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Parental metal exposures as potential risk factors for spina bifida in Bangladesh

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Competing Financial Interests

GT received a Student/New Investigator Travel Award of $750.00 to attend and present at the 2019 Environmental Mutagenesis and Genomics Society annual meeting in Washington DC from September 19–23, 2019. The other authors declare they have no actual or potential competing financial interests.

Data Availability

Data from this project are available upon request.

Ethics Approval

The Bangladesh Medical Research Council and the Human Research Committees at Boston Children's Hospital and the National Institute of Neurosciences & Hospital approved all study protocols, which were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from parents prior to enrollment.

Author Statement

Gwen Tindula: Conceptualization, Methodology, Software, Validation, Formal analysis, Data Curation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Sudipta Kumer Mukherjee:** Conceptualization, Investigation, Resources, Writing - Review & Editing, Project Administration. **Sheikh Muhammad Ekramullah:** Resources, Writing - Review & Editing. **DM Arman:** Resources, Writing - Review & Editing. **Subrata Biswas:** Resources, Writing - Review & Editing. **Joynul Islam:** Resources, Writing - Review & Editing. **John F. Obrycki:** Methodology, Data Curation, Writing - Original Draft. **David C. Christiani:** Conceptualization, Writing - Review & Editing, Supervision. **Liming Liang:** Writing - Review & Editing, Supervision. **Benjamin C. Warf:** Validation, Writing - Review & Editing. **Maitreyi Mazumdar:** Conceptualization, Methodology, Writing - Original Draft, Project Administration, Funding Acquisition.

Conflict of Interest Statement

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Abstract

Background: Neural tube defects are a pressing public health concern despite advances in prevention from folic acid-based strategies. Numerous chemicals, in particular arsenic, have been associated with neural tube defects in animal models and could influence risk in humans.

Objectives: We investigated the relationship between parental exposure to arsenic and 17 metals and risk of neural tube defects (myelomeningocele and meningocele) in a case control study in Bangladesh.

Methods: Exposure assessment included analysis of maternal and paternal toenail samples using inductively coupled plasma mass spectrometry (ICP-MS). A total of 278 participants (155 cases and 123 controls) with data collected from 2016–2020 were included in the analysis.

Results: In the paternal models, a one-unit increase in the natural logarithm of paternal toenail arsenic was associated with a 74% (odds ratio: 1.74, 95% confidence interval: 1.26–2.42) greater odds of having a child with spina bifida, after adjusting for relevant covariates. Additionally, paternal exposure to aluminum, cobalt, chromium, iron, selenium, and vanadium was associated with increased odds of having a child with spina bifida in the adjusted models. In the maternal models, a one-unit increase in the natural logarithm of maternal toenail selenium and zinc levels was related to a 382% greater (odds ratio: 4.82, 95% confidence interval: 1.32–17.60) and 89% lower (odds ratio: 0.11, 95% confidence interval: 0.03–0.42) odds of having a child with spina bifida in the adjusted models, respectively. Results did not suggest an interaction between parental toenail metals and maternal serum folate.

Discussion: Parental toenail levels of numerous metals were associated with increased risk of spina bifida in Bangladeshi infants. Paternal arsenic exposure was positively associated with neural tube defects in children and is of particular concern given the widespread arsenic poisoning of groundwater resources in Bangladesh and the lack of nutritional interventions aimed to mitigate paternal arsenic exposure. The findings add to the growing body of literature of the impact of metals, especially paternal environmental factors, on child health.

Keywords

Arsenic; folate; neural tube defects; heavy metals; Bangladesh

1. Introduction[1]

Neural tube defects are severe birth defects that occur when the neural plate, the embryonic precursor to the brain and spinal cord, fails to close around three to four weeks of gestation (Greene and Copp 2014; Mazumdar 2017). Health consequences depend on the location and severity of the neural tube defect, and include fetal death or complications including permanent spinal cord damage, neurological impairment, and gastrointestinal and genitourinary disorders (Greene and Copp 2014; Mazumdar 2017). Myelomeningocele, which refers to a specific neural tube defect in the spinal region, is the most severe type of spina bifida and can impact sensory and motor neurological functions in afflicted children (Copp et al. 2015). Folic acid supplementation of women before and during pregnancy, as well as folic acid fortification of food, have helped to reduce the prevalence of neural tube defects. For instance, research in South American countries, the United States, Canada, Costa Rica, and South Africa have reported reductions in the number of neural tube defects by 19–55% following folic acid fortification of food (Crider et al. 2011). However, folic-acid based interventions do not alone account for the global disparity in neural tube defect prevalence and affected pregnancies continue to occur in regions with widespread folic acid fortification and to women known to have taken folic acid supplements (Centers for Disease and Prevention 2010). In addition to folate status, other recognized risk factors for neural tube defects include obesity, ethnicity, and female infant sex; however, less than 50% of neural tube defects are estimated to be attributed to known risk factors (Agopian et al. 2013).

Increasing evidence suggests a role of environmental exposure in the etiology of neural tube defects, including prenatal exposure to arsenic. Arsenic is naturally occurring in soil and routes of exposure include inhalation of air that contains arsenic dusts and ingestion of contaminated food and water (Agency for Toxic Substances and Disease Registry (ATSDR) 2007). Chronic exposure to arsenic is associated with numerous health outcomes, including skin lesions, inflammation, intellectual impairments, and diabetes (Bozack et al. 2018; Naujokas et al. 2013). The 2012–2013 Multiple Indicator Cluster Survey in Bangladesh, where the current study takes place, estimated that roughly 24.8% of the population use household drinking water with arsenic levels above the 10 μg/L World Health Organization guideline (Bangladesh Bureau of Statistics (BBS) 2014), predominantly as a result of ubiquitous use of shallow tubewells (< 150 m) (BGS and DPHE 2001) contaminated with arsenic. Bangladesh also has relatively high occurrence of neural tube defects, with an estimated prevalence of 13.8 per 10,000 births (Dey et al. 2010; Zaganjor et al. 2016).

Arsenic induces neural tube defects in animal models (Beaudoin 1974; Carpenter 1987; Han et al. 2011; Hill et al. 2008). In addition to arsenic, heavy metals and trace elements are associated with neural tube defects in mice (Fernandez et al. 2004; H Li et al. 2018; Robinson et al. 2011; Stokes et al. 2017; Webster and Messerle 1980) and chickens (Kmecick et al. 2019; Papaconstantinou et al. 2003). Research in humans has examined the relationships between parental exposure to metals, including zinc, mercury, and lead, and the risk of neural tube defects in their children (Brender et al. 2006; Demir et al. 2019; Dey et al. 2010; Groenen et al. 2004; Mazumdar et al. 2015a; Ovayolu et al. 2020; Ozel et al. 2019; Van Brusselen et al. 2020; Yan et al. 2017; Zeyrek et al. 2009); however, most of the studies focus on maternal exposure and include a small subset of metals. In the

current study, we measured both maternal and paternal exposure to 18 metals, including essential trace elements, and metalloids using toenail samples, a non-invasive biomarker that represents exposures from the previous 3–12 months (Gutierrez-Gonzalez et al. 2019).

In a pilot study conducted between April and November of 2013, we reported previously on the relationship between arsenic exposure and neural tube defect risk in a Bangladeshi population of 57 children diagnosed with myelomeningocele and 55 control children. Although we did not observe a main effect between maternal arsenic exposure and disease risk, we identified an interaction between prenatal folic acid use and water arsenic concentrations, with decreasing protective effects of folic acid at higher water arsenic concentrations (Mazumdar et al. 2015a). The current study, which utilizes samples and data from a separate and larger case-control study from Bangladesh that was initiated in 2016, has two objectives. First, we will determine whether the relationships between arsenic, folate status, and risk of neural tube defects observed in the pilot study can be validated in a different case-control population from Bangladesh. Second, we will explore the influence of levels of trace elements and heavy metals in both mothers and fathers on spina bifida risk.

2. Materials and Methods

2.1 Study Population

Cases included children with myelomeningocele or meningocele who presented for evaluation of the neural tube defect at the National Institute of Neurosciences & Hospital (NINS), the primary center for spina bifida surgery in Bangladesh. Enrollment focused on recruiting children diagnosed with spina bifida rather than anencephaly, another type of neural tube defect, due to the inability to reliably identify anencephaly cases through our hospital-based case ascertainment system and the possibility that babies born at home with anencephaly would likely not survive to be seen at a hospital. Additionally, myelomeningocele, a severe form of spina bifida, was the most common neural tube defect observed in our pilot studies (Mazumdar et al. 2015a; Mazumdar et al. 2015b). Cases were eligible to enroll in the study if they were diagnosed with myelomeningocele or meningocele following examination by a physician, were less than a year old, received medical care from NINS, and whose parents were able to identify the primary water source used during early pregnancy. Controls were selected from individuals who presented at NINS or the adjacent children's hospital, Dhaka Shishu Hospital (DSH), with diagnoses with similar referral patterns and that are considered to be unrelated to arsenic exposure, such as craniosynostosis, trauma, and epilepsy. Six individuals originally classified as cases were later designated as controls following diagnosis with lipomeningocele. Cases and controls were matched on age (±6 months).

Between December 2016 and November 2020, 192 infants with spina bifida (myelomeningocele or meningocele) and 171 hospital-based controls were enrolled in the study. Out of the participants, 155 cases and 123 controls had data available for toenail metal exposures and relevant covariates and were included in the analysis. The Bangladesh Medical Research Council and the Human Research Committees at Boston Children's Hospital and NINS approved all study protocols, and informed consent was obtained from parents prior to enrollment.

2.2 Questionnaires and Physical Examination

Parents were interviewed by trained staff members. Information gathered at the interview included water source and intake, parental smoking, parental alcohol or drug use, parental education and occupation, and medical history, including the use of medications during pregnancy. Mothers were also asked to report multivitamin and folic acid supplementation during pregnancy. A food frequency questionnaire previously validated in rural Bangladeshi populations (Chen et al. 2004) was administered to the mothers to estimate pregnancy nutritional intake. The hospital visit also involved a physical examination of the child and measurement of the height, weight, and % body fat of the parents. A follow-up visit to each participant's home was conducted to obtain a sample from the water source the mother used when she discovered she was pregnant.

2.3 Toenail Metal Concentrations

Toenail samples from mothers and fathers were collected at the time of the initial study visit. Clippings from all ten toes were collected on a white sheet of paper and transferred to a small coin envelope (ULINE Model No. S-7798), which was sealed with tape. Toenail samples were stored at room temperature until specimens were shipped on dry ice to Boston, Massachusetts for storage and downstream analyses. Toenail samples were shipped at room temperature to the Dartmouth Trace Element Analysis Core for environmental metal exposure analysis. Specifically, the total concentrations of 18 elements in toenail specimens were measured, including aluminum (Al), antimony (Sb), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), uranium (U), vanadium (V), and zinc (Zn). Since the majority of the elements measured were metals, we will henceforth refer to the totality of toenail elemental exposures as metals, taking into consideration that arsenic and antimony are metalloids and selenium is a nonmetal.

Visible dirt was removed from the toenails by first placing all toenail samples from an individual into a 7 mL polyethylene tube, covering them in 2 mL of acetone, and placing them in an ultrasonic bath for 20 minutes. Following the initial ultrasonic bath, 2mL of a 1% solution of Triton X-100 was added and the tubes were returned to the ultrasonic bath for an additional 20 minutes. Upon removal from the bath, the samples underwent a series of five washes with 5 mL of trace metal grade deionized water (18 Ω). The nails were subsequently dried in a forced air oven set at 105°C.

Following cleaning, the samples were transferred to a pre-weighed 15 mL polypropylene centrifuge tube. The tube with the toenails was weighed and the mass of the sample was calculated by taking the difference between the 15 mL centrifuge tube with and without the nail sample; henceforth referred to as the sample mass. The toenails were digested in a volume of 9:1 optima nitric acid (67–69%) and hydrochloric acid (Fisher Scientific, USA). After overnight pre-digestion of the samples, 100 μl of hydrogen peroxide (H_2O_2) was added. The tubes were placed in a CEM MARS6 (CEM Corporation, Matthews, NC, USA) microwave digestion system and were heated to 105°C, with a 10 minute "time to temperature" and a 45 minute hold time. Once cooled, the tubes were removed from the microwave and hydrogen peroxide was added, followed by the addition of deionized

water. The mass of the tube was recorded, and the final dilution weight was calculated by subtracting the mass of the vial from the measured weight, a value that will subsequently be referred to as the final dilution mass. All digested toenail samples were analyzed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). The first set of toenail samples was run using the Agilent™ 7900 system, while sets two through four were analyzed with the Agilent™ 8900 system.

Quality control procedures included the incorporation of certified reference material, blanks, and spiked controls in the experimental runs. Each individual toenail metal measurement was compared to the method detection limit (MDL), which was calculated using $MDL_{ij} = (IDL_i \times \overline{DF_J})/1000$, where MDL_{ij} is the minimum detection limit for the i-th metal and the j-th toenail set (j=1, 2, 3, or 4), IDL_i is the instrument detection limit for the i-th metal in ppb, and $\overline{DF_J}$ is the average dilution factor for the j-th set. The dilution factor was calculated for each individual by dividing the final dilution mass of the toenails by the sample mass. For each toenail metal concentration, measurements that were below the set-specific MDL (Table S1) and were equal to or less than zero were assigned a value equal to the set detection limit divided by the square root of two. This resulted in six substitutions (substitutions by toenail metal: nickel=five, molybdenum=one). One individual was removed from the analysis because their toenail sample had an exceptionally large dilution factor.

2.4 Folate Assessment

Fasting blood samples were collected from mothers at the hospital visit via venipuncture. Mothers were instructed to fast for a minimum of 8 hours to facilitate assessment of fasting glucose. Blood was collected into a potassium oxalate and sodium fluoride coated blood collection tube, which was centrifuged, and the separated plasma was used to measure glucose and homocysteine. An additional blood collection tube with K2-EDTA anticoagulant was collected to determine hemoglobin and hematocrit levels in whole blood specimens. Finally, two tubes without anticoagulant were centrifuged to separate serum from clot. Aliquots from the first tube were processed to assess serum insulin, folate, and vitamin B12 levels. Serum folate was measured using a Chemiluminescent Microparticle Immunoassay (CMIA) assay on the ARCHITECT plus c4000 (Abbott Company, Abbott Park, IL, USA), adhering to the manufacturer's instructions. Folate, vitamin B12, glucose, and homocysteine were measured at NINS. Insulin was measured at the Bangabandhu Sheikh Mujib Medical University Lab.

2.5 Statistical Analysis

Descriptive statistics were generated for the study characteristics and parental toenail metal data. Data for cases and controls were initially compared using t-tests for continuous variables and chi-squared tests for binary variables. All parental toenail metal concentrations were natural log transformed prior to statistical analysis to account for the right-skewed distribution of the metals and to reduce the influence of outliers. We calculated Pearson correlation coefficients to determine the relationship between maternal and paternal concentrations for each metal.

Unconditional logistic regression models were used to calculate odds ratios (ORs) and 95% confidence intervals (CI) for the association between parental toenail metal exposures (predictors) and spina bifida case status (outcome). We utilized an unconditional, rather than conditional, logistic regression model because it has been shown to be an appropriate method in studies involving loose-matching on a few demographic variables (Kuo et al. 2018), such as the child age variable used in the matching in the current study. We generated three logistic regression models: the crude model; an adjusted model, and an interaction model. Each individual natural log transformed toenail metal concentration from a given parent was run separately for all models. The total number of models analyzed in the individual toenail metal analysis was 108 (18 toenail metals * 3 models [crude, adjusted, interaction] * 2 parents [mothers and fathers]). In the adjusted and interaction models, we included as additional predictor variables relevant covariates identified in our previous analysis. The covariates included continuous measures of parental ages (in years) and maternal serum folate levels (ng/mL). Since maternal serum folate was right-skewed, the variable was natural log transformed prior to analysis. Additionally, an infant sex variable and a birth place variable, coded as “1” for home birth and “0” for clinic or hospital birth, were included in the adjusted and interaction models. Although we observed significant age differences between cases and controls; specifically, controls were on average older than cases, we did not include child age as an additional predictor variable in our final models since we would not expect it to influence case diagnosis. In preliminary models that included age as a predictor, results for the metals were similar to those of the metals that were significant after adjusting for multiple hypothesis testing in the final models. The interaction model included the covariates and also accounted for the interaction between the parental toenail metal concentration and maternal serum folate levels. We adjusted P-values for multiple hypotheses testing using the Benjamini–Hochberg false discovery rate (FDR) threshold for significance of 0.05 (Benjamini and Hochberg 1995).

In addition to the individual metal exposure models, we performed elastic net logistic regression (Zou and Hastie 2005) for parental metal mixtures to select for metals with the greatest importance in predicting disease status. Elastic net regularization integrates both ridge regression and least absolute shrinkage and selection operator (LASSO) in variable selection. In ridge regression, coefficients of highly correlated variables are shrunk towards each other, but not to zero (Friedman et al. 2010). In contrast, LASSO reduces most coefficients to zero, while retaining a subset of non-zero coefficients (Friedman et al. 2010). Separate models were generated for maternal and paternal toenail metals. Natural log transformed toenail metals were scaled prior to analysis to facilitate comparisons between metals. Each parental model included the 18 natural log transformed and scaled toenail metal variables for the parent, as well as the covariates used in the individual metal logistic regression models. We also ran a combined parental model with maternal and paternal toenail metals included as predictor variables, along with the demographic covariates. Therefore, a total of three elastic net logistic regression models were generated (toenail metals from mothers, fathers, and mothers and fathers combined). The elastic net regression was implemented using the cv.glmnet function within the *glmnet* (Friedman et al. 2019) R package, using the default parameters and an elastic net mixing parameter α of 0.5, which falls between the lasso ($\alpha = 1$) and ridge regression ($\alpha = 0$) penalties. Penalized regression

coefficients were extracted when the lambda value used in the model fitting was equivalent to the lambda that produces the minimum mean cross-validated error; referred to as lambda min. As a sensitivity analysis, we also extracted the betas for which lambda was equal to the largest value of lambda such that the error is within 1 standard error of the minimum, henceforth referred to as the lambda 1SE, which gives us more conservative estimates.

All analyses were performed using STATA (v16.0), with the exception of the elastic net regression analysis, which was conducted in R statistical computing software (v3.6.2).

3. Results

3.1 Study Population Attributes

Parents and infants included in the study (278 parent-child pairs) did not differ from those that were excluded due to missing data (84 parent-child pairs) in the relevant covariates (i.e. serum folate, parental age, birth place, child sex). Characteristics of parents and children included in the study are presented in Table 1. Mothers and fathers of cases and controls had similar age distributions at the time of the study visit (maternal age range [years], cases: 17–37, controls: 18–35, t-test $P = 0.64$; paternal age range [years], cases: 21–48, controls: 19–52, t-test $P = 0.37$). Serum folate levels were similar between mothers of cases and mothers of controls (t-test $P = 0.33$). Most of the mothers in the study gave birth at a hospital or clinic. A greater proportion of the mothers of controls reported that this had been their first pregnancy (50%) than the mothers of the cases (36%). On average, control infants were older than case children (days; mean (SD), cases: 62.6 (75.3), controls: 183.3 (96.8), t-test $P < 0.001$); however, the range of child ages was similar (child age range [days], cases: 3–352, controls: 8–358). Of the 155 case and 123 control infants, 79 (51%) and 76 (62%) were male, respectively.

3.2 Parental Toenail Metal Concentrations

As shown in Table 2 and Figures S1–S4, concentrations varied across the 18 different metals measured in parental toenail samples. The metals with the greatest ranges observed in mothers and fathers of cases and controls were aluminum, iron, and zinc, whereas molybdenum, antimony, and uranium had the narrowest ranges in metal concentrations. On average, mothers of cases had lower concentrations of copper (geometric mean [GM]; cases: 3.59 μg/g, controls: 3.87 μg/g) and zinc (GM; cases: 100.76 μg/g, controls: 111.81 μg/g) and higher levels of selenium (GM; cases: 0.65 μg/g, controls: 0.62 μg/g) than mothers of controls ($P < 0.04$ for all t-tests). Differences between fathers of cases and controls were observed for concentrations of aluminum (GM; cases: 97.99 μg/g, controls: 80.42 μg/g), chromium (GM; cases: 0.80 μg/g, controls: 0.62 μg/g), iron (GM; cases: 222.08 μg/g, controls: 165.91 μg/g), cobalt (GM; cases: 0.09 μg/g, controls: 0.07 μg/g), arsenic (GM; cases: 0.72 μg/g, controls: 0.52 μg/g), vanadium (GM; cases: 0.24 μg/g, controls: 0.20 μg/g), and selenium (GM; cases: 0.74 μg/g, controls: 0.63 μg/g), with higher values reported in fathers of cases ($P < 0.05$ for all t-tests). Toenail concentrations for each metal were positively correlated between parents (Pearson r range: 0.15–0.69, Table S2).

3.3 Individual Assessments of Parental Toenail Metals

The primary interest of the analysis was to determine the relationship between arsenic, folate, and risk of neural tube defects. Maternal toenail arsenic concentrations were not associated with neural tube defect risk in the crude model (OR: 1.05, 95% CI: 0.82–1.34, p-value = 0.71) (Table 3). After accounting for parental age, child sex, maternal serum folate levels, and place of birth in the adjusted model and including an additional interaction term for the relationship between arsenic and serum folate in the interaction model, we did not observe an association of maternal arsenic with spina bifida in their children. In contrast, we observed a relationship between paternal arsenic exposure and neural tube defect case status. Specifically, a 1-unit increase in the natural logarithm of paternal toenail arsenic was associated with a 69% (OR: 1.69, 95% CI: 1.23–2.33, p-value = 0.001), 74% (OR: 1.74, 95% CI: 1.26–2.42, p-value = 0.001), and 312% (OR: 4.12, 95% CI: 1.25–13.58, p-value = 0.02) greater odds of being a case in the crude, adjusted, and interaction models, respectively.

A secondary goal of the analysis was to explore how additional metal exposures can influence the risk of having a child with spina bifida. Numerous toenail metals were associated with case status in the crude unconditional logistic regression models that were generated separately for each toenail metal exposure. In the maternal toenail metal models, ORs were 0.11 (95% CI: 0.03–0.41, $P = 0.001$), 0.41 (95% CI: 0.18–0.95, $P = 0.04$), and 4.65 (95% CI: 1.30–16.54, $P = 0.02$) for natural log transformed zinc, copper, and selenium, respectively. ORs from paternal models were 1.38 (95% CI: 1.02–1.86, $P = 0.04$), 1.44 (95% CI: 1.07–1.93, $P = 0.02$), 1.48 (95% CI: 1.12–1.96, $P = 0.01$), and 12.03 (95% CI: 4.26–34.00, $P < 0.001$) for natural log transformed cobalt, chromium, iron, and selenium, respectively. Selenium was the only metal associated with spina bifida case status in both the maternal and paternal crude models.

After adjustment for parental age, child sex, maternal serum folate levels, and place of birth, a 1-unit increase in the natural logarithm of maternal toenail selenium was associated with 382% greater odds of being a case (OR: 4.82, 95% CI: 1.32–17.60, $P = 0.02$) compared to being a control (Table 3, Figures S5). Additionally, maternal natural log transformed zinc exposure was associated with lower odds of having a child with spina bifida (OR: 0.11, 95% CI: 0.03–0.42, $P = 0.001$). In the paternal adjusted models, numerous metals were positively associated with disease status (Table 3, Figures S6). Specifically, ORs were 1.40 (95% CI: 1.05–1.88, $P = 0.02$), 1.42 (95% CI: 1.04–1.93, $P = 0.03$), 1.43 (95% CI: 1.06–1.92, $P = 0.02$), 1.46 (95% CI: 1.07–2.00, $P = 0.02$), 1.57 (95% CI: 1.17–2.11, $P = 0.003$), and 12.51 (95% CI: 4.33–36.18, $P < 0.001$) for vanadium, aluminum, chromium, cobalt, iron, and selenium, respectively.

The models did not suggest interactions between maternal serum folate and toenail metal concentrations, including the paternal toenail arsenic model. Only the relationships of case status with 1) maternal zinc, 2) paternal iron, 3) paternal arsenic and 4) paternal selenium in the crude and adjusted models remained significant after adjusting p-values for multiple hypothesis testing.

3.4 Elastic Net Regression

In the maternal toenail metal elastic net regression model (α=0.5, λ=lambda min), three toenail metals and the child sex variable were retained in the model and yielded non-zero penalized regression coefficients (Table 4). Specifically, selenium had a positive penalized elastic net beta (Se: 0.150), while copper and zinc had negative betas (Cu: −0.037, Zn: −0.261). In the paternal model, five toenail metals, paternal age, and the child sex variable were retained in the model. Arsenic, chromium, iron, and selenium where positively associated with spina bifida risk (betas; As: 0.154, Cr: 0.120, Fe: 0.148, Se: 0.469), whereas zinc was negatively related to case status (beta: −0.068). In the combined parental model, which included toenail metal concentrations from both parents as predictor variables, maternal copper and zinc, as well as paternal arsenic, chromium, iron, and selenium were retained in the model (maternal betas, Cu: −0.065, Zn: −0.222; paternal betas, As: 0.112, Cr: 0.112, Fe: 0.119, Se: 0.404).

In the sensitivity analysis, using the betas extracted when lambda was equal to the more conservative lambda 1SE, fewer predictor variables remained in the parental models (Table S3). In the maternal model, zinc (beta: −0.127) and selenium (beta: 0.035) remained. For the fathers, only arsenic (beta: 0.056), iron (beta: 0.034), and selenium (beta: 0.244) were retained in the model. In the combined parental model, the associations with maternal zinc (beta: −0.140) and paternal arsenic (beta: 0.065), chromium (beta: 0.041) iron (beta: 0.058), and selenium (beta: 0.296) remained in the model.

4. Discussion

We found that paternal arsenic, iron, and selenium exposure, measured in toenails, was associated with increased odds of myelomeningocele and meningocele in infants from Bangladesh after accounting for relevant covariates and adjusting for multiple hypothesis testing. Additionally, increased maternal zinc levels were related to decreased risk of having a child diagnosed with spina bifida in the adjusted individual regression analyses. The results did not suggest interactions between toenail metals in either parent and maternal serum folate levels. The coefficients that were retained in the elastic net regressions, which were generated to perform variable selection and to look at combined parental metal exposures, largely reflected the findings in the crude and adjusted models for the individual toenail metal analyses.

Our study is the first to report a relationship between paternal arsenic exposure, as measured from biological specimens, with neural tube defect risk in humans. Arsenic has been shown to induce neural tube defects in mice (Hill et al. 2008), rats (Beaudoin 1974), hamsters (Carpenter 1987), and chicks (Han et al. 2011). Only one other study has explored the influence of paternal arsenic exposure on the risk of neural tube defects in humans. Brender et al. (2006) examined whether paternal occupational exposure to arsenic and other heavy metals impacted risk of neural tube defects in a case control study in Texas. After classifying jobs into different metal exposure categories following review of occupational codes, material handled, and job tasks, they observed that paternal arsenic exposure around conception was not a significant risk factor (n=365, OR=1.5, 95% CI: 0.7–3.0). In contrast, we observed that paternal arsenic exposure measured in toenails was

related to increased odds of spina bifida after adjusting for parental age, place of birth, child sex, and maternal serum folate levels (n=278, OR=1.7, 95% CI: 1.3–2.4). Paternal toenail arsenic concentrations in our study were similar to values reported in men (Grashow et al. 2014; Wu et al. 2019) and in both genders (Al-Sabbak et al. 2012; Burgess et al. 2014; Kato et al. 2013; X Li et al. 2018; Slotnick et al. 2005; Unrine et al. 2019) in populations from the United States, Bangladesh, Vietnam, and Iraq. Our findings add to the growing body of evidence of the influence of paternal exposures and characteristics on child health (Buck Louis et al. 2016; Estors Sastre et al. 2019; Messerlian et al. 2017; Morkve Knudsen et al. 2019; Mustieles et al. 2020; Olsson et al. 2018; Snijder et al. 2011; Soubry et al. 2013).

Most of the existing literature assessing the relationship between arsenic exposure and risk of neural tube defects in children has focused on the role of maternal environmental exposure. In addition to estimating occupational exposures to heavy metals, Brender et al. (2006) measured urinary arsenic in 56 mothers of children diagnosed with neural tube defects and 74 mothers of controls. They did not observe differences in urinary arsenic between mothers of cases and controls (median urinary arsenic: case mothers=9.2 μg/L, control mothers=9.0 μg/L, Mann-Whitney test $P > 0.05$). Human studies that have measured arsenic levels in water samples and additional biological samples, such as hair, blood, and placenta, have reported either null findings (Jin et al. 2013; Mazumdar et al. 2015a; Ozel et al. 2019; Sanders et al. 2014; Wang et al. 2019) or higher levels of arsenic related to case status (Demir et al. 2019). A case-control study by Demir et al. (2019) in Turkey observed that 100 infants diagnosed with a neural tube defect and their mothers had higher plasma concentration of arsenic compared to 70 controls (average maternal plasma arsenic: controls=3.8 μg/L, neural tube defect cases=5.5 μg/L, $P<0.0001$; average infant plasma arsenic: controls=3.5 μg/L, neural tube defect cases=5.0 μg/L, $P<0.0001$). A pilot study conducted by our group in 2013 (Mazumdar et al. 2015a) did not identify a main effect of water arsenic concentrations on myelomeningocele risk, but did suggest a strong interaction between water arsenic and prenatal folic acid use. In the current study, maternal toenail arsenic was not a predictor of spina bifida risk (adjusted model, OR=1.04, 95% CI: 0.81–1.34) and we did not observe an interaction between folate and arsenic in either parental models. Arsenic levels in the toenails of mothers in our study were comparable to those observed in other studies conducted in Bangladesh during or after pregnancy (Obrycki et al. 2019; Rodrigues et al. 2015; Tauheed et al. 2017).

Although we did not observe an association between arsenic in mothers and case status, we identified significant relationships of maternal zinc and selenium with neural tube defects in children. In our study, maternal zinc was inversely associated with spina bifida in infants (adjusted model, coefficient=−2.22, OR=0.11, 95% CI: 0.03–0.42). The lower concentrations of zinc in mothers of infants with neural tube defects compared to mothers of controls (GM; cases: 100.76 μg/g, controls: 111.81 μg/g) that we observed has similarly been reported in study populations from Turkey (Zeyrek et al. 2009), the United Kingdom (Hinks et al. 1989), and Bangladesh (Dey et al. 2010). Deficiencies in zinc, which is critical during early development, may impact neural tube closure and interventions involving zinc supplementation should be explored (Dey et al. 2010).

Selenium was the only trace metal that was associated with case status in the models for both parents. In the adjusted models, ORs were 4.82 (95% CI: 1.32–17.60, $P = 0.02$) and 12.51 (95% CI: 4.33–36.18, $P < 0.001$) for the maternal and paternal analyses, respectively. Our study is the first to show a relationship between selenium status of fathers and neural tube defect risk. A study of 30 non-pregnant women, 69 women with a normal pregnancy, and 22 women with a fetus with either a neural tube defect or elevated levels of plasma alpha-fetoprotein with no detectable fetal abnormality demonstrated lower levels of leukocyte selenium in the latter groups compared to 31 women in the second trimester of a normal pregnancy ($P<0.05$) (Hinks et al. 1989). Demir et al. (2019) also explored the relationship between selenium and risk of neural tube defects, with higher levels of plasma selenium observed in controls compared to cases (average maternal plasma selenium: controls=8.0 μg/dL, neural tube defect cases=5.5 μg/dL, $P<0.0001$; average infant plasma selenium: controls=7.0 μg/dL, neural tube defect cases=5.3 μg/dL, $P<0.0001$). In a Spanish population, 44 women who gave birth to fetuses diagnosed with a neural tube defect had lower levels of plasma selenium on average than 181 control mothers (median maternal plasma selenium: controls=1.1 μmol/L, neural tube defect cases=0.8 μmol/L, $P<0.001$) (Martin et al. 2004). In contrast to the existing literature, our study identified higher levels of selenium in parents of cases compared to those of controls; however, the observed association with maternal selenium did not remain significant after accounting for multiple hypothesis testing. On average, the levels of selenium observed in maternal and paternal toenail samples in our study were comparable to other study populations from the United States (Burgess et al. 2014; Deyssenroth et al. 2018; Everson et al. 2017; Slotnick et al. 2005; Unrine et al. 2019) and Iraq (Al-Sabbak et al. 2012).

The biological mechanisms that could explain the associations observed in the current study involve oxidative stress and epigenetics. For maternal exposures, metals cross the placenta with varying degrees of efficiency (Bocca et al. 2019; Chen et al. 2014; Sakamoto et al. 2013), resulting in prenatal exposure to the developing fetus. The difference in placental transfer of metals could partially explain the limited number of metals that were associated with neural tube defect risk in the current study. Once in the fetus, metals may interact to induce oxidative stress. In the maternal metal models, increased zinc and selenium levels were related to lower and higher odds of having a child with spina bifida, respectively, in adjusted models. Zinc and selenium are considered to be important antioxidant and anti-inflammatory agents (Jarosz et al. 2017; Martin et al. 2004; Rayman 2000). However, selenium can function as a prooxidant at high levels (Bocca et al. 2019) and zinc deficiencies result in increased oxidative stress (Jarosz et al. 2017). Enhanced oxidative stress and diminished levels of antioxidants have been shown to be related to neural tube defects (Chandler et al. 2012; Chang et al. 2003; Han et al. 2011; Martin et al. 2004; Zhao et al. 2006). In mice, oxidative stress has been shown to alter the expression of genes involved in neural tube closure (Chang et al. 2003). It is possible that an imbalance in the levels of oxidants and antioxidants in response to environmental metal exposure could influence the risk of neural tube defects; however, more research on this potential biological mechanism, particularly in humans, is warranted.

In the paternal models, toenail levels of iron, selenium, and arsenic were positively related to child neural tube defect risk. Alterations to the epigenome, the multitude of

compounds that influence gene expression without changing the DNA sequence, may be the mechanism by which paternal metal exposures affect risk. These compounds include miRNAs, histone modifications, and the more commonly studied DNA methylation (Marcho et al. 2020). A growing body of literature, particularly from animal studies, supports links between environment exposures and epigenetic changes that can be transferred to the next generation through the male germline (Soubry et al. 2014). Specific sensitive periods to heritable epigenetic alterations in males include paternal embryonic development, prepuberty, spermatogenesis, and periconception and in the zygote (Soubry et al. 2014). For example, preconception exposure to environmental factors, including endocrine disrupting compounds and diet, has been shown to influence the epigenome of sperm (Marcho et al. 2020). A subset of the histone proteins present in sperm DNA are retained and modifications on these proteins can influence DNA methylation patterns in imprinted genes, which exhibit parental allele-specific methylation profiles and are critical during early development of the fetus (Hammoud et al. 2009; Miller et al. 2010; Soubry et al. 2014). In humans, maternal trace element and metal exposure has been associated with offspring imprinted genes methylation (Vidal et al. 2015). Additionally, exposure to arsenic has been associated with both global and site-specific DNA methylation (Argos et al. 2015; Hossain et al. 2017; Kile et al. 2012; Lambrou et al. 2012). More research is necessary to understand the potential role of paternal epigenetic modifications in the transfer of environmentally-induced epigenetic changes from one generation to another and the impact on birth outcomes, including neural tube defects.

Animal models and limited research in humans have suggested a role of DNA methylation in onset of neural tube defects (Mazumdar 2017). For instance, a case-control study conducted in China observed lower genomic DNA methylation in brain tissue samples from neural tube defect cases as compared to controls (Wang et al. 2010). Additionally, a study by Han et al. (Han et al. 2011) of chick embryos observed that altered reactive oxygen species levels, characteristic of oxidative stress, appear to mediate the relationship between arsenic exposure and DNA methylation, leading to neural tube defects. The combination of metal-induced oxidative stress and potential epigenetic perturbations could underly the observed associations between parental metal exposure and neural tube defect risk observed in the current study. In the future, we plan to explore the influence of metal and trace element levels on DNA methylation patterns of children with spina bifida, with a particular focus on imprinted genes.

One of the main strengths of the study is the availability of biological specimens to assess metal and folate levels, which allowed us to understand the body burden of exposure without having to rely on more subjective measures of exposure assessment. Another strength involved collection and analysis of paternal toenail samples for metal exposure. Whereas most of the research has focused on maternal metal exposures, we were able to identify novel associations between paternal toenail metal concentrations and risk of neural tube defects, which can inform targeted intervention strategies.

One limitation of the study was the rather small sample size; however, the number of study participants is comparable to existing literature assessing the relationships between parental metal exposure and neural tube defect risk. An additional limitation of the study is related

to the timing of exposure assessment. Controls were generally older than case infants; therefore, parental toenails collected around the time of the child visit may reflect different exposure windows for cases and controls. However, given the mass poisoning of water resources with arsenic in Bangladesh, we would anticipate that exposures are consistent across time. An additional study limitation includes the absence of toenail data from the infants included in the study. Previous studies have demonstrated correlations between level of metals in infant and postnatal maternal toenail samples (Davis et al. 2014; Rodrigues et al. 2015). Given the slow rate of toenail growth (1.6 mm/month) (Yaemsiri et al. 2010), the parental toenail measurements used in the current study reflect exposures from the past 3–12 months (Gutierrez-Gonzalez et al. 2019), thereby representing a proxy for the levels of metals crossing the placenta and reaching the developing fetus. An additional limitation of the study is the inability to confirm causal relationships between metal exposure and neural tube defects. This is a common concern for case-control studies. Although we identified novel relationships between metals and spina bifida risk, particularly in regards to paternal exposures, the results may be influenced by additional confounders that were not accounted for in the analysis and the study design does not permit causal interpretation of the results, limiting the ability to translate the findings into practical public health prevention strategies. Therefore, we hope the associations observed in this initial study will help inform future research to validate findings and to explore the usefulness of interventions that target fathers. Another limitation involves the lack of folate data for fathers in the study. Increasing evidence in humans suggests that periconceptional or postnatal paternal folate levels are related to embryonic growth (Hoek et al. 2019), gestation duration (Martin-Calvo et al. 2019), and risk of neural tube defects (Ratan et al. 2008). Given the importance of paternal metal exposures in this study and the potential role of folate in modifying the neurotoxicity of arsenic, we would like to measure paternal folate in future studies and to examine the epigenetic mechanisms, including DNA methylation of imprinted genes, that may facilitate the observed association between paternal arsenic and spina bifida in children.

In conclusion, our study is the first to report an association between paternal arsenic exposure and spina bifida risk in infants. Neural tube defects are severe birth defects with long-term health consequences in surviving children; therefore, understanding additional environmental risk factors that can be targeted for prevention is essential. In regions where folic acid fortification of food is improbable, the results from this study emphasize the need for additional research that explores the potential role of paternal nutritional status in influencing neural tube defect risk, including the assessment of paternal folate supplementation in altering the neurotoxicity of environmental exposures, such as arsenic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

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ATSDR	Agency for Toxic Substances and Disease Registry
BBS	Bangladesh Bureau of Statistics
BGS	British Geological Survey
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CMIA	Chemiluminescent Microparticle Immunoassay
DPHE	Department of Public Health Engineering
DSH	Dhaka Shishu Hospital
FDR	False Discovery Rate
GM	Geometric Mean
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LASSO	Least Absolute Shrinkage and Selection Operator
MDL	Method Detection Limit
NIEHS	National Institute of Environmental Health Science
NIH	National Institutes of Health
NINS	National Institute of Neurosciences & Hospital
OR	Odds ratio

Elemental abbreviations:

Al	Aluminum
Sb	Antimony
As	Arsenic
Cd	Cadmium

Cr	Chromium
Co	Cobalt
Cu	Copper
Fe	Iron
Pb	Lead
Mn	Manganese
Hg	Mercury
Mo	Molybdenum
Ni	Nickel
Se	Selenium
Sn	Tin
U	Uranium
V	Vanadium
Zn	Zinc

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Highlights

- Limited human research explores parental metal levels and neural tube defect risk
- Our novel study examined the relationship using 18 metals measured in both parents
- Maternal toenail Se and Zn were associated with spina bifida in adjusted models
- Elevated paternal metals (i.e. As, Fe, and Se) were linked to spina bifida in infants
- Research that examines interventions that target paternal exposure is needed

Table 1.

Characteristics of children with spina bifida, controls, and their parents (*n*=278 parent-child pairs) from a case-control study in Bangladesh (mean ± SD, except where noted).

Characteristic	Cases (*n*= 155)	Controls (*n*= 123)
Child		
Age (days)	62.6 ± 75.3	183.3 ± 96.8
Sex, % male	79 (51)	76 (62)
Gestational age (weeks)	36.9 ± 2.3	37.1 ± 2.3
Mothers		
Age (years)	24.8 ± 4.9	24.5 ± 4.8
Body Mass Index (kg/m^2)	23.7 ± 4.4	23.2 ± 4.3
Parity, % first born	56 (36)	62 (50)
Birth place, % home birth	40 (26)	34 (28)
Folate (ng/mL)	8.8 ± 5.2	8.0 ± 4.6
Fathers		
Age (years)	32.8 ± 6.5	32.1 ± 6.1

Table 2.

Distribution of toenail metal concentrations (μg/g) in mothers and fathers.

Toenail Metal (μg/g)	Cases(*n*= 155)				Controls (*n*= 123)			
	GM	IQR	Min	Max	GM	IQR	Min	Max
Maternal								
Aluminum (Al)	61.77	30.32 – 125.43	5.49	505.44	57.98	30.58 – 118.29	7.74	547.60
Arsenic (As)	0.55	0.28 – 0.99	0.12	10.61	0.53	0.29 – 0.83	0.09	12.30
Cadmium (Cd)	0.07	0.04 – 0.15	0.01	3.70	0.06	0.03 – 0.1	0.01	1.26
Cobalt (Co)	0.06	0.03 – 0.13	0.01	0.71	0.06	0.03 – 0.1	0.01	0.43
Chromium (Cr)	0.31	0.19 – 0.44	0.04	13.73	0.28	0.16 – 0.47	0.04	3.31
Copper (Cu)	3.59	3.02 – 4.2	1.74	11.06	3.87	3.12 – 4.56	2.08	24.47
Iron (Fe)	123.05	59.77 – 256.44	11.68	962.64	121.76	67.22 – 229.92	23.03	890.15
Mercury (Hg)	0.21	0.13 – 0.27	0.04	3.69	0.22	0.16 – 0.28	0.07	5.78
Manganese (Mn)	3.78	1.61 – 9.3	0.23	72.01	4.5	1.84 – 12.39	0.20	99.18
Molybdenum (Mo)	0.02	0.01 – 0.02	0.01	0.08	0.02	0.01 – 0.02	0.00	0.09
Nickel (Ni)	0.64	0.27 – 1.43	0.02	253.78	0.7	0.29 – 1.55	0.06	32.22
Lead (Pb)	0.85	0.48 – 1.34	0.14	58.89	0.9	0.59 – 1.43	0.17	3.42
Antimony (Sb)	0.01	0.01 – 0.02	0.00	0.33	0.01	0.01 – 0.02	0.00	0.09
Selenium (Se)	0.65	0.58 – 0.75	0.36	1.17	0.62	0.55 – 0.69	0.39	1.10
Tin (Sn)	0.14	0.08 – 0.26	0.03	3.05	0.15	0.09 – 0.23	0.02	2.87
Uranium (U)	0.01	0.01 – 0.02	0.00	0.09	0.01	0.01 – 0.02	0.00	0.08
Vanadium (V)	0.14	0.06 – 0.29	0.01	1.15	0.13	0.07 – 0.28	0.02	1.15
Zinc (Zn)	100.76	88.78 – 111.17	58.15	552.86	111.81	95.04 – 125.33	69.87	973.02
Paternal								
Aluminum (Al)	97.99	52.49 – 173.78	10.83	791.87	80.42	42.79 – 154.81	11.17	492.21
Arsenic (As)	0.72	0.42 – 1.15	0.18	7.17	0.52	0.33 – 0.71	0.12	4.12
Cadmium (Cd)	0.05	0.03 – 0.09	0.01	0.92	0.04	0.03 – 0.07	0.01	0.99
Cobalt (Co)	0.09	0.05 – 0.17	0.01	1.05	0.07	0.04 – 0.14	0.01	0.44
Chromium (Cr)	0.8	0.43 – 1.42	0.09	8.32	0.62	0.36 – 0.94	0.11	6.16
Copper (Cu)	3.39	2.88 – 3.96	1.60	66.77	3.42	2.76 – 4.13	1.86	11.22
Iron (Fe)	222.08	105.85 – 430.21	33.75	2152.45	165.91	90.88 – 325.69	31.03	900.35
Mercury (Hg)	0.17	0.12 – 0.24	0.05	1.39	0.17	0.12 – 0.22	0.03	1.67
Manganese (Mn)	5.88	2.73 – 13.45	0.65	82.91	4.81	2.19 – 10.25	0.38	104.53
Molybdenum (Mo)	0.02	0.01 – 0.03	0.01	0.23	0.02	0.01 – 0.02	0.01	0.41
Nickel (Ni)	0.85	0.43 – 1.53	0.03	85.32	0.65	0.29 – 1.43	0.05	28.02
Lead (Pb)	1.06	0.65 – 1.58	0.36	24.20	1.01	0.58 – 1.63	0.27	5.19
Antimony (Sb)	0.02	0.01 – 0.03	0.00	0.22	0.02	0.01 – 0.03	0.01	0.22
Selenium (Se)	0.74	0.6 – 0.91	0.38	1.34	0.63	0.55 – 0.7	0.30	1.04
Tin (Sn)	0.12	0.06 – 0.17	0.02	18.36	0.11	0.06 – 0.16	0.02	1.05
Uranium (U)	0.02	0.01 – 0.03	0.00	0.15	0.01	0.01 – 0.03	0.00	0.20
Vanadium (V)	0.24	0.13 – 0.45	0.02	1.32	0.2	0.11 – 0.38	0.03	1.53
Zinc (Zn)	95.64	85.44 – 109.03	46.48	251.57	97.87	83.14 – 110.89	55.49	378.38

Note: GM, geometric mean; IQR, interquartile range; Min, minimum; Max, maximum.

Table 3.

Individual unconditional logistic regression models examining the relationship between each parental toenail metal exposure and spina bifida risk in children (n=278).

	Crude[a]			Adjusted[b]			Adjusted + Interaction[c]		
Metal (ln transformed)	***β***	**OR (95% CI)**	***P***	***β***	**OR (95% CI)**	***P***	***β***	**OR (95% CI)**	***P***
Maternal									
Aluminum (Al)	0.078	1.082 (0.831, 1.408)	0.56	0.105	1.110 (0.841, 1.466)	0.46	−0.156	0.856 (0.331, 2.211)	0.75
Arsenic (As)	0.046	1.047 (0.819, 1.338)	0.71	0.042	1.043 (0.812, 1.339)	0.74	−0.054	0.947 (0.419, 2.142)	0.90
Cadmium (Cd)	0.116	1.124 (0.880, 1.435)	0.35	0.166	1.180 (0.909, 1.532)	0.21	0.116	1.123 (0.624, 2.023)	0.70
Cobalt (Co)	0.095	1.099 (0.842, 1.435)	0.49	0.118	1.126 (0.850, 1.490)	0.41	−0.049	0.952 (0.381, 2.377)	0.92
Chromium (Cr)	0.127	1.136 (0.845, 1.527)	0.40	0.162	1.176 (0.864, 1.599)	0.30	0.078	1.081 (0.407, 2.871)	0.88
Copper (Cu)	−0.896	0.408 (0.176, 0.946)	0.04	−0.817	0.442 (0.185, 1.058)	0.07	1.452	4.273 (0.372, 49.134)	0.24
Iron (Fe)	0.013	1.013 (0.779, 1.318)	0.92	0.033	1.033 (0.783, 1.364)	0.82	0.006	1.006 (0.393, 2.575)	0.99
Mercury (Hg)	−0.136	0.873 (0.621, 1.227)	0.44	−0.155	0.857 (0.607, 1.21)	0.38	0.194	1.214 (0.502, 2.939)	0.67
Manganese (Mn)	−0.117	0.890 (0.733, 1.080)	0.24	−0.106	0.900 (0.735, 1.102)	0.31	−0.014	0.986 (0.530, 1.832)	0.96
Molybdenum (Mo)	0.229	1.257 (0.762, 2.074)	0.37	0.266	1.305 (0.776, 2.194)	0.32	−1.324	0.266 (0.042, 1.679)	0.16
Nickel (Ni)	−0.047	0.954 (0.793, 1.148)	0.62	−0.043	0.958 (0.794, 1.155)	0.65	0.245	1.278 (0.674, 2.423)	0.45
Lead (Pb)	−0.103	0.902 (0.663, 1.228)	0.51	−0.106	0.899 (0.655, 1.235)	0.51	−0.403	0.668 (0.262, 1.703)	0.40
Antimony (Sb)	0.141	1.152 (0.824, 1.610)	0.41	0.123	1.131 (0.804, 1.591)	0.48	−0.294	0.745 (0.225, 2.463)	0.63
Selenium (Se)	1.536	4.645 (1.305, 16.536)	0.02	1.572	4.818 (1.319, 17.601)	0.02	−1.976	0.139 (0.002, 8.541)	0.35
Tin (Sn)	−0.022	0.978 (0.767, 1.248)	0.86	−0.017	0.983 (0.766, 1.262)	0.90	0.182	1.199 (0.521, 2.761)	0.67
Uranium (U)	0.182	1.200 (0.870, 1.655)	0.27	0.186	1.204 (0.863, 1.680)	0.28	−0.501	0.606 (0.201, 1.826)	0.37
Vanadium (V)	0.075	1.078 (0.838, 1.388)	0.56	0.103	1.108 (0.847, 1.449)	0.45	−0.053	0.948 (0.382, 2.355)	0.91
Zinc (Zn)	−2.183	0.113	0.001	−2.220	0.109	0.001	−1.832	0.160	0.35

	Crude[a]			Adjusted[b]			Adjusted + Interaction[c]		
Metal (ln transformed)	***β***	**OR (95% CI)**	***P***	***β***	**OR (95% CI)**	***P***	***β***	**OR (95% CI)**	***P***
		(0.031, 0.415)			(0.028, 0.415)			(0.004, 7.290)	
Paternal									
Aluminum (Al)	0.299	1.348	0.05	0.350	1.419	0.03	0.070	1.073	0.88
		(1.004, 1.810)			(1.045, 1.928)			(0.422, 2.731)	
Arsenic (As)	0.525	1.690	0.001	0.555	1.742	0.001	1.415	4.115	0.02
		(1.225, 2.332)			(1.256, 2.417)			(1.247, 13.575)	
Cadmium (Cd)	0.187	1.205	0.20	0.195	1.215	0.19	−0.076	0.927	0.87
		(0.908, 1.600)			(0.908, 1.626)			(0.377, 2.278)	
Cobalt (Co)	0.320	1.377	0.04	0.381	1.464	0.02	0.124	1.132	0.81
		(1.021, 1.857)			(1.072, 2.001)			(0.413, 3.100)	
Chromium (Cr)	0.363	1.437	0.02	0.355	1.427	0.02	0.198	1.219	0.63
		(1.072, 1.927)			(1.059, 1.921)			(0.544, 2.728)	
Copper (Cu)	−0.080	0.923	0.82	0.002	1.002	1.00	0.064	1.066	0.96
		(0.457, 1.863)			(0.488, 2.060)			(0.101, 11.280)	
Iron (Fe)	0.391	1.478	0.01	0.452	1.572	0.003	0.316	1.371	0.51
		(1.117, 1.957)			(1.171, 2.109)			(0.532, 3.532)	
Mercury (Hg)	0.145	1.156	0.52	0.151	1.163	0.51	−0.160	0.853	0.83
		(0.743, 1.799)			(0.741, 1.823)			(0.201, 3.623)	
Manganese (Mn)	0.157	1.170	0.14	0.195	1.215	0.08	−0.293	0.746	0.41
		(0.948, 1.445)			(0.975, 1.514)			(0.375, 1.486)	
Molybdenum (Mo)	0.237	1.268	0.22	0.299	1.348	0.14	0.111	1.118	0.85
		(0.867, 1.855)			(0.905, 2.008)			(0.367, 3.405)	
Nickel (Ni)	0.179	1.195	0.07	0.190	1.209	0.06	0.278	1.321	0.45
		(0.983, 1.454)			(0.991, 1.475)			(0.641, 2.721)	
Lead (Pb)	0.112	1.119	0.52	0.105	1.111	0.56	0.037	1.037	0.95
		(0.794, 1.576)			(0.782, 1.58)			(0.360, 2.990)	
Antimony (Sb)	0.239	1.270	0.17	0.222	1.249	0.21	0.208	1.231	0.72
		(0.906, 1.782)			(0.881, 1.770)			(0.403, 3.760)	
Selenium (Se)	2.488	12.033	<0.001	2.527	12.514	<0.001	1.282	3.604	0.43
		(4.259, 33.999)			(4.329, 36.175)			(0.152, 85.371)	
Tin (Sn)	0.141	1.151	0.32	0.152	1.164	0.30	0.549	1.732	0.29
		(0.870, 1.522)			(0.875, 1.550)			(0.628, 4.776)	
Uranium (U)	0.213	1.237	0.12	0.235	1.264	0.10	0.100	1.105	0.82
		(0.944, 1.622)			(0.954, 1.675)			(0.458, 2.666)	
Vanadium (V)	0.284	1.329	0.05	0.339	1.404	0.02	−0.014	0.986	0.98
		(1.003, 1.760)			(1.047, 1.883)			(0.406, 2.394)	
Zinc (Zn)	−0.376	0.686	0.44	−0.364	0.695	0.46	0.069	1.072	0.96
		(0.263, 1.789)			(0.263, 1.834)			(0.090, 12.828)	

Note: CI, confidence interval; ln, natural log; OR, odds ratio.

[a] Crude logistic regression models assessed the relationships between each natural log transformed toenail metal and spina bifida case status. Separate models were run for each parent and each metal.

[b] Adjusted logistic regression models examined the relationship between natural log transformed toenail metal concentrations and spina bifida status, adjusting for maternal age (years), paternal age (years), infant sex, place of birth, and maternal serum folate (ng/mL, natural log transformed).

[c] Interaction logistic regression models were the same as the adjusted models, with the addition of an interaction term for the relationship between toenail metals and maternal serum folate.

Table 4.

Results of elastic net regression models assessing the associations between all maternal or paternal toenail metal concentrations (separate parental models) or all parental toenail metals (combined parental model) with spina bifida status, using regression coefficients extracted for when lambda equals lambda min (n=278).

	Penalized Coefficient (Beta)			
	Separate Parental Models		Combined Parental Model	
	Maternal	Paternal	Maternal	Paternal
Aluminum (Al)[a]	0.000	0.000	0.000	0.000
Arsenic (As)[a]	0.000	0.154	0.000	0.112
Cadmium (Cd)[a]	0.000	0.000	0.000	0.000
Cobalt (Co)[a]	0.000	0.000	0.000	0.000
Chromium (Cr)[a]	0.000	0.120	0.000	0.112
Copper (Cu)[a]	−0.037	0.000	−0.065	0.000
Iron (Fe)[a]	0.000	0.148	0.000	0.119
Mercury (Hg)[a]	0.000	0.000	0.000	0.000
Manganese (Mn)[a]	0.000	0.000	0.000	0.000
Molybdenum (Mo)[a]	0.000	0.000	0.000	0.000
Nickel (Ni)[a]	0.000	0.000	0.000	0.000
Lead (Pb)[a]	0.000	0.000	0.000	0.000
Antimony (Sb)[a]	0.000	0.000	0.000	0.000
Selenium (Se)[a]	0.150	0.469	0.000	0.404
Tin (Sn)[a]	0.000	0.000	0.000	0.000
Uranium (U)[a]	0.000	0.000	0.000	0.000
Vanadium (V)[a]	0.000	0.000	0.000	0.000
Zinc (Zn)[a]	−0.261	−0.068	−0.222	0.000
Maternal Serum Folate (ng/mL)[b]	0.000	0.000	0.000	-
Birth Place, home birth	0.000	0.000	0.000	-
Mom Age (years)	0.000	0.000	0.000	-
Dad Age (years)	0.000	0.003	-	0.000
Child Sex, male	−0.119	−0.180	−0.118	-

Note: All estimates are from elastic net regression models adjusting for maternal age (years), paternal age (years), infant sex, place of birth, and maternal serum folate (ng/mL, natural log transformed).

[a]Toenail metal concentrations (in μg/g) were natural log transformed and scaled prior to analysis.

[b]Maternal serum folate was natural log transformed prior to analysis.