < 9.0 nmol/L)[a]	100 (31.3)
Plasma Hcys (μmol/L)	11.2 ± 12 (3.0–165.4)
Hyperhomocystinemia (Hcys > 13 μmol/L)	56 (17.5)
DPM per μg DNA	149,123 ± 23,932 (61,398–215,666)
Blood GSH (μmol/L)	491.0 ± 170.6 (104.6–1072.1)
Plasma GSH (μmol/L)	2.6 ± 0.7 (1.0–5.8)
Blood GSSG (μmol/L)	37.3 ± 18.8 (9.6–121.8)
Plasma GSSG (μmol/L)	2.1 ± 0.6 (0.8–4.8)
Blood GSH E_h (mV)	−198.0 ± 13.0 (–227.9 to –144.4)
Plasma GSH E_h (mV)	−98.5 ± 7.2 (–118.0 to −73.4)
Urinary 8-oxo-dG/urinary Cr (μm)	16.0 ± 14.2 (0.9–89.6)
Blood SAM[b] (μm)	1.29 ± 0.50 (0.44–3.38)
Blood SAH[b] (μm)	0.31 ± 0.17 (0.10–1.37)

[a]n = 319; [b]n = 312

decreased global DNA methylation through the depletion of SAM.[15] Under oxidizing conditions, cystathionine-β-synthase (CBS) activity increases to direct Hcys flux through the transsulfuration pathway for the generation of GSH.[16] Consequently, less Hcys is directed toward the regeneration of methionine, which may result in decreased SAM levels. In support of this hypothesis, treatment with the GSH-depleting hepatotoxin bromobenzene led to extensive depletion of liver methionine pools and hypomethylation of genomic liver DNA in Syrian hamsters.[17,18]

We tested the hypothesis that increased oxidative stress is associated with decreased global DNA methylation in a cohort of Bangladeshi adults by examining the associations of blood GSH concentrations and blood GSH redox state (E_h) with global methylation of peripheral blood mononuclear cell (PBMC) DNA. To evaluate whether the association of blood GSH redox state with global PBMC DNA methylation might be due to depletion of SAM, we also examined the associations of blood SAM with the blood GSH redox variables and DNA methylation to test for possible mediation.

Results

Demographic and clinical characteristics of the study participants are shown in **Table 1**. The average age was 43 y, and there were roughly equal numbers of males and females. Approximately 35% of the population was classified as underweight (BMI < 18.5 kg/m^2), and 31.3% of the participants were folate deficient (plasma folate < 9 nmol/L). Due to the Folate and Oxidative Stress (FOX) study sampling design, nearly 71% of the participants used wells with water As > 50 ug/L (the Bangladeshi standard) as their primary drinking source. Mean blood GSH and GSSG concentrations were 491.0 and 37.3 μmol/L, respectively, and the mean blood GSH E_h was –198 mV.

In our evaluation of potential covariates for inclusion in the regression models, we observed that water arsenic (As) was negatively correlated with blood GSH (Spearman $r = -0.15$, $p = 0.008$), as reported in an earlier study from our group.[19] Males had higher blood GSH concentrations, less oxidized blood GSH E_h values, and higher blood SAM levels than females ($p < 0.0001$). Ever-smokers also had higher GSH concentrations, less oxidized blood GSH E_h values, and higher SAM levels than never-smokers. Since most ever-smokers were male (113/123, 91.9%), we stratified the ever-smoking analyses by gender: No significant associations were found between GSH and smoking status after stratification, indicating that the observed differences in GSH, GSH E_h and SAM by smoking status were due to the preponderance of males in the ever-smoking group (data not shown).

To support the validity of whole blood GSH E_h as a marker of intracellular redox in blood cells, we examined relationships

Table 2. Spearman correlation coefficients of blood and plasma glutathione redox variables with plasma Hcys and blood SAH (n = 320)

		Blood GSH	Blood GSSG	Blood E_h GSH	Plasma E_h GSH
Plasma Hcys	*r*	0.22	−0.09	−0.27	0.07
	P	(< 0.0001)	(0.10)	(< 0.0001)	(0.22)
Blood SAH[a]	*r*	0.22	−0.38	−0.42	0.02
	P	(< 0.0001)	(< 0.0001)	(< 0.0001)	(0.74)

[a]n = 312.

Table 3. Unadjusted and adjusted regression coefficients for associations between predictors and [3H]-methyl incorporation of PBMC DNA (n = 320)

Predictor	Model	[3H]-methyl incorporation			
		B ± SE	*P*	R^2%	ΔR^2%
Blood GSH E_h	Unadjusted	303 ± 102	0.003	2.7	
	Adjusted[a]	271 ± 103	0.009	5.5	2.0
Blood GSH	Unadjusted	−13.0 ± 7.8	0.10	0.9	
	Adjusted[a]	−12.7 ± 8.0	0.11	4.2	0.7
Blood SAM[b]	Unadjusted	−4,799 ± 2,698	0.08	1.0	
	Adjusted[a]	−4,803 ± 2,747	0.08	5.2	1.7

[a]Adjusted for sex, age and well water As, n = 319; [b]n = 312.

of blood GSH redox variables with plasma Hcys and blood S-adenosylhomocysteine (SAH), shown in **Table 2**. Cellular oxidative stress has been shown experimentally to decrease plasma Hcys in vitro[20] and in vivo.[21] As expected, a more oxidized blood GSH E_h was negatively correlated with both plasma Hcys (Spearman r = −0.27, p < 0.0001) and blood SAH (Spearman r = −0.42, p < 0.0001). We did not find that plasma GSH E_h was correlated with plasma Hcys (Spearman r = 0.07, p = 0.22) or blood SAH (Spearman r = 0.02, p = 0.74).

A more oxidized blood GSH E_h was significantly correlated with increased [3H]-methyl incorporation in bivariate analyses (Spearman r = 0.17, p = 0.002). Since [3H]-methyl incorporation is inversely related to genomic DNA methylation, this indicated that a more oxidized blood GSH E_h is associated with reduced global DNA methylation. In covariate-adjusted linear regression models shown in **Table 3, a** 1 mV increase in blood GSH E_h was associated with an increase in [3H]-methyl incorporation of 271 disintegrations per minute (DPM) per μg DNA (p = 0.009). Similarly, a 1 μM decrease in blood GSH was associated with an increase in [3H]-methyl incorporation of 12.7 DPM per μg DNA, but this association was not statistically significant (p = 0.11).

For statistical evidence to support blood SAM as a mediator of the association between blood GSH redox and global DNA methylation, we examined associations of blood SAM with blood GSH, blood GSH E_h, and [3H]-methyl incorporation. Blood SAM was negatively associated with [3H]-methyl incorporation at borderline significance (B ± SE = −4803 ± 2747, p = 0.08, **Table 3**). Blood GSH concentration was a significant predictor of log-transformed blood SAM in a covariate-adjusted model (B ± SE = 0.026 ± 0.012 for a 100 μM increase in blood GSH, p = 0.04), but blood GSH E_h was not associated with blood SAM (p = 0.23). Since the association of blood GSH and [3H]-methyl incorporation did not reach statistical significance, and since blood GSH E_h and blood SAM were not associated, the result did not support the role of blood SAM as a mediator.

In exploratory analyses, we also examined associations of other variables with [3H]-methyl incorporation. Neither blood SAH nor the blood SAM/SAH ratio were significantly associated with [3H]-methyl incorporation in adjusted models (p = 0.65 and p = 0.79, respectively). An oxidized blood GSH E_h correlated positively with urinary 8-oxodG levels, per g urinary creatinine (Cr) (Spearman r = 0.17, p = 0.004); since oxidative damage of guanines in CpG dinucleotides has been hypothesized to decrease global DNA methylation,[22,23] we also examined the association between urinary 8-oxodG/Cr levels and [3H]-methyl incorporation. However, the covariate-adjusted association was not significant (B ± SE = 293 ± 207, p = 0.16).

Discussion

The objective of this study was to examine whether oxidative stress is associated with decreased global methylation of PBMC DNA in a cross-sectional study of Bangladeshi adults. We tested this hypothesis by examining the associations of blood GSH and blood GSH E_h with PBMC DNA methylation, and we also examined whether these associations were mediated by blood SAM levels. We observed that a more oxidized blood GSH E_h was associated with decreased DNA methylation. Decreased blood GSH was not significantly associated with decreased DNA methylation, indicating that the association of blood GSH E_h with global DNA methylation depended on the concentrations and balance of blood GSH and GSSG and was not simply explained by the concentration of blood GSH. Finally, although blood SAM was positively associated with PBMC DNA methylation at marginal significance, blood GSH E_h was not associated with blood SAM. Taken together, our observations are consistent with the hypothesis that an oxidized intracellular redox state, as measured by blood GSH E_h, is associated with decreased global DNA methylation, but blood SAM does not appear to mediate this association.

Only a few other epidemiologic studies have examined the association between oxidative stress and DNA methylation. A study of 45 infertile men found that seminal ROS production was negatively correlated with sperm global DNA methylation, and three-month supplementation with antioxidants produced a significant decrease in seminal ROS and significant increase in sperm global DNA methylation.[24] Additionally, in a study of 61 bladder cancer patients and 45 healthy controls, total antioxidant status in urine was positively correlated with methylation of long interspersed nuclear element-1 (LINE-1) in peripheral blood leukocyte (PBL) DNA from all subjects.[25] To our knowledge, this is the first study to find an association between blood glutathione redox status and global DNA methylation in a generally healthy population.

While methylation of DNA is known to be a dynamic process, a mechanism for demethylation has not been clearly established. Recent work has shown that the ten-eleven translocation (Tet) family of proteins can convert 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC),[26,27] which is believed to be an intermediary in passive and/or active DNA demethylation.[28,29] Interestingly, Chen et al. found that the de novo methyltransferases DNMT3a and DNMT3b act as both DNA methyltransferases and DNA dehydroxymethylases, depending upon the redox environment: treatment with dithiothreitol (DTT) or hydrogen peroxide (H_2O_2) inhibited and enhanced the dehydroxymethylation activities of the enzymes, respectively.[30] Chen et al. speculated that the dual methylation and dehydroxymethylation activities of the DNMT3 enzymes might explain the paradoxical observation that DMNT3 expression is upregulated and gene-specific DNA methylation is increased in cancer cells, yet global DNA methylation is decreased.[30]

Histone deacetylase (HDAC) inhibition has been shown experimentally to induce DNA hypomethylation,[31] and it has been hypothesized that HDAC activities might also be influenced directly by cellular redox.[32] In human neuroblastoma cells, exposure to H_2O_2 induces global DNA hypomethylation, along with downregulation of HDAC3, DNMT1 and DNMT3a expression.[33] One potential mechanism of redox inhibition of HDACs might occur through alteration of cysteine residues: Reactive carbonyl species inactivate HDAC1 through covalent modification of conserved cysteine residues,[34] and exposure to ROS-producing compounds induces intramolecular disulfide bond formation in conserved cysteine residues in HDAC4.[35]

We did not find that blood SAM was significantly associated with global DNA methylation or blood GSH E_h. Mathematical modeling of one-carbon metabolism predicts that the DNMT methylation rate is remarkably stable over wide fluctuations of SAM concentrations, primarily due to the low K_m for the DNMT reaction and long-range allosteric regulation of CBS and methylenetetrahydrofolate reductase (MTHFR) by SAM.[36] Furthermore, the hypothesis that increased demand for GSH production depletes SAM levels is complicated by the discovery that CBS is allosterically activated by SAM but destabilized under SAM-deficient conditions.[37] These findings, along with the observations in this study, do not lend strong support to the hypothesis that oxidative stress leads to DNA hypomethylation through SAM depletion.

It is not clear whether whole blood concentrations of SAM and GSH reflect intracellular concentrations in other tissues. Although in vitro evidence suggests that SAM is not easily transported across cell membranes,[38,39] in a cohort of healthy adults, the plasma SAM/SAH ratio was strongly correlated with the intracellular lymphocyte SAM/SAH ratio ($r = 0.73$).[40] A study in rats showed that an increase in ROS in liver initiated changes in the erythrocytic GSH/GSSG ratio; the authors concluded that the erythrocytic GSH/GSSG ratio reflects oxidative stress in liver and other tissues.[41] Additionally, an intact transsulfuration pathway has been identified in blood cells, including macrophages[42] and T cells,[43] and transsulfuration pathway flux is upregulated in naïve and activated T cells after exposure to peroxide.[43] Even if SAM and GSH measurements in blood do not directly reflect the absolute concentrations in other tissues, the presence of intact one-carbon metabolism and transsulfuration pathways in blood cells suggests that blood biomarkers might be appropriate to study perturbations of these pathways by oxidative stress.

We observed that an oxidized blood GSH E_h was correlated with decreased blood SAH and plasma Hcys, which is consistent with an increase in Hcys flux through the transsulfuration pathway under oxidized intracellular conditions.[16] Although some studies have used plasma GSH E_h as a marker of intracellular redox,[44,45] we did not observe significant correlations of Hcys or SAH with plasma GSH E_h, which suggests that whole blood GSH E_h might be a better indicator of the intracellular redox environment, at least within blood cells. However, an oxidized plasma GSH E_h is thought to reflect oxidative stress in other tissues[46] or systemic oxidative stress[47] and is observed in aging,[48] obesity[49] and disease states such as asthma[50] and heart disease.[51] Indeed, we found that plasma GSH E_h, but not blood GSH E_h, was positively correlated with age (Spearman $r = 0.14$, $p = 0.01$) and BMI (Spearman $r = 0.11$, $p = 0.05$). As such, the measurements of GSH redox pairs in both plasma and whole blood might be informative to fully understand the influence of "oxidative stress" in an organism.

Redox regulation is known to influence white blood cell cycle progression and proliferation,[52] and thus, it is possible that our observations are explained by redox-induced shifts in PBMC cell type distributions or counts. However, the overall level of global DNA methylation across cell types would have to vary dramatically for cell distribution shifts to explain our observations. While site-specific CpG methylation levels at certain loci have been found to be associated with blood cell type,[53] global DNA methylation levels were not significantly different among DNA samples from granulocytes, mononuclear cells, and white blood cells in adult women.[54] Furthermore, since PBMCs are a subset of total WBCs, the influence of cell type variability is reduced to some extent in our study.

Our study has several limitations. First, the cross-sectional design precludes us from establishing the directionality of the relationship between blood GSH redox state and global DNA methylation. Epigenetic alterations might induce changes in the cellular redox status: for example, mice exposed to d-β-hydroxybutyrate, an inhibitor of Class I HDACs, exhibit an increase in global H3 acetylation, increase in expression of oxidative stress resistance genes and a reduction in oxidative damage from ROS.[55] We also cannot establish whether the observed association with global DNA methylation is explained by an upstream factor (e.g., H_2O_2), the GSH redox state itself or another redox-associated event, e.g., alterations of the citric acid cycle and/or the NAD^+/NADH ratio.[32] Finally, we were unable to adjust for PBMC cell type counts or distributions in our regression models.

In conclusion, we observed that a more oxidized intracellular redox state, as measured by blood GSH E_h, was associated with a decrease in global methylation of PBMC DNA. Furthermore, blood SAM was marginally associated with an increase in global DNA methylation, but it did not mediate the association between blood GSH E_h and global DNA methylation. Future

research should examine whether alteration of intracellular redox is a mechanism through which environmental exposures and other factors influence DNA methylation. It might also be of interest to examine whether redox therapies might help to prevent progressive loss of global DNA methylation in cancer and other diseases.

Materials and Methods

Eligibility criteria and study design. The FOX study was initially designed to examine the dose-response relationship between arsenic exposure and markers of oxidative stress. In the FOX study, we recruited 379 men and women aged 35–65 y between April 2007 and April 2008 in Araihazar, Bangladesh. Participants were selected based on well water As (wAs) exposure such that the final study sample represented the full range of wAs concentrations in the region, as described previously.[19] Participants were excluded if they were pregnant and/or planned to become pregnant within 3 mo, were currently taking nutritional supplements (within the past 3 mo) or had known diabetes, cardiovascular or renal disease or other diseases known to be associated with oxidative stress.

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 institutional review boards of the Bangladesh Medical Research Council and Columbia University Medical Center.

Analytic techniques. *Sample collection and handling.* Blood and urine samples were collected and immediately processed at our field clinic in Araihazar. Blood samples were centrifuged at 3,000 × g for 10 min at 4°C, and red cells were separated from buffy coat and plasma. Blood and plasma aliquots were stored in freezers at −80°C. Processing for GSH and GSSG assays was performed on freshly drawn blood and plasma samples prior to storage in −80°C freezers. Urine samples were collected in 50 mL acid-washed polypropylene tubes and frozen at −20°C. All blood, plasma and urine samples were transported to Dhaka on dry ice and stored at −80°C (blood and plasma) and −20°C (urine). Samples were then packed on dry ice and flown to Columbia University for analysis.

Whole blood and plasma glutathione and glutathione disulfide. Whole blood and plasma GSH and GSSG were assayed by the method of Jones et al.,[56] as previously described.[19] Briefly, blood samples were collected in the field laboratory with a butterfly syringe, immediately processed for derivatization, and stored at −80°C until delivered to Columbia University for analysis. Of each sample, 20 μl was injected onto the HPLC, and metabolites were detected using a Waters 474 scanning fluorescence detector with 335 nm excitation and 515 nm emission (Waters Corp.). Intra-assay CVs were between 5 and 10%, and inter-assay CVs were between 11 and 18%.

Plasma folate and vitamin B_{12}. Plasma folate and vitamin B_{12} were analyzed using a radioproteinbinding assay according to the manufacturer's protocol (SimulTRAC-S, MP Biomedicals). This method requires heating the samples to 100°C to denature endogenous binding substances. To determine folate concentrations, folic acid as pteroylglutamic acid was used for calibration, and its ^{125}I-labeled analog was used as the tracer; for vitamin B_{12}, cyanocobalamin was used for calibration, and its ^{57}Co-labeled analog was used as the tracer. The intra- and inter-assay coefficients of variation for folate were 6% and 14%, respectively, and the intra- and inter-assay coefficients of variation for vitamin B_{12} were 9% and 4%, respectively.

Plasma total homocysteine. Plasma total Hcys was assayed using the method of Pfieffer et al.,[57] as previously described.[58] The intra- and inter-assay CVs for plasma total Hcys were 4 and 9%, respectively.

Blood S-adenosylmethionine and S-adenosylhomocysteine. SAM and SAH were measured in whole blood, as previously described.[59] Briefly, blood was thawed and vortexed, and 400 μl were added to an equal volume of 0.1 M sodium acetate, pH 6.0, plus 40% TCA. SAM and SAH were detected at 254 nm using a 996 Photodiode Array UV absorbance detector (Waters Inc.) and quantified relative to standard curves generated using purified compounds (Sigma). The inter-assay coefficient of variation was 9.6% for SAM and 16.1% for SAH.

Isolation of PBMC DNA. PBMC DNA was isolated from 4 mL PBMC lysate using 1 ml Protein Precipitation Solution (5-Prime), and standard isopropanol extraction was conducted using the manufacturer's protocol. DNA samples were then frozen at −20°C until further analysis.

Genomic DNA methylation. Genomic DNA methylation was measured using the [^{3}H]-methyl incorporation assay of Balaghi and Wagner,[60] as previously described.[61] The assay employs ^{3}H-labeled SAM and SssI methylase to add ^{3}H-labeled methyl groups to unmethylated CpG sequences. Thus, the DPM values are inversely related to global DNA methylation. PicoGreen dsDNA Quantitation Reagent (Molecular Probes) was used to quantify the amount of double-stranded DNA in each reaction. DPM values were expressed per μg DNA. The intra- and inter-assay coefficients of variation were 3.4% and 10.4%, respectively.

Urinary 8-oxodG. Urinary 8-oxodG was measured using the "New 8-OHdG Check" ELISA kit at the Genox Corporation's laboratory. Samples were measured in triplicate. The detection level for this assay was 0.64 ng/mL urine. A significant proportion of the study sample (n = 56 individuals, or 18%) had urinary 8-oxodG levels below the detection limit; levels for these individuals were set to one-half of the detection limit, or 0.32 ng/mL. The intra- and inter-assay coefficients of variation were 7.5% and 7.7%, respectively. Urinary Cr was analyzed with a colorimetric assay based on the Jaffe reaction[62] to adjust for urine concentration.

Water As. Procedures for field sample collection and laboratory analyses were described previously.[63,64] Briefly, water samples were collected in 20-mL polyethylene scintillation vials and acidified to 1% with high-purity Optima HCl (Fisher Scientific) at least 48 h before analysis.[65] 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 < 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 6.0% and 3.8%, respectively.

Calculation of the reduction potential (E_h). The reduction potentials of the blood and plasma GSH/GSSG redox pairs were calculated using the Nernst equation, $E_h = Eo + (RT/nF) \ln[(acceptor)/(donor)]$, where Eo is the standard potential for the redox couple at the defined pH, R is the gas constant, T is the absolute temperature, F is Faraday's constant, and n is the number of electrons transferred.[46] For GSH and GSSG, the equation simplifies to E_h (mV) = –264 – 30 log[$(GSH)^2/(GSSG)$], where (GSH) and (GSSG) are molar concentrations and the Eo value assumes a physiologic pH of 7.4.[46] A more positive E_h value reflects a more oxidized state.

Statistical methods. Descriptive statistics (means and standard deviations) were calculated for the characteristics of the sample. Spearman correlations were used to examine bivariate associations between blood GSH variables, [^{3}H]-methyl incorporation, and other continuous covariates. Wilcoxon rank-sum test was used to detect differences in continuous measures between categories of binary variables.

Linear regression analyses were constructed with blood GSH redox variables and blood SAM as predictors and [^{3}H]-methyl incorporation as the outcome, with and without controlling for confounding factors. Proper transformations were applied to the continuous independent variables with skewed distribution to reduce the impact of extreme values. Certain confounders (gender, age, cigarette smoking and water As) were selected a priori based on biologic plausibility and previous studies in the literature.[61] Other potential confounders (plasma folate, plasma Hcys, plasma vitamin B_{12}, betel nut chewing, BMI, television ownership, education) were considered by examining their bivariate associations with blood GSH redox variables, blood SAM and [^{3}H]-methyl incorporation. The control variables in the final models were those that were related to the outcome and main predictors and resulted in an appreciable (> 5%) change in the regression coefficient for the association between a predictor and the outcome. Since cigarette smoking did not predict [^{3}H]-methyl incorporation independently of gender, it was excluded from the final model. The final regression models contained gender, age and water As as covariates.

Blood SAM was considered to be a partial mediator of the association between blood GSH and [^{3}H]-methyl incorporation if it met the following criteria: (1) Blood GSH (predictor) was significantly associated with blood SAM (mediator); (2) Blood GSH was significantly associated with [^{3}H]-methyl incorporation (outcome); (3) Blood SAM was significantly associated with [^{3}H]-methyl incorporation; (4) inclusion of blood SAM in a model of blood GSH predicting [^{3}H]-methyl incorporation resulted in attenuation of the regression coefficient.

Values for [^{3}H]-methyl incorporation were excluded from the analysis if duplicates had coefficients of variation > 15% (n = 48), DNA assay inputs below 10 μg/ml (n = 8), or values that were extreme outliers, defined as values that exceeded the boxplot of the DPM values by more than three interquartile ranges (n = 2). Additionally, we were unable to isolate DNA from n = 1 lysate, resulting in a final sample size of 320. All statistical analyses were conducted using SAS (version 9.3; SAS Institute Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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