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Citation

Diao, Nancy. 2015. Prenatal Metals Exposure and Child Birth and Growth in Bangladesh. Doctoral dissertation, Harvard T.H. Chan School of Public Health.

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**PRENATAL METALS EXPOSURE AND CHILD BIRTH AND GROWTH IN
BANGLADESH**

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A Dissertation Submitted to the Faculty of
The Harvard T.H. Chan School of Public Health
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Science
in the Department of Environmental Health
Harvard University

Boston, Massachusetts

May 2015

**PRENATAL METALS EXPOSURE AND CHILD BIRTH AND GROWTH IN
BANGLADESH**

ABSTRACT

The objective of this dissertation is to contribute to ongoing research on prenatal metals exposure, in terms of arsenic, lead, and manganese, and infant health and growth, and to deepen the understanding of the complexity of such problems. We seek to do so in three parts. First we examine the association between combined prenatal metals exposure and infant birth weight and head circumference. Then, we look at the effect on birth weight from the HFE gene variants and its interaction effects with arsenic. Finally, we look at the association of prenatal metals exposure and child growth up to 36 months. The study populations of all three of our studies are taken from mothers enrolled in 2 hospitals affiliated with Dhaka Community Hospital in Bangladesh. They were given self-administered questionnaires at time of enrollment and are followed after birth. Child measurements were taken at time of birth, and the biomarker for these studies are cord blood metal measurements.

In the first part of this dissertation, through multivariate linear regression, including a metal interaction term, we found that prenatal arsenic and manganese exposure individually associated with lowered birth weight and birth head circumference. We also found evidence of interactions between the two metals, suggesting that joint exposure creates greater deficit in birth outcomes. In the next part, looking at gene-environment interactions, we found significant

modification effects of multiple SNPs on the HFE gene that increased the association between arsenic and birth weight. We also found direct effect of less studied HFE genes to lower birth weight. Finally, we assessed the effect of prenatal metals exposure on early growth in children through longitudinal analysis. In following the weight and height of the child from birth up to 36 months of age, our results indicated adverse association between arsenic and manganese on growth.

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ACKNOWLEDGEMENTS

There are any individuals and organizations that I am indebted to for making this dissertation possible.

First, I would like to express gratitude to my academic advisor and the chair of my research committee, Dr. David Christiani, for entrusting in me the opportunities that made my studies possible. I am grateful for the time and resources that Dr. Christiani has provided towards bettering my research, often going above and beyond the responsibilities of an advisor.

I would like to thank Dr. Liming Liang for giving me critical inputs on my analysis. Dr. Liang has always pushed me forward with his optimism and encouragements at times when I am nervous and confused. His reservoir of knowledge is truly an inspiration to me.

I am also grateful for all the help that Dr. Maitreyi Mazumdar has given me throughout my research projects, and for being the person whom I can always turn to for questions on data. She has given me relentless help, particularly during the weeks leading up to my defense time.

I would also like to acknowledge the time and efforts of all those who participated in the Superfund research study, the researchers from the Bangladesh sites and Harvard sites, and those with whom I have worked with on my analysis.

Further, I am grateful to Dr. Chensheng Alex Lu and Dr. Marc Weisskopf for serving on my oral examination committee.

Lastly and most importantly, I owe many thanks to my parents and grandparents. They have instilled in me the confidence to pursue my dreams, and have always been the ones I can fall back on. Their examples and encouragements have taught me to be a useful member of

society and to delve wholeheartedly into whichever path I choose. I hope my pursuits in public health would be the first steps in many more positive efforts to come.

Nancy Diao

Boston, Massachusetts

April 2015

CHAPTER 1

INTRODUCTION

BIRTH AND EARLY GROWTH AND HEALTH

The century old goal of improving health in populations continues to be the fundamental core of public health research. However, to understand and improve human health, the focus needs to be turned to much earlier on in life – from the moment we are born, or even before that. Birth parameters are important determinants of child health, and many studies have indicated that they project later health in adults [1-4]. Birth weight and early growth is an indicator of overall child health [5]. Low birth weight and slowed early growth has significant negative effects on adult health. Compared to their normal birth weight siblings, low birth weight children are 30 percent less likely to be in excellent or very good health in childhood [1]. They also score significantly lower in achievement tests [6]. Abnormally high increase in head circumference in the in the years after birth directly indicates brain growth, and decrease in the circumference predicts decreased cognitive skills later on in childhood [7]. Furthermore, slow growth in early stages of life predicts stunting in growth and shortness later in life [8-11].

PRENATAL METALS EXPOSURE AND INFANT HEALTH

What is affecting the health status and parameter of infants can already be taking place during the fetal stages. One of the arising concerning problems during pregnancy is the environmental metal exposure of the mothers, which can be passed to the fetus, affecting placental thickness and problematic birth outcomes [12, 13]. Bangladesh is one place where exposure to heavy metals in the environment is particularly widespread due to contaminated water sources, food, and industrial source [14]. Thus, it forms a good cohort for studying the adverse affects of metals on infant health and growth. The main metals of concern are arsenic, lead and manganese.

Source and arsenic exposure and pathology

Arsenic is an element found in soils, groundwater, surface water, air, and in diet. Arsenic occurs naturally in the earth's crust, with higher concentrations in some types of rocks and minerals. Chronic inorganic arsenic exposure is known to be associated with adverse health effects on several systems of the body, and increased risks of cancer of the lung and skin [15]. Other effects of chronic arsenic exposure reported for adults include kidney damage and failure, anemia, low blood pressure, and central nervous system symptoms such as headaches, weakness, and delirium [16]. Chronic arsenic exposure of children and adults has been associated with adverse liver and respiratory effects, including irritation of mucous membranes. During developmental stages, high inorganic arsenic exposure in humans was associated with increased incidence of preterm delivery, miscarriage, stillbirths, low birth weight, and infant mortality. Inorganic arsenic exposure during childhood was associated with decreased performance in tests of intelligence, and long-term memory [17]. Skin lesions associated with arsenic exposure have also been reported in children [6]. Arsenic has been shown in studies to be able to cross the placenta and has been found in fetal tissue [18].

Source of lead exposure and pathology

Lead is ubiquitous in the human environment as a result of many factors. Lead exposure present through industrial sources, leaded paint, soil, and drinking water deposits. Lead has no known physiologic value, and evidence is clear that in utero exposure to low levels of lead can affect infant and child neurodevelopment [3, 8].

It has also long been recognized as a reproductive toxin in both men and women. It's true today that the typical blood lead level among adults today is lower than in the past, so it is less possible to cause extreme effects on reproductive health such as miscarriage or stillbirth. But maternal lead can readily cross the placenta and enter fetal blood circulation starting around week 12–14 of pregnancy, making the fetus especially susceptible to lead poisoning [19]. It is biologically plausible that lead can induce low birth weight, preterm birth, and small for gestational age. Lead can potentially impair normal fetal bone growth by competing with calcium for deposition into bone because lead and calcium have similar chemical characteristics. Lead has been shown in experimental in vitro studies to be harmful to preterm birth. Lead impedes collagen synthesis and praline hydroxylation in mouse, which may have detrimental effects on membrane structure and cause its premature rupture [12]. Rats exposed to lead are shown to have reduced bone calcium content, reduced trabecular bone volume, altered growth plate morphology, and enhanced activities of spontaneous uterine contraction [20].

Source of manganese exposure and pathology

Manganese is a naturally-occurring metal that, in pure form, is silver-colored with no taste or smell. Manganese is normally found in the environment as a compound with oxygen, sulfur, or chlorine. Manganese is an essential nutrient, required in trace amounts for human health. Intake from diet is usually sufficient normally. Manganese compounds can be present as dust particles in the air or can dissolve into ground water. In children, higher manganese concentrations in hair or blood have been associated with learning disabilities and central neuro system related effects [13, 21]. Some subpopulations of children are at higher risk for overexposure to manganese via diet, including children with cholestatic liver disease with

decreased liver function and children who are given intravenous feeding. In experimental animal studies, very high doses of manganese were shown to affect male fertility and result in fetal toxicity. In some studies, manganese exposure in rats resulted in delayed bone formation, and decreased birth weight. Studies of early manganese exposure reported neurochemical and neurobehavioral changes in rats, such as increased spontaneous motor activity and increased brain dopamine levels. Few studies of manganese have been explored for human cohorts [22].

SIGNIFICANCE OF THIS DISSERTATION

The objective of this dissertation is to contribute to ongoing research on prenatal metals exposure and infant health and growth, and also to deepen the understanding of the complexity of such problems. The first part of this dissertation will focus on looking at main effect of the three metals on birth parameters and how their co-existence may alter the effects. The second part will assess gene-environment interactions of the HFE gene on arsenic association with birth weight. The third part will explore metals exposure on early child growth.

In Chapter 2, we conduct an analysis of the main effect as well as co-effects of prenatal metals exposure on birth parameters in terms of weight and head circumference. Our study population consists of 892 mother infant pairs with cordblood As, Mn, and Pb collected analyzed from Bangladesh. While other studies have examined the main effect of arsenic and lead and the child, few have collected data on manganese and its effects. Also, in the real world exposure scenario, metals present themselves in mixture of exposure rather than individually [23]. Co-exposure can manifest combined effects that differ from single exposures [24].

In Chapter 3, we analyze the effect of HFE (hemochromatosis) gene variants on birth weight, and its effect modification on the association between prenatal arsenic exposure and birth weight. The HFE gene is responsible for regulating iron uptake in the body, alterations in that can increase iron content in the body, constituting to possible toxicity and effect on other mechanisms in the body. This manuscript would be the first to explore the interaction between HFE gene and arsenic effects on birth weight.

In Chapter 4, we explore the association of prenatal metals exposure on childbirth and growth in early years in a longitudinal analysis. We followed a sub-cohort of our Bangladesh data with 819 children followed from birth up to 36 months age, with recorded weight and height. Slowed and stunted growth early on has been documented in various studies to indicate problematic health in later life. Height-for-age and weight-for-age z score more than 2 standard deviations below the median reference set by the World Health Organization has been shown to have increased later behavioral issues, cardiovascular diseases and cognitive deficits [10].

A summary of the overall findings, in addition to the conclusions and implications of our inquiry is presented in Chapter 5. Our research aims ultimately to supplement the current knowledge of the effect of prenatal metals exposure and child health, and explore the complexities of their relationships. It is our hope that these findings will be used to increase knowledge and ultimately promote health for vulnerable maternal populations that continue to be affected by environmental metals.

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CHAPTER 2

The association of combined prenatal metals exposure on newborn birth parameters in Bangladesh

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ABSTRACT

Objective: The adverse effect of arsenic, lead, and manganese is well alerted in adults.

However, limited information is available to shed light on the effect on children. In particular, fetal development is of concern because it can predict later growth and developmental problems. In this study, we seek to characterize the association of prenatal metals exposure with newborn birth parameters.

Methods: Study cohort includes 892 women recruited between 2004 and 2011 in Bangladesh. Birth weight and birth head circumference were collected. Umbilical cord blood measurements were collected at the time of delivery and analyzed via ICP-MS. The associations between prenatal metals exposure, as well as pairwise co-exposure, and newborn birth weight and head circumference were analyzed via multivariate linear models.

Results: From this study, we found that arsenic indicated a strong association with decrease in birth head circumference (-1.15 cm, +/- 0.04cm) and with decrease in birth weight (-0.20 kg, +/- 0.08 kg). Exploring combined effects between the metals indicated that there was a significant interaction between high arsenic and high manganese on changes in birth head circumference (-0.10cm, P-value=0.04). There was also a significant interaction between high arsenic and high manganese on changes in birth weight (-0.04kg, P-value =0.05).

Conclusion: Results indicate that prenatal arsenic exposure adversely affects the birth head circumference and birth weight of newborns. The affect is increased when high arsenic is combined with high manganese.

INTRODUCTION

Environmental exposure to heavy metals is a threat to countries around the world, especially Bangladesh. Approximately 133 million Bangladeshis are in danger of metals exposure through water, food and industrial sources [1]. Pregnancy has been established as a potentially critical period of vulnerability for exposure to numerous chemicals. Many metals have been shown to be able to cross the placenta and affect the fetus [2-7]. Studies show that chronic exposure to is associated with significantly higher adverse pregnancy outcomes of spontaneous abortion, stillbirth, preterm birth rate and fetal loss [8].

Main environmental metals monitored in Bangladesh that projects a worldwide concern include arsenic, lead, and manganese. Arsenic enters the water supply from agricultural or industrial sources or natural deposits in the Earth, and prolonged consumption of this contaminated water can cause bladder, kidney, lung, or skin cancer, as well as chronic skin conditions [9]. Other pathways of exposure include inhalation of dusts or ingestion of contaminated soil and food, especially rice consumption [10]. The World Health Organization's (WHO) safe limit of arsenic in water supplies is 10 parts per billion (ppb), and Bangladesh's safe drinking water level is 50 ppb [11]. Despite these boundaries, one environmental survey found that 56% of water samples exceed WHO's arsenic limitations and 37% surpass Bangladesh standards [1]. Lead is a known hazard that has plagued humans for centuries. Its effect on neurological and behavioral development is well studied in both adult and children, but little is explored in terms of its early effects on birth health [12-14]. Even less investigation has been placed on the effect of manganese on human development, as most effects were tested on animals. The metal is known to target the nervous system and liver functions, and its presence in the body is readily absorbed [14-16]. We seek to quantify the effects of these metal exposures on

newborn health by looking at birth weight and birth head circumference. Birth weight is a measurement of overall child health, as lowered birth weight has significant negative effects on adult health. Low birth weight children are nearly twice as likely as their normal birth-weight siblings to be in problematic health by ages 40-50 (23 percent versus 12 percent) [17]. Head circumference has been shown to have direct association with neurodevelopment of children. Smaller than normal head circumference in early life projected significantly lowered standardized test scores in teen years [18, 19].

The focus of this study is not only to understand the effect on the new born after of being exposed prenatally to these metals in the environment, but to explore how they their effect changes in combination. The real world exposure case rarely has metals present themselves individually. Rather, they are present in a mixture of exposures [20]. Observational as well as experimental evidence indicated that such co-exposure may give rise to combined effects that are different from what can be noted when considering the effects of chemicals individually [21]. The focus of many present studies are on understanding the toxicity and effect of individual metals on health, very few has pried into exploring in terms of co-existence. Thus, our study examines the effect of prenatal metals exposure on birth parameters of weight and head circumference, and also assesses the co-presence of these metals on child health.

MATERIALS AND METHODS

Study Population

Our study spans form 2004 to 2011. We recruited participants through a series of community meetings held in Pabna, Bangladesh. Pregnant women were primarily recruited as potential participants if they were long-term residents and obtained primary health care from the

Pabna and its affiliate Dhaka community Hospital. These two Upazilas location were selected for the study area because a national survey conducted by the British Geological Survey indicated that the average concentration of metal in the ground water of these areas was more moderate than mother regions in Bangladesh yet spanned a wide range of concentrations [22].

Additionally, Dhaka Community Hospital Trust (DCH) operates these two rural health clinics that offer prenatal care and promote exposure awareness by encouraging metal to only drink water from wells that comply with the Bangladesh drinking water standard.

Women were eligible to participate in the study if they were 18 years or older with a singlet pregnancy of less than 28 weeks' gestation confirmed by ultrasound at the time of enrollment, did not have a history of diabetes mellitus or of using oral hypoglycemics, were planning to continue receiving prenatal care through DCH, had used the same drinking water source for at least the previous six months at the time of enrollment, and intended to live at their residence with the same water source throughout pregnancy. Infants delivered pre-term will be excluded from the sample population. In all, 1,782 women were recruited into the study. Of these, 963 have cord blood measurements collected and tested up to date.

Participating women are offered prenatal care during the course of their pregnancies including two pre-natal clinic visits, two ultrasounds, and a supply of pre-natal multivitamins. Participants are seen three times by study staff during the course of their pregnancies: at or prior to the 12th week of gestation, at or around the 28th week of gestation, and at delivery. At the first study visit, self reported questionnaires are administered in person by a trained interviewer fluent in Bangla. The questionnaire includes questions on socio-demographic characteristics, lifestyle and personal habits, medical history, occupational history and a nutritional survey and fluid consumption.

Outcome Measurement

Measurements were taken by trained DCH nurses or midwives at time of delivery. The infant's weight is measured on a pediatric scale, calibrated before each measurement using a 5-kg weight, and is recorded to the nearest 10 grams. Head circumference is obtained using measuring tape and is also recorded to the nearest centimeter.

Exposure Measurement

Cord blood measurements are used in this study to better capture the exposure level in the uterus. Umbilical cord blood is sampled at birth delivery. The blood is extracted after the umbilical cord blood is clamped at two sites and then detached from the mother. All samples collected are stored and extracted into metal free tubes. Collection tubes are checked for trace element contamination prior to use. Also, a second blue top blood collection tube is uncapped prior to sample collection and re-capped at the finish of the collection to be used as field blank samples to ensure that no external metal contamination is present. Serum is separated by centrifugation at 3000g for 20 minutes and frozen at -80 degrees Celsius. Samples of metals are analyzed at HSPH via ICP-MS. Approximately 1 g of blood is dissolved in 2 mL of Trace Select Ultra nitric acid (HNO₃) (Fisher Scientific, Pittsburgh, PA) at room temperature for 24 hours. After 24 hours the samples are treated with 1 mL of hydrogen peroxide (H₂O₂) and allowed to digest over 2-3 days. Samples are then diluted to 10 mL with deionized water. Samples are analyzed via ICP-MS for Cd and total As. Standard reference material blood (NIST 955c, Toxic Metals in Caprice Blood, National Institute of Standards and Technology, Gaithersburg, MD) and

method blanks are used to validate the performance of the ICP-MS and of the digestion method, respectively.

Statistical Analysis

We modeled arsenic, lead, and manganese separately with birth weight and birth head circumference to first monitor the main effects of each. We then monitored pairwise interactions between the metals to assess if we see any interaction effects when the metals were treated linearly. We modeled the metals as a continuous variable, centered at the arithmetic mean value of the distribution. Although manganese, lead, and arsenic were all moderately skewed, the Gauss-Markov model requires that only the model residuals be normally distributed. Because this assumption was satisfied, we did not log transform the exposure variables. Skewness may also arise from presence of outliers. The influences of high tail-end outliers were examined in sensitivity analysis. Birth weight and birth head circumference were approximately normally distributed and modeled as continuous outcomes.

After examining linear interactions between pairwise metals, we then selected metal pairs that we observed any significant interaction terms present and performed quartile assessments to see if there are any non-linear interactions between the metals. Each metal of concern was broken into quartiles and then the interacting metal was further monitored in quartile increases upon each quartile of the main metal. This is to ensure a thorough examination of the effect of one metal on the other.

We adjusted all models for the same set of covariates. Covariates included in our analysis from maternal measurements included site of data collection (Pabna or Sirajdikhan clinic), maternal age, maternal BMI at birth, average monthly income (categorized into low, medium,

high), smoking status (past, current, never), maternal education and paternal education (both categorized in to 6 levels from illiterate to post-graduate), nutrition intake measured in terms of a total protein intake variable that was created based on the sum of frequencies of meat, fish, and fowl intake. Child measurements controlled for included gestational age, gender. Covariates are selected based on biological plausibility and prior literature. To the model with a priori covariates, we added, one at a time, covariates that ere associated in bivariate models ($P < 0.10$) with exposures and outcomes at any time point. Additional covariates were included in the final model if the manages or lead effect estimates changed $>10\%$.

We conducted sensitivity analysis by additionally looking at the pairwise metal interactions using splines. We also examined the influence of extreme observations, which we previously excluded from the study population, by fitting models with and without outliers. Analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC) and R 3.1.2 (The R Foundation for Statistical Computing, Boston, MA).

RESULTS

From the 1782 women who met the enrollment criteria to be part of the maternal-infant pair outcome study, 963 had complete umbilical cord blood metals measurements up to date. Others are still in the process of analysis. Of those complete with cord blood metal measurements, 7% were further excluded due to extreme outliers that greatly shifts the distribution of the variables. In total, 892 participants are in our cohort of examining the effect of prenatal metals effect on birth parameters. In Table 2.1, we present the major characteristics of the mother and children included in the cohort. The children were equally distributed in gender, and had mean gestational age that's comparably normal when referred to national standards.

Maternal age appears to be mostly of young mothers (means 23 years of age, SD 5 years), with the oldest mother being only 42 years old. All women are married, and none had ever smoked. These women consists of mostly lower education, only 4% completed college and beyond, similarly with their husbands (7% completing college and beyond). They are also comprised of mostly low to medium income.

The arithmetic mean (\pm SD) concentration for birth weight is 2.8 ± 0.3 Kg (range, 0.8-4.5), and for birth head circumference is 32.3 ± 1.9 Kg (range, 27-37). Linear regression testing the main effects of the three metals with birth weight and head circumference (Table 2.2) indicated that there is a significant association in arsenic with decrease in birth head circumference (-1.15 cm, \pm 0.04cm) and with decrease in birth weight (-0.20 kg, \pm 0.08 kg). There is also a good correlation between manganese and negative birth outcomes, though borderline significance (with birth head circumference, -0.09 cm, $p=0.08$; with birth weight, -0.12 kg, $P=0.08$). No significant effect was evident from lead on birth weight or head circumference.

In determining which pair-wise metals to explore in depth, the linear interactions between the metals indicated a significant interaction term was possibly present between arsenic and manganese when treated linearly (-0.03, $P=0.06$), appearing to create further deficit in the outcome measures. In exploring this interaction in detail, arsenic, the strong interaction of the two, was treated into quartiles. Cord blood arsenic quartiles ranges Q1: 0.07-0.29ug/dL, Q2: 0.29-0.49ug/dL, Q3: 0.49-1.07ug/dL, Q4:1.07-11.8ug/dL. Within each quartile range of arsenic, manganese was treated in quartiles, compared to the first quartile to assess its affect. The outputs of the interaction terms on head circumference were presented in Table 2.3. In the third quartile of arsenic, the highest quartile of manganese significantly interacted with arsenic by further

decreasing the effect on head circumference by -0.09 cm (P=0.05). In the fourth quartile of arsenic, the highest quartile of manganese interacted with arsenic significantly to lower head circumference ($\beta = -0.10$, P=0.04). In looking at arsenic and manganese interactions on birth weight, we observed significant interaction only in the highest quartile of arsenic. Here, manganese exhibited interactions in the third and fourth quartile to lower birth weight further (Q3: $\beta = -0.05$, P=0.05; Q4: $\beta = -0.04$, P=0.05).

Sensitivity analysis in looking at the distribution of the interaction between arsenic and manganese presented a similar pattern of interaction peaking when both metal concentrations are high. When including extreme outlier values, the quartile in which significant interaction appeared did not change, but the interaction term did become stronger compared to the original analysis (with head circumference, $\beta = -0.13$, P=0.03, with birth weight, $\beta = -0.05$, P=0.05).

DISCUSSION

In our study, we found that interaction is present in the higher quartiles of arsenic and manganese that created greater deficits in both birth weight and birth head circumference, compared to effects of exposure to either metal alone.

Our study is an important step into looking at a more realistic presentation of metal exposure from the environment. Most toxicological studies focus on a single agent and usually do not measure or do not adjust for potential intermediate or modifying effects of other chemicals [23]. Although such an approach has identified toxicities associated with various metals, it does not reflect real world scenarios in which humans are in exposure to multiple chemicals [24, 25].

The combined interaction effect of arsenic and manganese manifested an additional change of up to 40g decrease in birth weight per unit increase in each metal. This is a large added negative effect compared to that the effect of arsenic alone, which is -200g per unit increase in arsenic level. The change in head circumference when manganese is present alongside arsenic is merely up to 0.1cm decrease in head circumference per unit increase in arsenic. While these differences represent a small decrease in head circumference measurement for an individual, they may have large implications for the population level because the entire distribution of birth head circumference may shift, increasing the number of infant with very low head circumference that are of clinical concern. Prior studies of manganese noted that manganese tends to exhibit a U shaped pattern in its exposure effects [26], as it is an essential nutrient for the human body in trace amounts but too low of a presence can equally be detrimental to health [27-29]. However, we observed no significant interaction between manganese and arsenic at low levels of manganese, both in quartile terms and in spline. The lack of an interaction with arsenic at low manganese levels may be because the mechanisms by which manages deficiency causes adverse effects may not overlap with those for arsenic. Also, there is no known standard to how low is too low in terms of necessary manganese level. Thus, the lowest level observed in our population may not be low enough to produce an effect.

We found no significant combined effects of arsenic or manganese with lead. To our knowledge, there has been no evidence of lead interacting with arsenic on head circumference or birth weight. There are varying studies on the co-effect of lead and manganese on child neurodevelopment. One epidemiologic study reported significant inverse associations of lead with verbal IQ among children with high manganese concentrations present [30]. However, this study is conducted on intelligence status with school children past the age of 6 years. While we

attempt to capture the effect on birth head circumference, which may reflect intelligence and health and child in later year, it may be too early for any effect to be evident, as such effects may present themselves only in later years of growth.

Limitation in our study can arise from our covariates are collected by self-administered questionnaires. There may be under report of certain covariates such as smoking status since women are expected to be non-smokers in Bangladesh [31], however, such variations should be considerably small to not have significant changes on our findings. There may be questions on bias in the number of cord blood samples used, as we have only 892 cord blood metal samples while 1700 mothers were recruited. This is because not all blood measurements were analyzed up to date, but the selection process of measurements was random given time of birth. Also, this is a significantly large sample size compared to other studies with similar attempts. The strength of our study also lies in that this is first prospective epidemiologic study to examine the interactions between arsenic, lead, and manganese on birth outcomes. Birth is a crucial point that projects later developmental and health problems. Finally, our study population was stable, with little attrition and relative homogeneity in demographics that could act as confounders.

In conclusion, the synergism observed in joint metals exposure during fetal stages prompts attention to environmental health awareness during maternal pregnancy.

Table 2.1: Participant Characteristics (n=892)

Characteristics	Parameters		
	mean (sd)	min	max
Mother age at delivery	23.1 (4.7)	18	42
Birthweight (kg)	2.8 (0.3)	0.8	4.5
Birth head circumference (cm)	32.3 (1.9)	27	37
Birth length (cm)	46.1 (3.0)	28	56
gestational age (weeks)	38.5 (2.0)	28	42
	NO. (%)		
Infant gender			
Male	448 (51)		
Female	444 (49)		
Maternal education			
illiterate	69 (2)		
able to write	160 (19)		
secondary education	269 (38)		
higher secondary education	266 (37)		
college/graduate	68 (3)		
post graduate	59 (1)		
Paternal education			
illiterate	82 (5)		
able to write	203 (26)		
secondary education	235 (32)		
higher secondary education	219 (30)		
college/graduate	86 (5)		
post graduate	56 (2)		
Marital status			
Married or with partner	892 (100)		
Smoking Status			
Never smoker	892 (100)		
Current smoker	0		
Ever smoker	0		
Family Income(Taka)			
Low	241 (23)		
Medium	545 (63)		
Higher	86 (3)		

Table 2.2: Linear analysis of main effect* of cord blood metals on head circumference and birth weight

Exposure (ug/dL)	Effect Estimate	S.E.	P-value
<i>head circumference</i>			
Lead	-0.04	0.01	0.06
Arsenic	-1.15	0.04	0.03
Manganese	-0.09	0.08	0.08
<i>birth weight</i>			
Lead	-0.03	0.07	0.26
Arsenic	-0.20	0.08	0.02
Manganese	-0.12	0.10	0.08

*All models control for site of data collection, maternal age, BMI, income, education, smoking status, maternal nutrition, child gender, delivery type, gestational age.

Table 2.3: Change in head circumference by interactions* between Arsenic and Manganese

	β estimate, P-value			
	Manganese ^b			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Arsenic ^a				
Quartile 1		-0.08 (0.12)	-0.05 (0.23)	-0.05 (0.19)
Quartile 2		-0.12 (0.35)	-0.08 (0.22)	-0.10 (0.12)
Quartile 3		-0.06 (0.11)	-0.08 (0.09)	-0.09 (0.05)
Quartile 4		-0.09 (0.10)	-0.11 (0.08)	-0.10 (0.04)

*All models control for site of data collection, maternal age, BMI, income, education, smoking status, maternal nutrition, child gender, delivery type, gestational age, and birth weight.

^aCord blood arsenic Quartile ranges: 0.07-0.29ug/dL, 0.29-0.49ug/dL, 0.49-1.07ug/dL, 1.07-11.8ug/dL.

^bCord blood Manganese centered at mean: 13.7ug/dL.

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CHAPTER 3

Modifications of the HFE gene variants on prenatal arsenic exposure and infant birth weight in Bangladesh

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ABSTRACT

Objective: Fetal growth is a complex process that subject to both the effects of genetic and environmental factors. Increased prenatal arsenic exposure has been shown to be associated with significant decrease in newborn birth weight, which is an indication of decreased overall health. Polymorphisms in the hemochromatosis (HFE) genes has been shown to disrupt iron regulatory abilities in the body, which in turn increases toxicity burden and can possibly lead to increased transport of other metals. In our study, we explore the effect of HFE gene variants on the effect of birth weight, and its modifying effect on the burden of arsenic on birth weight.

Methods: We investigated the effect of 7 SNPs from the infant HFE gene in modifying the association of arsenic on birth weight in 755 Bangladesh women and infant pairs. Subjects were recruited between 2004 and 2011. Birth weight measurements were obtained at the time of delivery. Umbilical cord blood measurements were taken at time of delivery, and from there we extracted arsenic levels and HFE gene variants. The association between HFE gene variants on birth weight, and its modification effects on arsenic on birth weight was analyzed via multivariate linear regression.

Results: 7 of a total of 15 SNPs on the infant HFE gene were selected due to their higher than 10% minor allele frequency. Multivariate linear regression revealed that SNPs rs2704720 and rs1572982 all predicted a lowered birth weight. In the modification analysis, interaction models suggested that on the infant HFE gene, SNPs rs2704720, rs9366637 and rs1572982 strengthened the negative association between arsenic and birth weight.

Conclusion: Results indicate that variants on the HFE gene not only pose an effect in decreasing birth weight, but they also cause an negative association between arsenic and birth weight.

INTRODUCTION

Birth weight of the newborn child is a particularly important measurement of infant and later life health. Decreased birth weight has been established as a predictor of infant mortality, birth complications, developmental outcome such as cognitive performance, and chronic disease even into adults [1, 2]. Both genetic and environmental factors play a role in affecting the newborn birth weight. On the environmental side, some examples include maternal health, nutrition status and exposure to pollutants [3]. Arsenic contamination in particular through water and food has been a major threat to countries around the world. Bangladesh is very susceptible to such risk, as more than 100 million Bangladeshis are in danger of arsenic poisoning. Decrease in birth weight has been shown in other studies and in our previous studies to be independently associated with arsenic exposures [4-7].

Genetic variations are equally important to consider. A few recent population studies have estimated that up to 50% of the variation in birth weight is due to hereditary factors and genetic mutations [8, 9]. Genetics components can individually affect birth weight but can also come into play in gene environment interactions. Studies have indicated that birth weight become altered in the cases of extreme iron status [6, 10, 11]. Currently, the HFE gene is of particular interest in public health because of its variants that play a role in iron regulations in the body. Mutation in the HFE protein would prevent the protein from reaching cell surface to interact with transferrin receptor and as a result iron regulation in the body is disrupted and too much iron gets absorbed from the diet [12].

Studies have indicated that well known HFE variants H63D and C282Y have had negative effects on infant birth weight [13, 14]. However, other polymorphisms of the HFE are

not well studied. The aim of our study is to examine all of the polymorphisms of HFE available to us in our cohort to look at the effect of HFE gene variants on birth weight. We also aim to examine the effect modification of HFE gene variants on the established effects arsenic and birth weight. No previous studies have examined that effect in humans. There has been cases where arsenic has been attempted to be leached from the body in the case of arsenic poisoning by using a natural zeolite containing high iron. The essential idea is for arsenic to be absorbed onto iron compounds to create larger particles that can be eliminated easily [15]. While not fully tested on the natural occurrence of iron in the body, this may suggest that the presence of HFE gene may foster a protective modification effect on the association of arsenic and birth weight.

METHODS

Study Population

The study cohort was recruited in the Sirajdikhan and Pabna Sadar Upazilas of Bangladesh from years 2008 to 2011. These two area clinics forms the Dhaka Community Hospital Trust (DCH), which offers prenatal care and promotes exposure awareness. Pregnant women were recruited by the DCH-trained female community health care workers who live in the villages serviced by the clinics. Eligibility criteria included: more than 18 years of age, ultrasound confirmed singleton pregnancy less than 16 weeks gestation, used a consistent water supply, planned continued current residence and continued prenatal health care with DCH, and agreed to deliver at DCH or at home with a DCH trained midwife. Medical questionnaires were administered on medical history and demographics during scheduled visits from the mothers. Of

the total 1782 women that remained the study, 985 cord blood measurements were analyzed up to date.

Outcome measurement

The infant's weight is measured on a pediatric scale, calibrated before each measurement using a 5-kg weight, and is recorded to the nearest 10 grams.

Infant weight was measured at time of delivery.

Exposure measurement

Cord blood arsenic measurements

Umbilical cord blood, a biomarker capturing exposures to the in utero environment [16], is collected at the time of delivery. After the infant is delivered but before the delivery of the placenta, the umbilical cord is clamped at two sites and then detached from the mother. Approximately 6 mL of the enclosed cord blood is extracted into a blue-top blood collection tube containing the anticoagulant K₂EDTA (BD Vacutainer) and inverted 10-20 times. Samples are stored at 4°C until shipped to HSPH for storage and analysis. A second blue-top blood collection tube is uncapped prior to sample collection and is re-capped at its conclusion. These field blank samples are collected to measure and account for the introduction of external metal contamination during cord blood collection.

Samples of metals are analyzed at HSPH via ICP-MS. Approximately 1 g of blood is dissolved in 2 mL of Trace Select Ultra nitric acid (HNO₃) (Fisher Scientific, Pittsburgh, PA) at room temperature for 24 hours. After 24 hours the samples are treated with 1 mL of hydrogen peroxide (H₂O₂) and allowed to digest over 2-3 days. Samples are then diluted to 10 mL with

deionized water. Samples are analyzed via ICP-MS for Cd and total As. Standard reference material blood (NIST 955c, Toxic Metals in Caprine Blood, National Institute of Standards and Technology, Gaithersburg, MD) and method blanks are used to validate the performance of the ICP-MS and of the digestion method, respectively.

Genotyping methods

DNA extraction and genotyping were performed at the Broad Institute, from archived umbilical cord blood samples. DNA was extracted from sampled using the Puregene DNA Isolation Kit (Gentra Systems). Genotyping was done using the Illumina HumanOmniExpress BeadChip (whole genome coverage with >730,000 SNPs) and Illumina HumanExome BeadChip (whole exome coverage with >240,000 SNPs). In our data, we have located 11 SNPs on the HFE gene that is over 5% minor allele frequencies. None of these SNPS are in complete linkage disequilibrium with each other according to the CEU HapMap data.

Statistical Analysis

Our genotyping results presented to us 15 SNPS on the HFE gene with sufficient data. From this, we located 7 SNPF that is over 10% minor allele frequencies (MFA) for sufficient comparison between the wildtype and heterozygous variant. We had the common H63D variant, SNP rs1799945 present in our data, but due to its low MAF, it was removed from further studies. We checked the linkage disequilibrium for the SNPs on the CEU HapMap data.

Demographic characteristics and arsenic levels by genotype were examined and mean difference were tested by chi-square test for continuous and categorical variables as appropriate. Potential non-linearity between continuous predictor variables and birth weight was explored by

plotting results from generalized additive models. Resulting non-linear associations were controlled for as covariates. Multiple linear regressions were then used to model the relationship between birth weight, HFE gene variants, and cord blood arsenic exposure, after controlling for potential confounders. The potential confounding variables considered in our models were based on biological plausibility and based on literature reviews. Covariates included in our analysis from maternal measurements included site of data collection (Pabna or Sirajdikhan clinic), maternal age, maternal BMI at birth, average monthly income (categorized into low, medium, high), smoking status (past, current, never), maternal education and paternal education (both categorized in to 6 levels from illiterate to post-graduate), nutrition intake measured in terms of a total protein intake variable that was created based on the sum of frequencies of meat, fish, and fowl intake. Child measurements controlled for included gestational age and gender. To examine the potential modifying effect of the variants, we initially ran separate multiple linear regression models stratified by infant genotype. In further stratified models, we fitted multiple linear regression models that included an interaction term between the genotype and blood arsenic.

Analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC) and R 3.1.2 (The R Foundation for Statistical Computing, Boston, MA).

RESULTS

Our genotyping results presented 16 SNPs on the HFE gene that are present for all 755 subjects. However, after examining minor allele frequency, we excluded 5 SNPs due to minor allele frequencies below 5%. (Table 3.1) We later excluded another 2 more SNPs whose MAF was below 10%, because due to our smaller sample size, there were not enough variant to wildtype ration to deem worth of exploration. None of the SNPs are in complete linkage

disequilibrium with each other according to the CEU HapMap data. It should be noted that in our data, we did have the H65D variant present (rs1799945). Although this SNP was below 10% MAF, we kept it in the analysis due to many studies have found association between birth outcomes and H65D variant.

Table 3.2 presents the demographics of our study population (N=755) in terms of median and interquartile range of cord blood lead, arsenic, and manganese. The distribution of the data showed that there are slightly elevated levels of the metals for women in the age group above 35 years. Lower education and lower income mothers exhibited higher arsenic and lead exposures. The difference is less apparent in manganese levels. The level of metals exposure is equal between infant genders.

With the 7 SNPs we selected, we ran multivariate linear adjusted regression between the HFE SNP and birth weight individually in Table 3. We found SNPs rs2704720 and rs1572982 all predicted a lowered birth weight (rs2704720: $\beta=-0.12$, 90% CI=-0.23, -0.02; rs1572982: $\beta=-0.17$, 95% CI =-0.35, -0.03). There was no observed significant association on birth weight from the H65D (rs1799945) variant. In Table 3.4, we checked the effect of adding an interaction of the SNPs with arsenic on birth weight. The results indicated that. In the modification analysis, interaction models suggested that on the infant HFE gene, SNPs rs2704720 ($\beta=-0.06$), rs9366637 ($\beta=-0.10$) and rs1572982 (-0.09) strengthened the negative association between Arsenic and birth weight.

DISCUSSION

Our study indicated that the presence of SNPs rs2704720 and rs1572982 on the HFE gene predicted a lowered birth weight. There were previously established negative associations

of arsenic on infant birth weight. In the modification analysis, interaction models suggested that on the infant HFE gene, SNPs rs2704720, rs9366637 and rs1572982 strengthened the negative association between arsenic and birth weight. This an indication that there is interaction between the HFE genotype and arsenic exposure and birth weight. This is significant finding as to our knowledge this study is the first to observe effect modification of the association between arsenic exposure and birth weight by HFE genotype status.

The effect of the HFE modifiers on birth weight is contributed mostly to iron absorption [17]. The HFE protein regulates iron levels in the body. It is located on chromosome 6, position 21.3. It's of 9kb in length, ranging from base pairs 26087422~26096438. Changes in the HFE gene can cause iron overload, which can maybe lead to cognitive performance decline to due cellular oxidative damage [18-20]. The HFE gene provides instructions for producing the hepcidin protein that is located on the surface of cells, primarily liver and intestinal cells. The HFE protein is also found on some immune system cells. The HFE protein also interacts with other proteins on the cell surface to detect the amount of iron in the body. Hepcidin is produced by the liver, and it determines how much iron is absorbed from the diet and released from storage sites in the body. When the proteins involved in iron sensing and absorption are functioning properly, iron absorption is strictly regulated. On average, the body absorbs about 10 percent of the iron obtained from the diet. Mutations on the gene would prevent the altered HFE protein from reaching cell surface to interact with transferrin receptor and as a result iron regulation is in disruption and too much iron gets absorbed from the diet. [20]

Our study results where alterations in the SNPs of the HFE gene negatively affect birth weight is in line of previous studies. A recent publication using a cohort in Mexico on the effect of HFE gene variant H63D, SNP rs1799945, has indicated an effect on decreasing infant birth

weight. Previous research has shown the iron tends to exhibit a U-shaped relation where both extreme iron deficiency and iron excess can create risk in the decrease in infant birth weight [21-25]. Other studies have shown that low iron and anemic conditions in the early and late stages of pregnancy can directly affect the placenta. These changes leads to a larger weight placenta with increases in capillary surface areas, which changes the mechanisms in gas exchange and was thought to be a response to condition that will cause limitations in growth as a result [26]. On the other hand, reactive oxygen species resulting from reactive iron species is thought to be one of the major causes of the damage done by iron excess [27].

No other study has looked at the combined effect of arsenic and iron effect. The mechanism behind interactive effect of HFE variants on arsenic exposure and birth weight remains elusive. One simple explanation may be that the increased iron toxicity induces additive toxicity effect to the effect already present in arsenic, increasing toxicity level in the body. Other studies that examined arsenic indicated that arsenic binds to iron compounds to produce large particles [28]. Such formation may form a problem in excretion from the body.

The strength of our study comes from examining other less well studies SNPs of the HFE gene in examining the overall effect of the HFE variants on health outcomes. Also, we are likely the first study to examine the effect of HFE gene on arsenic and birth weight associations. While other metals such as lead have been well studied, the effect on arsenic has never been touched mainly due to uncertainties in the mechanism. However, arsenic remains one of the major environmental pollutions in Bangladesh and indicates the strongest negative association with birth outcomes in our cohort. As with all studies, there are limitations. In our study, we are not yet able to examine both maternal and infant iron levels, nor through looking at various sources such as hemoglobin and ferritin. Usually, considering multiple biomarkers is a more accurate

way to determine exposure status. Given such, our study results in terms of iron and arsenic co-exposure should only be considered preliminary.

Table 3.1: Infant genotype frequencies

SNP	All1	All2	MAF	Wildtype (%)	Variant (%)
rs2794720	C	G	0.41	83	17
rs2794719	C	A	0.41	83	17
rs9366637	A	G	0.21	96	4
exm521702	G	C	0.07	99	1
rs1799945	G	C	0.07	99	1
exm521704	T	A	0.0006	100	0
rs2071303	G	A	0.34	90	10
exm521728	A	G	0.001	100	0
exm521729	A	G	0.002	100	0
exm521730	G	A	0.001	100	0
rs1800708	G	A	0.21	96	4
rs2858996	A	C	0.11	99	1
rs2071302	G	A	0.04	100	0
rs1572982	G	A	0.43	82	18
rs707889	A	G	0.10	99	1
rs17596719	A	G	0.22	96	4

Table 3.2: Prenatal metals exposure levels by study population characteristics (N=755)

Characteristics	Blood Pb (ug/dL)		Blood As (ug/dL)		Blood Mn (ug/dL)	
	median	25th~75th percentile	median	25th~75th percentile	median	25th~75th percentile
<i>age (years)</i>						
<20	3.75	5.05	0.49	0.75	7.10	6.61
20-35	3.75	4.32	0.48	0.74	7.15	6.63
>35	4.02	6.01	0.53	0.65	7.35	5.62
<i>Infant gender</i>						
Male	3.56	4.32	0.44	0.65	6.51	5.69
Female	4.06	6.12	0.49	0.69	7.01	6.66
<i>Maternal education</i>						
illiterate	4.21	5.12	0.52	0.65	7.33	6.59
able to write	4.12	5.55	0.56	0.63	7.35	5.45
secondary education	4.01	5.21	0.42	0.72	7.12	6.42
higher secondary education	3.98	4.38	0.43	0.76	7.33	5.21
college/graduate	3.95	4.36	0.39	0.77	6.85	7.10
post graduate	3.52	5.64	0.39	0.66	6.65	6.59
<i>Family Income(Taka)</i>						
Low	4.09	5.12	0.53	0.65	7.12	6.40
Medium	3.68	4.35	0.48	0.75	7.65	6.23
Higher	3.72	5.67	0.44	0.70	7.22	5.49

Table 3.3: Adjusted results of the association of genotype and birth weight

Genotype	Effect Estimate	95% CI
rs2794720	-0.12	-0.23, -0.02
rs2794719	0.09	-0.02, 0.21
rs9366637	-0.16	-0.31, -0.03
rs2071303	-0.06	-0.14, 0.08
rs1800708	-0.04	-0.13, 0.11
rs1572982	-0.17	-0.35, -0.03
rs17596719	0.02	-0.08, 0.12

^a All models control for site of data collection, maternal age, BMI, income, education, smoking status, maternal nutrition, child gender, delivery type, gestational age

Table 3.4: Effect modification of genotype on the association of prenatal Arsenic exposure and birthweight

Genotype	Genotype Variant	Blood Arsenic	Interaction
rs2794720	-0.13 *	-0.21 *	-0.06 **
rs2794719	0.09	-0.20 *	0.03
rs9366637	-0.15 *	-0.19 *	-0.10
rs2071303	-0.05	-0.17 *	-0.01
rs1800708	-0.05	-0.18	-0.06
rs1572982	-0.18 *	-0.21 **	-0.09 **
rs17596719	0.02	-0.17	-0.06

^a All models control for site of data collection, maternal age, BMI, income, education, smoking status, maternal nutrition, child gender, delivery type, gestational age

*P<0.05

**P<10⁻³

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CHAPTER 4

The association of prenatal metals exposure with children's growth in Bangladesh

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ABSTRACT

Objective: Arsenic, lead, and manganese are all known health hazards. However, most information of these metals exposure come from studies on adults. The effects of exposure to metals during fetal life on early growth and development still remain uncertain. In this study, we seek to characterize the association of prenatal metals exposure with childhood growth parameters.

Methods: Study population was taken from 819 women recruited between 2004 and 2011 in Bangladesh. Height and weight measurements were taken at birth, 12 months and again between 24-36 months. Umbilical cord blood measurements were collected at the time of delivery and analyzed via ICP-MS. The association between prenatal metals exposure and child height and weight was investigated using multivariate linear and mixed models.

Results: In this study, we showed that, among the three cord blood metals tested, early arsenic (As) exposure had the strongest and most consistent inverse association with infant parameters of birth weight (beta=-0.13 kg +/- 0.05 kg) and birth height (beta=-0.09 cm +/-0.06 cm). Manganese (Mn) also indicated statistically significant inverse relationship with birth height, however only at higher quartile levels compared to the reference. There is inverse association found with child height changes up to 36 months of age due to early exposure to arsenic (beta= - 0.14 cm +/- 0.08 cm) and at high levels of manganese (Mn: beta=-0.09cm, p=0.05 at highest quartile). No association has been apparent with Lead.

Conclusion: Results indicate that prenatal arsenic and manganese exposure adversely affect child height and weight development at birth and up to 36 months of age.

INTRODUCTION

Growth restriction in early life is associated with a number of poor health outcomes later in life. In particular, stunting, or height-for-age z score more than 2 standard deviations below the median reference set by the World Health Organization, has been associated with later behavioral problems, cognitive deficits, and increased risk of hypertension and cardiovascular disease. Also, studies indicated that stunted growth reported by 24 months of age has been associated with shortness in stature in adult life. Underweight, or weight-for-age z score more than 2 standard deviations below the World Health Organization median, is associated with increased risk of death from diseases such as pneumonia, diarrhea, and malaria [1-3]. Many factors play a role in growth restriction in children, and the most important risk factors for stunting and underweight happen early on in life, including maternal nutrition issues during pregnancy and problems in breastfeeding. However, early-life environmental exposures have also been noted to possibly play a role in growth restriction [4].

Currently, over 100 million people in Bangladesh are at risk of drinking contaminated drinking water, and are constantly facing environmental exposure routes exposing them to elevated levels of arsenic and manganese, as well as lead (Pb) from non-water sources. Studies have indicated that all three metals can cross the placenta and have been found in fetal tissue [5-9]. All three metals are associated with adverse effects on health. Increased exposures are associated with cognitive, physical and psychological diseases, and toxicity is cumulative and irreversible [10].

Even merely moderate exposure to arsenic during pregnancy is found to be associated with fetal loss and increased infant morbidity [11]. There is much less information on effects of arsenic on child growth. Two cross-sectional studies in Bangladesh reported associations of

arsenic in drinking water with low weight and height at a later age. There has also been a cross sectional study in China suggesting elevated water arsenic concentrations with growth of children at 12 years of age [12]. However, almost no association has been tested on longitudinal studies and on early ages. Early growth is subject to less other environmental bias than later growth and would give a more accurate representation of the effect.

Childhood lead exposure has been shown in some studies to be associated with stunting in pre-school children, and studies in animals demonstrate that lead may affect growth via toxic effects on bone tissue [13]. Rats exposed to lead have reduced bone calcium content, reduced trabecular bone volume, altered growth plate morphology and enhanced activities of spontaneous uterine contraction [9].

Almost no studies have examined the effect of manganese on child growth. In fact, very few papers have looked at manganese's effect on children at all. Those that did focused on manganese's effect on neurological development only [14, 15].

Thus, there is a critical need to explore in detail the effect of prenatal metals measurements of As, Pb and Mn on early child growth. In this study, we examine the effect of these prenatal metals exposure on child growth up to 36 months of age in Bangladesh.

MATERIALS AND METHODS

Study Population.

Our study population is taken from two regions of Bangladesh, where we recruited women between December 2004 and December 2011 who were seeking prenatal care at two of Dhaka's affiliate community hospitals. The clinics, Pabna Community Clinic (PCC) and Sirajdikhan Community Clinic (SCC), are located approximately 50 km southwest and 200 km

northwest of Dhaka, respectively, and serve demographically similar populations. Women were eligible to participate in the study if they were 18 years or older with a singlet pregnancy of less than 28 weeks' gestation confirmed by ultrasound at the time of enrollment, did not have a history of diabetes mellitus or of using oral hypoglycemics, were planning to continue receiving prenatal care through DCH, had used the same drinking water source for at least the previous six months at the time of enrollment, and intended to live at their residence with the same water source throughout pregnancy. Infants delivered pre-term will be excluded from the sample population. In all, 1,782 women were recruited into the study. Of these, 985 has cord blood measurements collected and tested, and 825 newborns were followed up for measurements and tests after birth.

Participating women were offered prenatal care during the course of their pregnancies including two pre-natal clinic visits, two ultrasounds, and a supply of pre-natal multivitamins. Participants are seen three times by study staff during the course of their pregnancies: at or prior to the 12th week of gestation, at or around the 28th week of gestation, and at delivery. At the first study visit, questionnaires are administered to record the mother's medical and family histories, and an ultrasound is performed to confirm the gestational age of the fetus.

Outcome measurement.

Child height and weight are used as measurements of growth separately. It is too early of age to examine the children in terms of BMI. The infant's birth weight and birth length were recorded at the time of delivery, 12 months and another at between 20-36 months of age. The infant's weight was measured on a pediatric scale, calibrated before each measurement using a 5-kg weight, and was recorded to the nearest 10 grams. Length was measured using a measuring board and is recorded to the nearest centimeter.

Exposure measurement.

For our analysis, we examined metals measurements in cord blood. Previous papers mostly looked at drinking water. However, exposures are not just through water, but can be through inhalation, dermal routes and other ingestions. We used cord blood as a biomarker because it captures total exposures in the utero environment and has shown good correlation with drinking water metal measurements in Bangladesh [16]. Umbilical cord blood, a biomarker capturing exposures to the in utero environment, is collected at the time of delivery. After the infant was delivered but before the delivery of the placenta, the umbilical cord was clamped at two sites and then detached from the mother. Approximately 6 mL of the enclosed cord blood is extracted into a metal-free blood collection tube containing the anticoagulant K₂EDTA (BD Vacutainer) and inverted 10-20 times. Samples were stored at 4°C until shipped to HSPH for storage and analysis. A second blue-top blood collection tube is uncapped prior to sample collection and was re-capped at its conclusion. These field blank samples are collected to measure and account for the introduction of external metal contamination during cord blood collection.

Samples of metals were analyzed at HSPH via ICP-MS. Approximately 1 g of blood is dissolved in 2 mL of Trace Select Ultra nitric acid (HNO₃) (Fisher Scientific, Pittsburgh, PA) at room temperature for 24 hours. After 24 hours the samples are treated with 1 mL of hydrogen peroxide (H₂O₂) and allowed to digest over 2-3 days. Samples are then diluted to 10 mL with deionized water. Samples are analyzed via ICP-MS for Cd and total As. Standard reference material blood (NIST 955c, Toxic Metals in Caprine Blood, National Institute of Standards and

Technology, Gaithersburg, MD) and method blanks are used to validate the performance of the ICP-MS and of the digestion method, respectively.

Statistical Analysis

Covariates included in our analysis from maternal measurements included site of data collection (Pabna or Sirajdikhan clinic), maternal age, maternal BMI at birth, average monthly income (categorized into low, medium, high), smoking status (past, current, never), maternal education and paternal education (both categorized in to 6 levels from illiterate to post-graduate), nutrition intake measured in terms of a total protein intake variable that was created based on the sum of frequencies of meat, fish, and fowl intake. Child measurements controlled for included gestational age, gender, whether breastfed up to a year after birth, and child age calculated by exact different from date of parameter measurement and date of birth.

We used Pearson's correlations to evaluate the pairwise relationship between the three metals. We additionally adjusted for the exposure metals in our models for a more rigorous analysis. The possibility of a nonlinear association between outcome measures and the predictor variables was explored graphically. Cord blood metal measurements were categorized into quartiles. Multivariate linear regression analyses was performed to assess the association between birth weight and cord blood metal levels. The p-value for the trend was obtained by running linear regression with the exposure as a linear variable. We then used multivariate linear mixed model to examine the effect of cord blood metals on child weight up to 36 months of age. Similar analysis was performed for height.

We further examined if any effect of cord blood metals on later growth was mediated through birth measurements [17]. Multivariate linear regression was used to determine is a

mediation effect was present through birth measurements. Bootstrapping of was used to estimate the indirect effect through the repeated sampling of data. Analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

RESULTS

After removing the 6 extreme outliers in the data, our cohort consisted 819 participants for analyses. Participant characteristics (Table 4.1) showed a cohort of women who are mostly of low to medium income, and mostly of lower education levels (only 4% completed college and beyond). The women consisted of a mean of 23.1 years at delivery (range 18 to 42 years). The children of this cohort of mothers are equally distributed in gender (51% male, 49% female). All women are married and all breastfed their children up to 1 year of age.

Pearson's correlation coefficients table (Table 4.4) indicated that there was association between our exposure variables As and Mn ($p < 0.001$), and As and Pb ($p < 0.001$). We avoided such correlation from affecting out results by adjusting for all the metals.

The association of the cord blood metals with birth parameters is shown in Table 4.2. There was a significant inverse association between As and birth weight ($\beta = -0.13 \text{ kg} \pm 0.05 \text{ kg}$, $p = 0.03$), As and birth length ($\beta = -0.09 \text{ cm} \pm 0.04 \text{ cm}$, $p = 0.02$). There appears to be an inverse dose-response affect between As and birth weight and height. Mn and birth length also exhibited a significant overall inverse effect ($\beta = -0.05 \text{ cm} \pm 0.04 \text{ cm}$, $p = 0.05$). However, the association with Mn appears to be only apparent in the higher quartiles of exposure.

As for the association of prenatal metals with later growth parameters (Table 4.3), we noted an overall inverse association between As and child height up to 36 months of age ($\beta = -0.14 \text{ cm} \pm 0.08 \text{ cm}$, $p = 0.04$). There was also an overall inverse effect of Mn on child height up

to 36 months of age ($\beta=-0.06 \pm 0.04$ cm, $p=0.05$), however, this association appears mostly driven by significance in the higher levels of Mn. While no significant P -value for trend was observed in the association between As and child weight up to 36 months of age, there was statistically significant negative association exhibited in the higher quartiles of As (quartile 3: $\beta=-0.14$, $P=0.05$; quartile 4: $\beta=-0.21$, $P=0.05$).

We examined the association of birth length as a mediator in the total effect of arsenic on height after birth. (Figure 4.1) We originally found a direct negative effect of As on height at 12 months ($\beta=-0.16$, $p=0.04$). However, we found also that there were significant direct negative effects of As on birth length ($\beta=-0.09$, $p=0.02$), as were the direct positive effects of birth length on height at 12 months ($\beta=0.78$, $p=0.01$). When accounting for the effect of birth length, As at 12 months was reduced to non-significance ($\beta=-0.04$, $p=0.08$). Results of the bootstrap analysis indicated that mediation was present ($M=0.07$. 95% CI= 0.03 to 0.16).

DISCUSSION

In this study, we showed that, among the three cord blood metals tested, early arsenic exposure had the strongest and most consistent inverse association with infant parameters of birth weight and birth height. Manganese also indicated statistically significant inverse relationship with birth height, however only at higher quartile levels compared to the reference. There is inverse association found with child height changes up to 36 months of age due to early exposure to arsenic and manganese. No association was apparent for lead.

While our studies have concluded no association between lead and early child growth, there have been conflicting reports elsewhere on the association of lead with early growth. A study on lead exposure lead exposure and growth on early preschool children up to 33 years of

age has indicated an association between lead and lower height. That study showed that the greatest effects of lead on child height are seen by 2-3 years of age. This association was, however, monitored only among those children who had mean blood lead levels greater than the cohort median. This effect was based also only on 35 (15% of the total subjects). Alternatively, a later, much larger cohort (n=1505) study on urinary lead and child growth up to 5 years of age found significant effect of lead associated with adverse height development.

It is uncertain how arsenic and manganese affects early child growth in our results. Possible explanations are that arsenic exposure is associated with increased risk of anemia. Growth retardation is one known result of anemia in children [18]. In some papers, improving iron and zinc status in undernourished children below 4 years improves growth in later life [19]. In addition, arsenic has endocrine-disrupting properties, which may disrupt growth in young children. Literature noted it happened usually in a sex-specific fashion. Arsenic may affect insulin signaling and glucose metabolism eventually leading to glucose intolerance and diabetes in exposed populations. Early disruption of glucose uptake by tissues can sometimes lead to impaired growth [20].

In humans, manganese blood levels increase through pregnancy, and manganese crosses the placenta actively [3]. Postnatally, infants would have increased absorption of ingested manganese and decreased elimination mechanisms, which indicates that this increases their susceptibility to high manganese exposure [21].

Manganese is a critical to formation of the bone matrix-Impaired growth and bone abnormalities have been documented in animals suffering from severe manganese deficiency or of excess [14]. Thus, Manganese tends to exhibit a U-shaped dose response curve [15]. In our study, we observed higher and significant effects on height inhibitions mostly at higher quartiles of

Manganese. This is likely due to that the level of cord blood Manganese in our study population is not low enough to observe any effect at low doses. Another possible explanation for this effect would be oxidative stress caused by high manganese levels. Oxidative stress can cause detrimental effects in cellular function and growth [22].

A strength in our study comes from the inclusion of Mn, which is rarely considered in literature when looking at growth parameters. Also, the use of cord blood takes in account all routes of exposure and what is directly passed on the child. A limitation of our study lies in possible misclassification of covariates as they are self-administered questionnaires. However, such deviations are too small to affect our results and would likely bias our results towards the null. Also, because our study population is demographically homogenous, we are limited with generalizability of our results from this one population alone; however, this also plays to our advantage as there would be good internal validity of our results.

In conclusion, this study provided evidence that increasing levels of pre-natal Arsenic exposure and high levels of Manganese exposure can negatively impact child birth weight and height, and negatively affect early child growth, especially height changes, up to 3 years of age. Since exposure to these metals prenatally can affect child growth so early on, these results prompts concern for reducing environmental exposures during pregnancy.

Table 4.1: Participant Characteristics (n=819)

Characteristics	Parameters		
	mean (sd)	min	max
Mother age at delivery	23.1 (4.7)	18	42
Birthweight (kg)	2.8 (0.3)	0.8	4.5
Birth head circumference (cm)	32.3 (1.9)	27	37
Birth length (cm)	46.1 (3.0)	28	56
gestational age (weeks)	38.5 (2.0)	28	42
	NO. (%)		
Infant gender			
Male	411 (51)		
Female	408 (49)		
Maternal education			
illiterate	58 (2)		
able to write	148 (19)		
secondary education	256 (38)		
higher secondary education	254 (37)		
college/graduate	57 (3)		
post graduate	46 (1)		
Paternal education			
illiterate	70 (5)		
able to write	190 (26)		
secondary education	223 (32)		
higher secondary education	208 (30)		
college/graduate	73 (5)		
post graduate	54 (2)		
Marital status			
Married or with partner	819 (100)		
Family Income(Taka)			
Low	217 (23)		
Medium	530 (63)		
Higher	72 (3)		
Breastfed up to one year of child age	819 (100)		

Table 4.2: Adjusted* result of the association of cord blood metals concentration quartiles with birth parameters

	Birth Parameter	Quartile 1	Quartile 2	Quartile 3	Quartile 4	<i>P</i> value for trend
		β estimate (<i>P</i> -value)				
Arsenic	Birth Weight	[reference]	-0.06 (0.05)	-0.09 (0.03)	-0.12 (0.03)	0.02
	Birth Height	[reference]	-0.05 (0.05)	-0.07 (0.06)	-0.09 (0.03)	0.02
Lead	Birth Weight	[reference]	-0.09 (0.14)	-0.15 (0.24)	-0.11 (0.21)	0.24
	Birth Height	[reference]	-0.007 (0.21)	-0.005 (0.15)	-0.01 (0.09)	0.13
Manganese	Birth Weight	[reference]	-0.01 (0.10)	-0.01 (0.07)	-0.04 (0.05)	0.08
	Birth Height	[reference]	-0.005 (0.08)	-0.004 (0.05)	-0.01 (0.05)	0.05

*All models control for site of data collection, maternal age, BMI, income, education, smoking status, nutrition, child gender, delivery type, gestational age, and other metals.

Table 4.3: Adjusted* result of the association of cord blood metals concentration quartiles with growth parameters up to 36 months of age

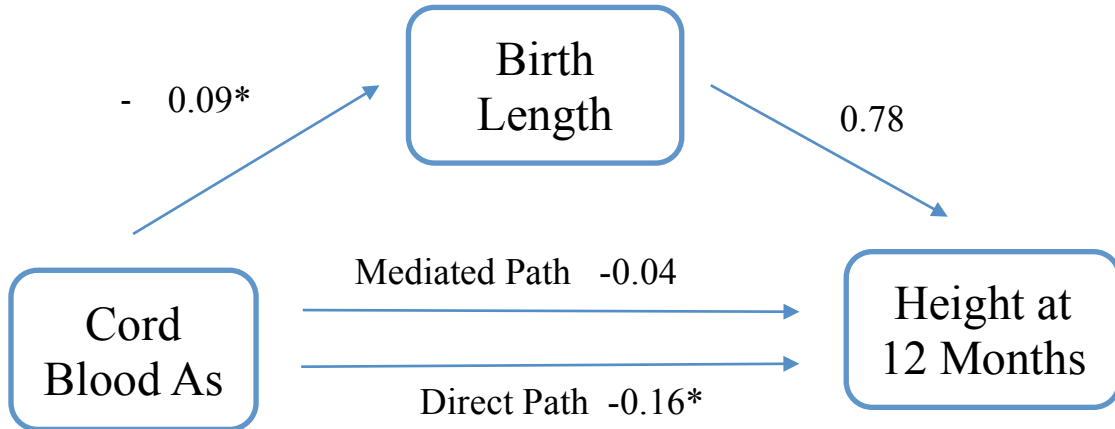
	Growth Parameter	Quartile 1	Quartile 2 β estimate (<i>P</i> -value)	Quartile 3	Quartile 4	<i>P</i> value for trend
Arsenic	weight 0-36 months	[reference]	-0.008 (0.08)	-0.02 (0.05)	-0.03 (0.05)	0.07
	height 0-36 months	[reference]	-0.02 (0.10)	-0.05 (0.06)	-0.06 (0.03)	0.04
Lead	weight 0-36 months	[reference]	-0.09 (0.31)	-0.11 (0.22)	-0.15 (0.35)	0.35
	height 0-36 months	[reference]	-0.19 (0.32)	-0.21 (0.26)	-0.24 (0.45)	0.42
Manganese	weight 0-36 months	[reference]	-0.06 (0.42)	-0.10 (0.48)	-0.08 (0.33)	0.46
	height 0-36 months	[reference]	-0.03 (0.10)	-0.04 (0.05)	-0.06 (0.05)	0.05

*All models control for site of data collection, maternal age, BMI, income, education, smoking status, nutrition, child gender, child age, delivery type, gestational age, and other metals.

Table 4.4: Correlation between cord blood metals.

	Arsenic	Lead	Manganese
Arsenic	1.00	0.14 ($P < 0.001$)	0.50 ($P < 0.001$)
Lead		1.00	0.004 ($P = 0.91$)
Manganese			1.00

Figure 4.1. Cord blood As as a predictor of height at 12 months mediated through birth length



* $P < 0.05$

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CHAPTER 5

SUMMARY AND CONCLUSION

We found that prenatal metal exposure to be a significant determinant of child health and development.

In Chapter 2, we examined the effect of prenatal exposure of metals and their combined influence on infant birth parameters. We found prenatal arsenic and manganese exposure individually associated with lowered birth weight and birth head circumference. We also found evidence of interactions between the two metals, suggesting that joint exposure creates greater deficit in birth outcomes. The interactions were nonlinear, existing only in high levels of exposure, and would have been missed simply using main effects regression models. This study serves an important step into looking a more realistic presentation of metal co-exposures that exist in the environment. The synergism observed in our joint metal toxicity shifts the focus to explore further into multi-metal exposures.

In Chapter 3, we found significant modification effects of multiple SNPs on the HFE gene that increased the association between arsenic and birth weight. The detrimental effect of arsenic on birth weight has been documented in previous papers and confirmed in initial analysis of Chapter 2. The HFE gene variants can introduce excess iron in the body due to disrupted iron regulation and uptake. This study is one of the first to examine the change of the HFE gene variants on the effect of arsenic and birth weight. We also included less common SNPs not examined in literature. Our study sheds light on the changes manifested through gene-environment interactions.

In Chapter 4, we assessed the effect of prenatal metals exposure on early growth in children. In this longitudinal study, we followed the weight and height of the child from birth up to 36 months of age. Our results indicated adverse association between arsenic and manganese on growth. It is an important find as this is one of the first studies to examine the effect of

manganese on child growth. The attrition in early childhood growth from prenatal exposures underscores the need for awareness of environmental exposures during pregnancy, and call for future research to explore the mechanism further.