Published in final edited form as:

Birth Defects Res A Clin Mol Teratol. 2015 September ; 103(9): 754–762. doi:10.1002/bdra.23399.

Polymorphisms in maternal folate pathway genes interact with arsenic in drinking water to influence risk of myelomeningocele

Maitreyi Mazumdar^{1,2}, Linda Valeri³, Ema G. Rodrigues^{1,2}, Md Omar Sharif Ibne Hasan⁴, Rezina Hamid⁵, Ligi Paul⁶, Jacob Selhub⁶, Fareesa Silva¹, MdGolam Mostofa⁴, Quazi Quamruzzaman⁴, Mahmuder Rahman⁴, and David C. Christiani²

¹Department of Neurology, Boston Children's Hospital, Boston, MA, USA

²Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

³Department of Biostatistics, Harvard School of Public Health, Boston, MA, USA

⁴Dhaka Community Hospital Trust, Dhaka, BANGLADESH

⁵Bangladesh Medical College, Dhaka, BANGLADESH

⁶Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, USA

Abstract

Background—Arsenic induces neural tube defects in many animal models. Additionally, studies have shown that mice with specific genetic defects in folate metabolism and transport are more susceptible to arsenic-induced neural tube defects. We sought to determine whether 14 single-nucleotide polymorphisms in genes involved in folate metabolism modified the effect of exposure to drinking water contaminated with inorganic arsenic and posterior neural tube defect (myelomeningocele) risk.

Methods—Fifty-four mothers of children with myelomeningocele and 55 controls were enrolled through clinical sites in rural Bangladesh in a case-control study of the association between environmental arsenic exposure and risk of myelomeningocele. We assessed participants for level of myelomeningocele, administered questionnaires, conducted biological and environmental sample collection, and performed genotyping. Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure inorganic arsenic concentration in drinking water. Candidate single-nucleotide polymorphisms were identified through review of the literature.

Results—Drinking water inorganic arsenic concentration was associated with increased risk of myelomeningocele for participants with 4 of the 14 studied single-nucleotide polymorphisms in genes involved in folate metabolism: the AA/AG genotype of rs2236225 (*MTHFD1*), the GG genotype of rs1051266 (*SLC19A1*), the TT genotype of rs7560488 (*DNMT3A*), and the GG genotype of rs3740393 (*AS3MT*) with adjusted OR of 1.13, 1.31, 1.20, and 1.25 for rs2236225, rs1051266, rs7560488, and rs3740393, respectively.

Corresponding Author: Maitreyi Mazumdar, Department of Neurology, Boston Children's Hospital, 300 Longwood Avenue, BCH 3123, Boston, MA 02115, Phone: (617) 355-2758, Fax: (617) 730-0285, maitreyi.mazumdar@childrens.harvard.edu.

Competing Financial Interests Declaration: The authors report that they have no actual and/or potential competing financial interests.

Conclusions—Our results support the hypothesis that environmental arsenic exposure increases the risk of myelomeningocele via interaction with folate metabolic pathways.

Keywords

myelomeningocele; neural tube defect; arsenic; environmental health; folate

INTRODUCTION

Neural tube defects are debilitating birth defects characterized by high rates of mortality and lifelong disabilities in surviving children. They occur when the neural plate fails to fold in the first 3 to 4 weeks of gestation, causing death or permanent damage to the spinal cord. The causes of human neural tube defects are largely unknown, but are most certainly multifactorial, consisting of both genetic and environmental components (Finnell and others, 2000).

Both observational studies and randomized trials have provided evidence that folic acid intake reduces the risk of neural tube defect-affected pregnancies (Obican and others, 2010). Fortification of folic acid in the U.S. food supply has been associated with a 20% decline in anencephaly and a 34% decline in spina bifida (Canfield et al., 2005; Honein et al., 2001; Williams et al., 2002). Despite the accumulating evidence regarding the protective benefits of periconceptional folic acid supplementation, the mechanisms by which this effect is achieved remain unknown.

Folate is the center of a one-carbon metabolic pathway. The observed protective effects of folic acid on neural tube defect risk have led to intense research into the genes that encode enzymes in this pathway. According to one hypothesis, polymorphisms within these genes affect the amount of one-carbon units, which are critical for a variety of methylation reactions that form the basis of normal neural tube development (van der Linden and others, 2006).

This one-carbon hypothesis regarding the protective effects of folate receives support from another direction, that is, from current understanding about the toxicity of arsenic exposure. Arsenic is listed as number 1 on the Substance Priority List (SPL) of the 275 most hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR), highlighting the significant potential threat to human health due to its toxicity and potential for human exposure (Agency for Toxic Substances & Disease Registry, 2014). Arsenic metabolism depends on the availability of one-carbon donors, and as noted above, genetic variations in folate pathways affect this availability as well as neural tube defect risk. It is therefore biologically plausible that variations in folate pathway genes influence the associations between environmental arsenic exposure and risk of neural tube defect.

Studies have shown that arsenic induces neural tube defects (exencephaly, spina bifida occulta) in several animal models, including mouse (Hill and others, 2008), rat (Beaudoin, 1974), hamster (Carpenter, 1987), and chick (Han et al., 2011). In addition, arsenic's effects exhibit a typical dose response (Golub et al., 1998; Holson et al., 2000; Shalat et al., 1996). Genetic defects in folate metabolism increase the teratogenicity of arsenic in animal models.

For example, mice with specific defects in folate transport have higher rates of neural tube defects after *in utero* arsenic exposure than wild-type mice similarly exposed (Hill et al., 2008; Włodarczyk et al., 2014). In addition, mice nullizygous for genes encoding proteins involved in cellular uptake of folate are more susceptible to arsenic-induced neural tube defects (Spiegelstein et al., 2005).

No study in humans has definitely demonstrated a direct association between environmental arsenic exposure and human neural tube defects. However, our recent study of 57 cases of myelomeningocele (a specific type of neural tube defect characterized by failure of posterior neural tube closure) and 55 controls in Bangladesh suggested that arsenic interferes with folic acid's protective effects in neural tube defect prevention (Mazumdar and others, 2015). In that study, a significant interaction was observed between drinking water inorganic arsenic and reported periconceptional folic acid use; as drinking water inorganic arsenic concentration increased from 1 to 25 µg/L, the estimated protected effect of folic acid use declined and was not protective at higher concentrations of arsenic.

Bangladesh is experiencing an epidemic of chronic arsenic poisoning from contaminated drinking water. According to survey data from 2000 to 2010, an estimated 35 to 77 million people in Bangladesh have been chronically exposed to arsenic in their drinking water in what has been described as the largest mass poisoning in history (Smith et al., 2000). No data are available on the incidence and prevalence of neural tube defects in Bangladesh, although these malformations are fairly common in clinical practice (Dey et al., 2010). Our pilot study results, referenced above, raise concern that the combination of arsenic poisoning, folate deficiency and possible genetic susceptibility in Bangladesh may be a setting for an unrecognized epidemic of neural tube defects.

The aim of our current study was to use data from our previous case-control study in Bangladesh in order to determine whether the associations between environmental arsenic exposure and neural tube defects were modified by polymorphisms in candidate genes from folate metabolic pathways in humans.

METHODS

Study population

This case-control study was conducted in the Pabna, Barisal, Rajshahi, Munshiganj, Dhaka, and Chittagong Districts of Bangladesh, using data from our previously described case-control study of environmental arsenic exposure and myelomeningocele risk (Mazumdar et al., 2015). Cases included live births, stillbirths, and infants less than one year of age with myelomeningocele. Case diagnoses were confirmed by physical examination by a physician. Controls were selected from pregnancy registries from Dhaka Community Hospital-affiliated community health centers that were located in the same areas as the cases and randomization procedures used for selection of controls have been previously described (Mazumdar et al., 2015). Cases and controls were frequency matched on sex and birth quarter. Participation was 98% among potential cases and 83% among potential controls.

Informed consent was provided by parents before enrollment. The Human Research Committees at Boston Children's Hospital and at Dhaka Community Hospital approved this study.

Questionnaires and Medical History

Once a case or control was enrolled into the study, trained interviewers asked parents about their medical histories, including the use of medications during pregnancy, family history, water consumption, and reproductive history. Nutritional intake of mothers during pregnancy was assessed using a food frequency questionnaire previously validated in rural Bangladeshi populations (Chen et al. 2004). Periconceptional folic acid use was defined as report of taking any folic acid containing supplement within the two months before becoming aware of the pregnancy. Children underwent a standardized physical examination including evaluation for level of myelomeningocele, presence of ventriculoperitoneal shunt, neurocutaneous stigmata, and other congenital abnormalities. Study data were collected and managed using REDCap electronic capture tools hosted at Boston Children's Hospital (Harris et al., 2009).

Arsenic Exposure

Drinking Water—At the time of enrollment, a water sample was collected from the tube well each mother identified as her primary source of drinking water at the time she became aware of her pregnancy. As mothers often return to their parents' homes after learning of pregnancy, we attempted to find and test the well that mothers used in the very beginning of pregnancy. Water samples were collected in 50 ml polypropylene tubes (BD Falcon, BD Bioscience, Bedford, MA), and preserved with Reagent Grade nitric acid (Merck, Germany) to a pH <2 and stored at room temperature. Inorganic arsenic concentration was measured using inductively coupled plasma mass-spectrometry (ICP-MS) following US EPA method 200.8 (Spectrum Analytical, Inc., Agawam, MA USA). For quality control, instrument performance was validated by a spiked laboratory control sample (ICP, Analytical Mixture 12 Solution A, High-Purity Standards, Charleston, SC USA) with recoveries ranging from 98% to 107%. Of the 109 samples included in this analysis, 45 (41.3%) had an inorganic arsenic concentration below the 0.15 µg/L limit of detection (LOD). These samples were reassigned half the value of the LOD for statistical analyses. Families found to have drinking water inorganic arsenic concentrations ≥ 50 µg/L were advised to seek alternative drinking water.

Blood Collection and Processing

Maternal blood samples were collected after enrollment, at the time of the study visit, via venipuncture into two EDTA tubes and transported on ice to Dhaka within one day of collection, after which they were centrifuged to separate blood cells and plasma. Plasma was collected into microcentrifuge tubes and stored at -20°C. Blood/plasma samples were shipped to Harvard School of Public Health on dry ice.

Folate Analysis

Folate analyses were performed at the Vitamin Metabolism Laboratory at the Jean Mayer USDA Human Nutrition Research Center at Tufts University. We measured total folate of the plasma samples by microbial assay with the use of *Lactobacillus casei*, as we have described previously (Horne and Patterson, 1988). Briefly, we serially diluted 5 μ L of each plasma sample and plated the samples in triplicate onto a 96-microtiter well plate with 150 μ L of *L. casei* growth medium. We incubated the plates overnight in a 37°C humid incubator and measured the absorbance, which indicated microbial growth, with the use of a 96-well plate reader (Power Wave HT; BioTek Instruments Inc, Winooski, VT USA) at 595 nm. To test if any arsenic in plasma affected the microbial assay, we spiked 3 random samples with 5 ng/mL folic acid, and there were no inhibitory components detected in the plasma. The coefficients of variability (CVs) for the assay using one plasma sample with high folate concentration and one sample with low folate concentration were 6.78% and 4.73%, respectively.

DNA Extraction, Maternal Candidate Genes and Single Nucleotide Polymorphisms

Multiplex polymerase chain reaction amplifications were performed from genomic DNA extracted from whole blood following the Puregene Protocol (Gentra Systems, Minnesota). Selected genes and single-nucleotide polymorphisms (SNPs) were those associated with both neural tube defects and arsenic toxicity in genome-wide association studies, or those with supporting evidence from candidate gene studies. In total, 14 SNPs from 11 maternal candidate genes were selected: *MTHFR* (rs1801133, rs1801131); *MTHFD1* (rs2236225); *MTR* (rs1805087); *MTRR* (rs1801394); *SLC19A1* (rs1051266); *XPB/ERCC2* (rs1799793, rs13181); *NCAM1* (rs2298526); *DNMT1* (rs2228612); *DNMT3B* (rs6058896); *DNMT3A* (rs7560488); and *AS3MT* (rs11191439, rs3740393).

Samples were genotyped at the High-Throughput Polymorphism Detection Core Laboratory at the Dana-Farber/Harvard Cancer Center. All samples were genotyped using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA), in 384-well format. The 5' nuclease assay (TaqMan®) was used to distinguish the two alleles of a gene. PCR amplification was carried out on 5–20 ng DNA using 1 X TaqMan® universal PCR master mix (No Amp-erase UNG) in a 5 μ L reaction volume. Amplification conditions on an AB 9700 dual plate thermal cycler (Applied Biosystems, Foster City, CA) were as follows: 1 cycle of 95°C for 10 min, followed by 50 cycles of 92°C for 15s and 60°C for 1 min. TaqMan® assays were ordered using the ABI Assays-on-Demand service for custom assays. Genotyping was performed by laboratory personnel blinded to case-control status.

To determine whether the distribution of genotypes deviated from expected Hardy-Weinberg equilibrium (HWE) frequencies, the genotypes control mothers were tested using a χ^2 goodness-of-fit test (Rodriguez et al., 2009). All selected SNPs passed the HWE χ^2 test with p-value > 0.05. The analytic subset comprised 100% of the controls and 95% of the cases and represent the full population that had both drinking water inorganic arsenic concentrations and DNA samples available.

Statistical Analysis

Data were analyzed using SAS (version 9.3; SAS Institute, Inc., Cary, NC, USA). We used unconditional logistic regression to calculate crude and adjusted odds ratios (ORs) and 95% confidence intervals (CI). Conditional methods were not used due to the uneven number of cases and controls, but instead we forced the matching variables, child age and sex in all models as suggested by Rothman and Greenland (Rothman and Greenland, 1998a; b). Data exploration suggested that the log-odds of case identification varied exponentially with levels of inorganic arsenic in drinking water. Consequently log-transformed inorganic arsenic concentration was treated as a continuous variable in regression models. Potential confounders included smoking status of each parent, medication use, betel nut use, birth order, periconceptional folic acid use, family history of congenital defect, use of pesticides during pregnancy, place of birth (home, birth center or hospital), and report of undergoing an ultrasound during pregnancy. We obtained an adjusted estimate of risk of myelomeningocele by adjusting for variables that had p-values <0.05 in multivariable models.

We used inverse probability weighting to correct for unequal sampling fractions of our control populations, a method recently and succinctly reviewed (Seaman and White, 2013). Using inverse probability weighting, each individual in the study population was given a sampling weight that was the inverse of the probability of selection, in this case the inverse of the proportion of controls from the site from which the control was recruited.

We studied how interaction between a SNP and drinking water inorganic arsenic concentration affected myelomeningocele risk by using interaction terms in our weighted logistic regression models. The effect of each factor is first determined. Then, a common way to assess interactions between the effects is to use both terms in a multiple logistic regression model and to include the effect of each factor along with a term that multiplies the two factors (Khoury and Wagener, 1995). The coefficient of this interaction term then determines whether an interaction is present:

$$G(Z)=\alpha+\beta_xX+\beta_yY+\beta_{xy}XY$$

In this equation, Z is the odds of disease, α is a constant, X is the environmental exposure, Y is the genotype, and XY is the interaction term. The coefficients β_x , β_y , and β_{xy} are determined by regression analysis. Using the parameters derived from our logistic regression models, we estimated the OR for drinking water inorganic arsenic concentration on myelomeningocele risk for each SNP. The adjustments of multiple comparisons were conducted using a modified Bonferroni method. Thus, any two-sided p-values <0.00018 were considered statistically significant.

RESULTS

The 54 cases of neural tube defects in these analyses included 46 cases of lumbar and 8 cases of cervical myelomeningocele. No other congenital defects were reported. None of the mothers reported alcohol consumption, smoking, betel nut use, or use of medications other

than vitamins/folic acid supplements (specifically, all mothers denied use of anticonvulsant drugs) during pregnancy. As shown in Table 1, mothers of controls were younger than mothers of cases (23.1 years versus 25.1 year, $p = 0.06$). Mothers of controls were more likely to report use of periconceptional folic acid than mothers of cases (71% versus 50%, $p = 0.03$). Plasma folate concentrations were higher at the time of study visit among women who reported periconceptional folic acid use (mean 12.7 nmol/L vs 7.4 nmol/L, $p=0.02$). Drinking water inorganic arsenic concentration was higher in controls than in cases (Median 6.86 $\mu\text{g/L}$ vs. 0.72 $\mu\text{g/L}$; though this appeared to be due to the contribution of controls from one site only, Birhampur Community clinic (BCC) in Pabna ($n=22$). Data exploration showed that the median inorganic arsenic drinking water arsenic concentration for controls who from sites aside from BCC was below the limit of detection (Supplementary Table 1). The drinking water inorganic arsenic concentration in our studies reflected the epidemic of arsenic poisoning in Bangladesh. Among all controls, over 25% percent of the samples had inorganic arsenic concentration greater than the current Bangladeshi reference level of 50 $\mu\text{g/L}$ and the maximum drinking water inorganic arsenic was over 10 times that threshold at 506 $\mu\text{g/L}$.

Supplementary Table 2 shows the summary characteristics of the SNPs genotyped in this population. The minor allele frequency of the 14 SNPs ranged from 3% to 48%, indicating that these are common variants in a Bangladeshi population. We observed that three SNPs (rs1801394, rs2228612, and rs3740393) respectively in *MTRR*, *DNMT1*, and *AS3MT* were associated with an increased risk of myelomeningocele. After adjustment for drinking water inorganic arsenic concentration, child sex, child age, mother's age, father's age, periconceptional folic acid use, and birth place, the adjusted ORs from the weighted logistic regression models were 2.02 (95% CI: 1.30–3.14, $p\text{-value} < 0.002$), 5.94 (95% CI: 3.60–9.80, $p < 0.0001$) and 1.69 (95% CI: 1.09–2.63, $p\text{-value} = 0.02$) for rs1801394, rs2228612, and rs3740393, respectively. After adjustment for multiple comparisons, the association for rs1801394 and rs3740393 were no longer statistically significant. One SNP, rs13181 in *XPB/ERCC2*, was associated with a decreased risk of myelomeningocele. After adjustment for drinking water inorganic arsenic concentration, age and sex of child, mother's age, father's age, periconceptional folic acid use, and birth place, the adjusted OR for rs13181 was 0.42 (95% CI: 0.27–0.64, $p\text{-value} < 0.0001$).

In order to assess the effect of interaction between SNPs and drinking water arsenic on risk of myelomeningocele, we modeled for each SNP the effect of arsenic exposure using interaction terms in the weighted logistic regression models. Four SNPs were found to have significant interactions with drinking water arsenic: rs1051266 (*SLC19A1*); rs7560488 (*DNMT3A*); rs3740393 (*AS3MT*); and rs2236225 (*MTHFD1*) (Table 2).

The ORs for exposure to drinking water inorganic arsenic and myelomeningocele risk that were estimated using parameters from the logistic regression models are presented in Table 2. For rs2236225 (*MTHFD1*), the AA/AG genotype, drinking water inorganic arsenic concentration was associated with an increased risk of myelomeningocele (OR = 1.13, 95% CI: 1.04, 1.23). For rs1051266 (*SLC19A1*), rs7560488 (*DNMT3A*), and rs3740393 (*AS3MT*), increased risk of myelomeningocele with increasing arsenic exposure was observed for the

wild type (ORs 1.31, 95% CI: 1.14, 1.50; 1.20, 95% CI: 1.09, 1.32; and 1.25, 95% CI: 1.14, 1.38) for rs1051266, rs7560488, and rs374039, respectively).

DISCUSSION

Using a candidate SNP approach, we found that 4 SNPs in maternal folate pathway genes modified the effect of drinking water inorganic arsenic exposure on myelomeningocele risk. Drinking water inorganic arsenic concentration was positively associated with increased risk of myelomeningocele for participants with the AA/AG genotype of rs2236225 (*MTHFD1*), the GG genotype of rs1051266 (*SLC19A1*), the TT genotype of rs7560488 (*DNMT3A*), and the GG genotype of rs3740393 (*AS3MT*) with adjusted OR of 1.13, 1.31, 1.20, and 1.25 for rs2236225, rs1051266, rs7560488, and rs3740393, respectively. Each of these genes and SNPs will be discussed below after a brief summary of our findings and their potential significance.

Our data suggest that individuals in the population who have certain genetic polymorphisms in folate metabolism may be more susceptible than others to arsenic. When this observation is added to the known but unexplained relationship between folate and neural tube defect risk, a plausible hypothesis that human exposure to environmental arsenic affects the risk of myelomeningocele. The corollary hypothesis in animal models has a wealth of support (Shalat et al., 1996). Our data also suggest possible mechanisms to explain arsenic's effect on myelomeningocele risk. Folate provides precursors for synthesis of DNA and methyl groups for synthesis of S-adenosyl methionine, the universal methyl donor for all biological methylation reactions. Cell division and tissue growth and DNA methylation are connected to these two roles of folate. One possible explanation for the observed interaction between arsenic exposure and polymorphisms in genes involved in folate metabolism is that particular polymorphisms are associated with less efficient production of methyl groups. Metabolism of arsenic may deplete the supply of necessary methyl groups, thus adversely affecting neural tube development.

The first of the 4 genes we will discuss in more detail is the 5, 10-methylenetetrahydrofolate dehydrogenase gene, *MTHFD1*, which plays a central role in folate metabolism. *MTHFD1* is a cytoplasmic enzyme dependent upon nicotinamide adenine dinucleotide phosphate (NADP). *MTHFD1* catalyzes three sequential reactions in the conversion of one-carbon derivatives of tetrahydrofolate, which are substrates for methionine synthesis and *de novo* purine and pyrimidine synthesis (Barlowe and Appling, 1990); thus, *MTHFD1* plays a central role in folate pool maintenance. The maternal and infant genotypes that include the rs2236225 A allele have been associated with increased risk of neural tube defects (ORs 1.5–1.8) in some studies (Brody et al., 2002; De Marco et al., 2006; Pangilinan et al., 2012; Parle-McDermott et al., 2006), but not all (Hol et al., 1998; van der Linden et al., 2007). A recent meta-analysis of seven studies demonstrated an overall significant correlation between the presence of this SNP in mothers and the finding of neural tube defects in their children (Jiang et al., 2014). *MTHFD1* has also been identified as a human orthologue to proteins in yeast models that confer resistance to arsenic-induced toxicity (Vujcic and others, 2007).

The metabolic basis for the association between arsenic exposure and rs2236225 on myelomeningocele risk is unknown, as the polymorphism does not appear to modify plasma or red blood cell folate or plasma homocysteine levels (Brody et al., 2002; Carroll et al., 2009; Konrad et al., 2004). However, *in vitro* studies have shown that the *MTHFD1* rs2236225 AA genotype is associated with reduced enzymatic function and impairs *de novo* purine biosynthesis (Christensen et al., 2009).

Second, the reduced folate carrier gene *SLC19A1* (formerly known as *RFC*) encodes a membrane protein that is involved in the transport of folate into cells. Thus, functional change in the protein encoded by *SLC19A1* could modulate folate metabolism by increasing or decreasing intracellular folate availability. Polymorphisms in this gene, including the A80G variant (rs1051266), have been associated with increased susceptibility to myelomeningocele in Chinese populations (Etheredge et al., 2012; Pei et al., 2009; Pei and others, 2015; Shang et al., 2008). Other variants in this gene have been found to be associated with neural tube defects in Caucasians of European descent and Hispanics of Mexican descent in the United States (O'Byrne et al., 2010). Animal studies have not shown that this gene is sensitive to arsenic exposure. In mice with disrupted *RFC* genes, no genotype-related differences in susceptibility to arsenic-induced neural tube defects were observed (Spiegelstein et al., 2005).

Third, polymorphisms in the arsenic (III) methyltransferase (*AS3MT*) gene encode the main methyltransferase in arsenic metabolism and affect an individual's efficiency in detoxifying ingested arsenic. The initial steps of arsenic metabolism involve methylation, and are catalyzed by *AS3MT*; these reactions require a methyl group from S-adenosyl methionine (Buchet and Lauwerys, 1985). As reviewed above, synthesis of S-adenosyl methionine depends on folate supply. Polymorphisms in *AS3MT* have been consistently associated with urine arsenic methylation patterns in populations from Bangladesh (Rodrigues et al., 2012), Argentina (Schlawicke et al., 2007; Schlawicke Engstrom et al., 2009), Chile (Hernandez et al., 2008), Mexico (Gomez-Rubio et al., 2010), and Central Europe (Lindberg et al., 2007), as well as U.S. Native American populations (Tellez-Plaza et al., 2013). It is plausible that genetic variation in *AS3MT* affects myelomeningocele risk by modulating 1) the relative concentrations of methylated (generally thought to be less toxic) species of arsenic, and/or 2) the amount of remaining folate available for important reactions in neural tube development.

Finally, DNA (cytosine-5)-methyltransferase-3A (*DNMT3A*) belongs to a family of genes that encode enzymes involved in *de novo* methylation during development. The folate pathway generates S-adenosyl methionine, which is used by *DNMT3A* as a methyl donor. *DNMT3A* rs7560488 has been previously found to be associated with neural tube defect risk in an Irish population (Pangilinan et al., 2012). Decreased expression of *DNMT3A* has been observed as a consequence of low-dose arsenic exposure (Reichard et al., 2007), suggesting that arsenic's toxicity may also involve epigenetic effects. Recent *in vitro* functional studies suggest that variations in rs7560488 substantially alters transcriptional activity of the gene via influencing binding of a number of as of yet, not well defined transcriptional factors (Wu et al., 2014).

This study is the first in humans to provide evidence that arsenic increases myelomeningocele risk through direct involvement in folate metabolic pathways, possibly by depleting the methyl pool necessary for fundamental biological processes, including DNA synthesis and DNA methylation. This hypothesis is biologically plausible, and builds on a wealth of literature in animal models.

The strengths of our study include the use of individual measures of exposure, i.e., measurements of inorganic arsenic in the drinking water from wells that mothers identified as their primary source of well water at the time they became aware of their pregnancy—a time point very close to the crucial time point in neural tube defect pathogenesis. Another reason our study may have found associations is that the arsenic concentrations in our study were much higher than those observed in studies performed in the U.S. For example, among controls in a 2006 study conducted along the Texas/Mexico border, less than 2% of women were found to have inorganic arsenic concentrations greater than 10 µg/L in drinking water (Brender et al., 2006), whereas in this study, the drinking water inorganic arsenic concentrations was greater than 10 µg/L in 55.5% of controls, and the maximum drinking water arsenic concentration (506 µg/L) was over 50 times that threshold.

Preventive strategies that promote the use of folic acid supplements for women of childbearing age the fortification of grain products have been successful in reducing the incidence of neural tube defects in many countries (Canfield et al., 2005; Honein et al., 2001; Williams et al., 2002). Supplementation usually requires the procurement and purchase of micronutrients in a relatively expensive form as well as a high degree of adherence. Another strength of our study is that we minimized the potential recall bias by validating arsenic exposure and folic acid use reports with biomarkers. Though we attempted to validate reports of periconceptional folic acid use by measuring current plasma folate, this is imperfect as the plasma was collected after pregnancy. While the plasma folate measurements corroborate the accuracy of reports, biomarkers were collected following pregnancy and are not by themselves not the best estimates of exposure or folate status during early pregnancy.

Important limitations include the retrospective assessment of maternal arsenic exposure as well as potential for recall bias regarding folic acid use. An additional limitation is the use of maternal SNPs only, as variation in infants' DNA may confer additional susceptibility to environmental exposures. Though this is a limitation, in studies that have both maternal and infant DNA, maternal genetic variations have a stronger link with neural tube defects than those of infants (Etheredge et al., 2012). We also tested SNPs individually in our analyses, and did not have the power to investigate gene-gene interactions.

CONCLUSIONS

Environmental arsenic exposure may increase the risk of neural tube defects through interactions with folate metabolic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this study was provided by the Child Neurology Foundation and the Harvard School of Public Health NIEHS Center (ES000002). Dr. Mazumdar was supported by a Mentored Career Development Award from the NIEHS, National Institutes of Health (K23 ES017437). Additional support was provided by NIEHS grant P42 ES16454.

References

- Agency for Toxic Substances & Disease Registry. CERCLA Priority List of Hazardous Substances. Centers for Disease Control and Prevention; 2014.
- Barlowe CK, Appling DR. Molecular genetic analysis of *Saccharomyces cerevisiae* C1-tetrahydrofolate synthase mutants reveals a noncatalytic function of the ADE3 gene product and an additional folate-dependent enzyme. *Molecular and cellular biology*. 1990; 10(11):5679–5687. [PubMed: 2233711]
- Beaudoin AR. Teratogenicity of sodium arsenate in rats. *Teratology*. 1974; 10(2):153–157. [PubMed: 4428424]
- Brender JD, Suarez L, Felkner M, Gilani Z, Stinchcomb D, Moody K, Henry J, Hendricks K. Maternal exposure to arsenic, cadmium, lead, and mercury and neural tube defects in offspring. *Environ Res*. 2006; 101(1):132–139. [PubMed: 16171797]
- Brody LC, Conley M, Cox C, Kirke PN, McKeever MP, Mills JL, Molloy AM, O'Leary VB, Parle-McDermott A, Scott JM, Swanson DA. A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. *Am J Hum Genet*. 2002; 71(5):1207–1215. [PubMed: 12384833]
- Buchet JP, Lauwerys R. Study of inorganic arsenic methylation by rat liver in vitro: relevance for the interpretation of observations in man. *Arch Toxicol*. 1985; 57(2):125–129. [PubMed: 4026571]
- Canfield MA, Collins JS, Botto LD, Williams LJ, Mai CT, Kirby RS, Pearson K, Devine O, Mulinare J, National Birth Defects Prevention N. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. *Birth defects research Part A, Clinical and molecular teratology*. 2005; 73(10):679–689.
- Carpenter SJ. Developmental analysis of cephalic axial dysraphic disorders in arsenic-treated hamster embryos. *Anatomy and embryology*. 1987; 176(3):345–365. [PubMed: 3631535]
- Carroll N, Pangilinan F, Molloy AM, Troendle J, Mills JL, Kirke PN, Brody LC, Scott JM, Parle-McDermott A. Analysis of the MTHFD1 promoter and risk of neural tube defects. *Hum Genet*. 2009; 125(3):247–256. [PubMed: 19130090]
- Chen Y, Ahsan H, Parvez F, Howe GR. Validity of a food-frequency questionnaire for a large prospective cohort study in Bangladesh. *The British journal of nutrition*. 2004; 92(5):851–859. [PubMed: 15533275]
- Christensen KE, Rohlicek CV, Andelfinger GU, Michaud J, Bigras JL, Richter A, Mackenzie RE, Rozen R. The MTHFD1 p.Arg653Gln variant alters enzyme function and increases risk for congenital heart defects. *Hum Mutat*. 2009; 30(2):212–220. [PubMed: 18767138]
- De Marco P, Merello E, Calevo MG, Mascelli S, Raso A, Cama A, Capra V. Evaluation of a methylenetetrahydrofolate-dehydrogenase 1958G>A polymorphism for neural tube defect risk. *Journal of human genetics*. 2006; 51(2):98–103. [PubMed: 16315005]
- Dey AC, Shahidullah M, Mannan MA, Noor MK, Saha L, Rahman SA. Maternal and neonatal serum zinc level and its relationship with neural tube defects. *J Health Popul Nutr*. 2010; 28(4):343–350. [PubMed: 20824977]
- Etheredge AJ, Finnell RH, Carmichael SL, Lammer EJ, Zhu H, Mitchell LE, Shaw GM. Maternal and infant gene-folate interactions and the risk of neural tube defects. *Am J Med Genet A*. 2012; 158A(10):2439–2446. [PubMed: 22903727]
- Finnell RH, Gelineau-van Waes J, Bennett GD, Barber RC, Wlodarczyk B, Shaw GM, Lammer EJ, Piedrahita JA, Eberwine JH. Genetic basis of susceptibility to environmentally induced neural tube defects. *Ann N Y Acad Sci*. 2000; 919:261–277. [PubMed: 11083116]

- Golub MS, Macintosh MS, Baumrind N. Developmental and reproductive toxicity of inorganic arsenic: animal studies and human concerns. *J Toxicol Environ Health B Crit Rev*. 1998; 1(3): 199–241. [PubMed: 9644328]
- Gomez-Rubio P, Meza-Montenegro MM, Cantu-Soto E, Klimecki WT. Genetic association between intronic variants in AS3MT and arsenic methylation efficiency is focused on a large linkage disequilibrium cluster in chromosome 10. *Journal of applied toxicology : JAT*. 2010; 30(3):260–270. [PubMed: 20014157]
- Han ZJ, Song G, Cui Y, Xia HF, Ma X. Oxidative stress is implicated in arsenic-induced neural tube defects in chick embryos. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*. 2011; 29(7):673–680. [PubMed: 21723934]
- Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *Journal of biomedical informatics*. 2009; 42(2):377–381. [PubMed: 18929686]
- Hernandez A, Xamena N, Sekaran C, Tokunaga H, Sampayo-Reyes A, Quinteros D, Creus A, Marcos R. High arsenic metabolic efficiency in AS3MT287Thr allele carriers. *Pharmacogenet Genomics*. 2008; 18(4):349–355. [PubMed: 18334919]
- Hill DS, Wlodarczyk BJ, Finnell RH. Reproductive consequences of oral arsenate exposure during pregnancy in a mouse model. *Birth defects research Part B, Developmental and reproductive toxicology*. 2008; 83(1):40–47.
- Hol FA, van der Put NM, Geurds MP, Heil SG, Trijbels FJ, Hamel BC, Mariman EC, Blom HJ. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylene tetrahydrofolate dehydrogenase, methenyl tetrahydrofolate cyclohydrolase, formyl tetrahydrofolate synthetase) in patients with neural tube defects. *Clinical genetics*. 1998; 53(2):119–125. [PubMed: 9611072]
- Holson JF, Stump DG, Clevidence KJ, Knapp JF, Farr CH. Evaluation of the prenatal developmental toxicity of orally administered arsenic trioxide in rats. *Food Chem Toxicol*. 2000; 38(5):459–466. [PubMed: 10762732]
- Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong LY. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA*. 2001; 285(23):2981–2986. [PubMed: 11410096]
- Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clinical chemistry*. 1988; 34(11):2357–2359. [PubMed: 3141087]
- Jiang J, Zhang Y, Wei L, Sun Z, Liu Z. Association between MTHFD1 G1958A polymorphism and neural tube defects susceptibility: a meta-analysis. *PloS one*. 2014; 9(6):e101169. [PubMed: 24977710]
- Khoury MJ, Wagener DK. Epidemiological evaluation of the use of genetics to improve the predictive value of disease risk factors. *Am J Hum Genet*. 1995; 56(4):835–844. [PubMed: 7717394]
- Konrad C, Muller GA, Langer C, Kuhlbaumer G, Berger K, Nabavi DG, Dziewas R, Stogbauer F, Ringelstein EB, Junker R. Plasma homocysteine, MTHFR C677T, CBS 844ins68bp, and MTHFD1 G1958A polymorphisms in spontaneous cervical artery dissections. *J Neurol*. 2004; 251(10):1242–1248. [PubMed: 15503105]
- Lindberg AL, Kumar R, Goessler W, Thirumaran R, Gurzau E, Koppova K, Rudnai P, Leonardi G, Fletcher T, Vahter M. Metabolism of low-dose inorganic arsenic in a central European population: influence of sex and genetic polymorphisms. *Environmental health perspectives*. 2007; 115(7): 1081–1086. [PubMed: 17637926]
- Mazumdar M, Ibne Hasan MO, Hamid R, Valeri L, Paul L, Selhub J, Rodrigues EG, Silva F, Mia S, Mostofa MG, Quamruzzaman Q, Rahman M, Christiani DC. Arsenic is associated with reduced effect of folic acid in myelomeningocele prevention: a case control study in Bangladesh. *Environ Health*. 2015; 14(1):34. [PubMed: 25885259]
- O'Byrne MR, Au KS, Morrison AC, Lin JI, Fletcher JM, Ostermaier KK, Tyerman GH, Doebel S, Northrup H. Association of folate receptor (FOLR1, FOLR2, FOLR3) and reduced folate carrier (SLC19A1) genes with meningocele. *Birth defects research Part A, Clinical and molecular teratology*. 2010; 88(8):689–694.

- Obican SG, Finnell RH, Mills JL, Shaw GM, Scialli AR. Folic acid in early pregnancy: a public health success story. *Faseb J*. 2010; 24(11):4167–4174. [PubMed: 20631328]
- Pangilinan F, Molloy AM, Mills JL, Troendle JF, Parle-McDermott A, Signore C, O'Leary VB, Chines P, Seay JM, Geiler-Samerotte K, Mitchell A, VanderMeer JE, Krebs KM, Sanchez A, Cornman-Homonoff J, Stone N, Conley M, Kirke PN, Shane B, Scott JM, Brody LC. Evaluation of common genetic variants in 82 candidate genes as risk factors for neural tube defects. *BMC medical genetics*. 2012; 13:62. [PubMed: 22856873]
- Parle-McDermott A, Kirke PN, Mills JL, Molloy AM, Cox C, O'Leary VB, Pangilinan F, Conley M, Cleary L, Brody LC, Scott JM. Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. *European journal of human genetics : EJHG*. 2006; 14(6):768–772. [PubMed: 16552426]
- Pei L, Liu J, Zhang Y, Zhu H, Ren A. Association of reduced folate carrier gene polymorphism and maternal folic acid use with neural tube defects. *Am J Med Genet B Neuropsychiatr Genet*. 2009; 150B(6):874–878. [PubMed: 19105199]
- Pei L, Zhu H, Ye R, Wu J, Liu J, Ren A, Li Z, Zheng X. Interaction between the SLC19A1 gene and maternal first trimester fever on offspring neural tube defects. *Birth defects research Part A, Clinical and molecular teratology*. 2015; 103(1):3–11.
- Reichard JF, Schnekenburger M, Puga A. Long term low-dose arsenic exposure induces loss of DNA methylation. *Biochem Biophys Res Commun*. 2007; 352(1):188–192. [PubMed: 17107663]
- Rodrigues EG, Kile M, Hoffman E, Quamruzzaman Q, Rahman M, Mahiuddin G, Hsueh Y, Christiani DC. GSTO and AS3MT genetic polymorphisms and differences in urinary arsenic concentrations among residents in Bangladesh. *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals*. 2012; 17(3):240–247.
- Rodriguez S, Gaunt TR, Day IN. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. *American journal of epidemiology*. 2009; 169(4):505–514. [PubMed: 19126586]
- Rothman, KJ.; Greenland, S. *Modern Epidemiology*. 2. Rothman, KJ.; Greenland, S., editors. New York: Lippincott, Williams & Wilkins; 1998a. p. 401-434.
- Rothman, KJ.; Greenland, S. *Modern Epidemiology*. 2. Rothman, KJ.; Greenland, s, editors. New York: Lippincott, Williams & Wilkins; 1998b. p. 147-161.
- Schlawicke Engstrom K, Broberg K, Concha G, Nermell B, Warholm M, Vahter M. Genetic polymorphisms influencing arsenic metabolism: evidence from Argentina. *Environmental health perspectives*. 2007; 115(4):599–605. [PubMed: 17450230]
- Schlawicke Engstrom K, Nermell B, Concha G, Stromberg U, Vahter M, Broberg K. Arsenic metabolism is influenced by polymorphisms in genes involved in one-carbon metabolism and reduction reactions. *Mutation research*. 2009; 667(1–2):4–14. [PubMed: 18682255]
- Seaman SR, White IR. Review of inverse probability weighting for dealing with missing data. *Statistical methods in medical research*. 2013; 22(3):278–295. [PubMed: 21220355]
- Shalat SL, Walker DB, Finnell RH. Role of arsenic as a reproductive toxin with particular attention to neural tube defects. *J Toxicol Environ Health*. 1996; 48(3):253–272. [PubMed: 8656449]
- Shang Y, Zhao H, Niu B, Li WI, Zhou R, Zhang T, Xie J. Correlation of polymorphism of MTHFRs and RFC-1 genes with neural tube defects in China. *Birth defects research Part A, Clinical and molecular teratology*. 2008; 82(1):3–7.
- Smith AH, Lingas EO, Rahman M. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull World Health Organ*. 2000; 78(9):1093–1103. [PubMed: 11019458]
- Spiegelstein O, Gould A, Wlodarczyk B, Tsie M, Lu X, Le C, Troen A, Selhub J, Piedrahita JA, Salbaum JM, Kappen C, Melnyk S, James J, Finnell RH. Developmental consequences of in utero sodium arsenate exposure in mice with folate transport deficiencies. *Toxicol Appl Pharmacol*. 2005; 203(1):18–26. [PubMed: 15694460]
- Tellez-Plaza M, Gribble MO, Voruganti VS, Francesconi KA, Goessler W, Umans JG, Silbergeld EK, Guallar E, Franceschini N, North KE, Kao WH, MacCluer JW, Cole SA, Navas-Acien A. Heritability and preliminary genome-wide linkage analysis of arsenic metabolites in urine. *Environmental health perspectives*. 2013; 121(3):345–351. [PubMed: 23322787]

- van der Linden IJ, Afman LA, Heil SG, Blom HJ. Genetic variation in genes of folate metabolism and neural-tube defect risk. *The Proceedings of the Nutrition Society*. 2006; 65(2):204–215. [PubMed: 16672082]
- van der Linden IJ, Heil SG, Kouwenberg IC, den Heijer M, Blom HJ. The methylenetetrahydrofolate dehydrogenase (MTHFD1) 1958G>A variant is not associated with spina bifida risk in the Dutch population. *Clinical genetics*. 2007; 72(6):599–600. [PubMed: 17894836]
- Vujcic M, Shroff M, Singh KK. Genetic determinants of mitochondrial response to arsenic in yeast *Saccharomyces cerevisiae*. *Cancer research*. 2007; 67(20):9740–9749. [PubMed: 17942904]
- Williams LJ, Mai CT, Edmonds LD, Shaw GM, Kirby RS, Hobbs CA, Sever LE, Miller LA, Meaney FJ, Levitt M. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology*. 2002; 66(1):33–39. [PubMed: 12115778]
- Wlodarczyk BJ, Zhu H, Finnell RH. Mthfr gene ablation enhances susceptibility to arsenic prenatal toxicity. *Toxicol Appl Pharmacol*. 2014; 275(1):22–27. [PubMed: 24384392]
- Wu H, Zhang K, Gong P, Qiao F, Wang L, Cui H, Sui X, Gao J, Fan H. A novel functional TagSNP Rs7560488 in the DNMT3A1 promoter is associated with susceptibility to gastric cancer by modulating promoter activity. *PLoS One*. 2014; 9(3):e92911. [PubMed: 24667323]

Characteristics of cases of myelomeningocele and controls in Bangladesh (mean $\pm$ SD, except where noted)

Table 1

	Controls (n=55)	Cases (n=54)	P value
Age (months)	8.0 $\pm$ 4.9	6.1 $\pm$ 5.4	0.51
Male [n (%)]	32 (58)	32 (59)	0.91
Mother's age at delivery (years)	23.1 $\pm$ 4.1	25.1 $\pm$ 5.3	0.06
Father's age at delivery (years)	31.5 $\pm$ 5.4	32.2 $\pm$ 6.4	0.23
Reported periconceptional folic acid use [n(%)]	39 (71)	27 (50)	0.03
Mother's plasma folate concentration at study visit (nmol/L)	10.4 $\pm$ 6.5	10.6 $\pm$ 13.7	0.95
First born	25 (46)	23 (43)	0.76
Cesarian section [n(%)]	24 (44)	23 (43)	0.91
Reported undergoing ultrasound during pregnancy [n(%)]	50 (91)	46 (85)	0.35
Birth site			0.21
Hospital	23 (42)	25 (46)	
Birth center/clinic	11 (20)	15 (28)	
Home	21 (38)	14 (26)	
Level of myelomeningocele			
Lumbar [n (%)]	-	46 (85)	
Cervical [n (%)]	-	8 (15)	

Table 2

Estimated OR for ln-water inorganic arsenic concentration on myelomeningocele risk for different genotypes

Genotype	OR*	95% CI
<i>MTHFD1</i> (rs2236225)		
GG	0.78	(0.67, 0.92)
AG + AA	1.13	(1.04, 1.23)
<i>SLC19A1</i> (rs1051266)		
GG	1.31	(1.14, 1.50)
GA + AA	0.82	(0.75, 0.92)
<i>DNMT3A</i> (rs7560488)		
TT	1.20	(1.09, 1.32)
CT + CC	0.84	(0.75, 0.94)
<i>AS3MT</i> (rs3740393)		
GG	1.25	(1.14, 1.38)
GC + CC	0.54	(0.54, 0.65)

* ORs are estimated from weighted logistic regression models adjusted for, child sex, mother's age, father's age, child's age in months, periconceptional folic acid use. Models use inverse probability weighting to correct for clinic sites.