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

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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; Eh, reduction potential of redox couple, in millivolts; FOX, folate and oxidative stress study; GFAA, graphite furnace atomic absorption; GSH, glutathione; GSSG, glutathione disulfide; H2O2, 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 leucocyte; 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 [3H]-methyl incorporation assay; values are inversely related to global DNA methylation. Whole blood GSH redox status (Eh) was calculated using the Nernst equation. We found that a more oxidized blood GSH Eh 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 cells5 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...
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. 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, $E_n$ (in mV). 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). 

Increased oxidative stress and aberrant DNA methylation often co-occur in carcinogenesis. 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. Additionally, the activity of methionine adenosyltransferase (MAT), which catalyzes the enzymatic addition of the methyl group of 5-methyltetrahydrofolate (5mTHF) to form methionine (Met) and tetrahydrofolate (THF), can be regulated by oxidative stress. Under conditions of oxidative stress, MAT activity is upregulated to direct Met 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 gamma-glutamyltransferase (GGT). GGT transfers the gamma-glutamyl group to an amino acid, producing cysteinyl-glycine (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$_-^+$ antiporter can import CySS using a transmembrane Glu gradient, and the b$_+$ system can directly import CySS, which can be converted back to Cys to maintain the intracellular Cys pool.

Second, it has been hypothesized by others that GSH depletion under conditions of chronic oxidative stress may lead to
decreased global DNAmethylation through the depletion of SAM.\textsuperscript{15} Under oxidizing conditions, cystathionine-\(\beta\)-synthase (CBS) activity increases to direct Hcys flux through the transsulfuration pathway for the generation of GSH.\textsuperscript{16} Consequently, less Hcys is directed toward the regeneration of methionine pools and hypomethylation of genomic liver DNA in Syrian hamsters.\textsuperscript{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\(\text{h}\)) 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 \(\mu\)g/L (the Bangladeshi standard) as their primary drinking source. Mean blood GSH and GSSG concentrations were 491.0 and 37.3 \(\mu\)mol/L, respectively, and the mean blood GSH E\(\text{h}\) 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.\textsuperscript{19} Males had higher blood GSH concentrations, less oxidized blood GSH E\(\text{h}\) values, and higher blood SAM levels than females (\(p < 0.0001\)). Ever-smokers also had higher GSH concentrations, less oxidized blood GSH E\(\text{h}\) 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\(\text{h}\) 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\(\text{h}\) as a marker of intracellular redox in blood cells, we examined relationships...
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 and in vivo. As expected, a more oxidized blood GSH Eh 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 Eh was correlated with plasma Hcys (Spearman \( r = 0.07, p = 0.22 \)) or blood SAH (Spearman \( r = 0.17, p = 0.002 \)). Since \([3H]\)-methyl incorporation 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 Eh 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 Eh was associated with decreased DNA methylation. Decreased blood GSH was not significantly associated with decreased DNA methylation, indicating that the association of blood GSH Eh 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 Eh 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 Eh, 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 a 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 leucocyte (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.

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

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Blood GSH</th>
<th>Blood GSSG</th>
<th>Blood GSH Eh, GSH</th>
<th>Plasma GSH Eh, GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Hcys</td>
<td>( r = 0.22 )</td>
<td>(-0.09)</td>
<td>(-0.27)</td>
<td>0.07</td>
</tr>
<tr>
<td>( p &lt; 0.0001 )</td>
<td>(0.10)</td>
<td>(0.0001)</td>
<td>(0.22)</td>
<td></td>
</tr>
<tr>
<td>Blood SAH( ^a )</td>
<td>( r = 0.22 )</td>
<td>(-0.38)</td>
<td>(-0.42)</td>
<td>0.02</td>
</tr>
<tr>
<td>( p &lt; 0.0001 )</td>
<td>(0.0001)</td>
<td>(0.0001)</td>
<td>(0.74)</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) n = 312.

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

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Model</th>
<th>([3H])-methyl incorporation</th>
<th>( B \pm SE )</th>
<th>( P )</th>
<th>( R^2 % )</th>
<th>( \Delta R^2 % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood GSH Eh,</td>
<td>Unadjusted</td>
<td>303 ± 102</td>
<td>0.003</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood GSH</td>
<td>Adjusted</td>
<td>271 ± 103</td>
<td>0.009</td>
<td>5.5</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Blood GSH</td>
<td>Unadjusted</td>
<td>-13.0 ± 7.8</td>
<td>0.10</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood GSH</td>
<td>Adjusted</td>
<td>-12.7 ± 8.0</td>
<td>0.11</td>
<td>4.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Blood SAM( ^a )</td>
<td>Unadjusted</td>
<td>-4,799 ± 2,698</td>
<td>0.08</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood SAM( ^a )</td>
<td>Adjusted</td>
<td>-4,803 ± 2,747</td>
<td>0.08</td>
<td>5.2</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Adjusted for sex, age and well water As, n = 319; \( ^a n = 312 \).
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), which is believed to be an intermediary in passive and/or active DNA demethylation. 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 (H2O2) inhibited and enhanced the dehydroxymethyltransferase activities of the enzymes, respectively. 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.

Histone deacetylase (HDAC) inhibition has been shown experimentally to induce DNA hypomethylation, and it has been hypothesized that HDAC activities might also be influenced directly by cellular redox. In human neuroblastoma cells, exposure to H2O2 induces global DNA hypomethylation, along with downregulation of HDAC3, DNMT1 and DNMT3a expression. 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, and exposure to ROS-producing compounds induces intramolecular disulfide bond formation in conserved cysteine residues in HDAC4.

We did not find that blood SAM was significantly associated with global DNA methylation or blood GSH Eh. 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 Km for the DNMT reaction and long-range allosteric regulation of CBS and methylenetetrahydrofolate reductase (MTHFR) by SAM. 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. 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, in a cohort of healthy adults, the plasma SAM/SAH ratio was strongly correlated with the intracellular lymphocyte SAM/SAH ratio (r = 0.73). 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. Additionally, an intact transsulfuration pathway has been identified in blood cells, including macrophages and T cells, and transsulfuration pathway flux is upregulated in naive and activated T cells after exposure to peroxide. 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 Eh 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. Although some studies have used plasma GSH Eh as a marker of intracellular redox, we did not observe significant correlations of Hcys or SAH with plasma GSH Eh, which suggests that whole blood GSH Eh might be a better indicator of the intracellular redox environment, at least within blood cells. However, an oxidized plasma GSH Eh is thought to reflect oxidative stress in other tissues and systemic oxidative stress and is observed in aging, obesity and disease states such as asthma and heart disease. Indeed, we found that plasma GSH Eh, but not blood GSH Eh, 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, 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, global DNA methylation levels were not significantly different among DNA samples from granulocytes, mononuclear cells, and white blood cells in adult women. 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. We also cannot establish whether the observed association with global DNA methylation is explained by an upstream factor, e.g., H2O2, the GSH redox state itself or another redox-associated event, e.g., alterations of the citric acid cycle and/or the NAD+/NADH ratio. 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 Eh, 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 Eh and global DNA methylation. Future studies examining a larger sample size and other tissues will be informative to better understand the connection between redox environment, intracellular concentrations of SAM and GSH, and epigenetic alteration.
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. 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 polystyrene 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. as previously described. 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 B12.** Plasma folate and vitamin B12 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 125I-labeled analog was used as the tracer; for vitamin B12, cyanocobalamin was used for calibration, and its 57Co-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 B12 were 9% and 4%, respectively.

**Plasma total homocysteine.** Plasma total Hcys was assayed using the method of Pfeifer et al. as previously described. 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. 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 [3H]-methyl incorporation assay of Balaghi and Wagner, as previously described. The assay employs 3H-labeled SAM and SssI methylase to add 3H-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 to adjust for 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-
inter-assay coefficients of variation (CVs) for this standard were 6.0% and 3.8%, respectively.

**Calculation of the reduction potential (Eh)**. The reduction potentials of the blood and plasma GSH/GSSG redox pairs were calculated using the Nernst equation, $E_h = E_0 + (RT/nF) \ln[(\text{acceptor})/(\text{donor})]$, where $E_0$ 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 $E_h (mV) = -264 - 30 \log([\text{GSH}]/[\text{GSSG}])$, where (GSH) and (GSSG) are molar concentrations and the Eh 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, [3H]-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 [3H]-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 $B_6$, betel nut chewing, BMI, television ownership, education) were considered by examining their bivariate associations with blood GSH redox variables, blood SAM and [3H]-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 [3H]-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 [3H]-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 [3H]-methyl incorporation (outcome); (3) Blood SAM was significantly associated with [3H]-methyl incorporation; (4) inclusion of blood SAM in a model of blood GSH predicting [3H]-methyl incorporation resulted in attenuation of the regression coefficient.

Values for [3H]-methyl incorporation were excluded from the analysis if duplicates had coefficients of variation > 15% (n = 48), DNA assay inputs below 10 $\mu$g/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.

**Acknowledgments**

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gene transcription in A induces DNA demethylation and histone acetylation...

