INVESTIGATION OF NUTRITIONAL BIOMARKERS ASSOCIATED WITH METABOLISM OF INORGANIC ARSENIC AND INFANT BIRTHWEIGHT

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ABSTRACT

Jessica E. Laine: Investigation of Nutritional Biomarkers Associated with Metabolism of
Inorganic Arsenic and Infant Birthweight
(Under the direction of David B. Richardson)

Exposure to inorganic arsenic (As) in utero represents a critical window of susceptibility
for iAs associated adverse birth outcomes. Ingested iAs undergoes hepatic methylation
generating mono- and di-methyl arsenicals (MMA and DMA, respectively), a process that
facilitates urinary As elimination. Differences in pregnant women's metabolism of As (e.g.
increases in %MMAs and decreases in %DMAs) are a risk factor for adverse birth outcomes.
One carbon metabolism (OCM), the nutritionally-regulated pathway essential for supplying
methyl groups, plays a role in As metabolism and is understudied during the prenatal period. In
this cross-sectional study from the Biomarkers of Exposure to ARsenic (BEAR) pregnancy
cohort in Gómez Palacio, Mexico, we assessed the relationships among OCM indicators (e.g.
maternal biomarkers of serum B12, folate, and homocysteine (Hcys)), and levels of iAs and its
metabolites in maternal urine and in neonatal serum. We also estimated the relationship between
OCM indicators, iAs metabolism, and infant birthweight using a causal mediation approach,
where we measured the total effect (TE) and the natural direct effects (NDE) of OCM indicators
on infant birthweight, and controlled direct effects (CDE) of both OCM indicators and maternal
metabolism of iAs on infant birthweight.

Interestingly, the prevalence of folate sufficiency (serum folate levels > 9 nmol/L) in the
cohort was high 99%, and hyperhomocysteinemia (Hcys levels >10.4 μmol/L) was low (8%).
However, 74% of the women displayed a deficiency in B12 (serum levels < 148 pmol/L).

Differences in lower B12 levels and higher Hcys were associated with increases in total arsenic levels in urine (U-tAs). In unadjusted comparisons, infants born to mothers in the lowest tertile of serum folate had significantly higher mean levels of C-%MMA relative to folate replete women. Furthermore, beta regression results demonstrated that maternal Hcys was positively associated with both C-tAs and %C-MMAs. Interestingly, levels of folate modify the effects of B12 deficiency on infant birthweight. The causal mediation results demonstrated that there is evidence of interaction between OCM indicators and iAs metabolism. The results from this study indicate that maternal OCM status may influence neonatal As metabolites, and interactions of OCM with iAs metabolism may influence infant birthweight.

To all the women in science that came before me, and to those that will come after me: Let us continue to defy stereotypes, let us continue to overcome marginalization, let us embody respect and equality in our work, research, and life- but, most of all, as Vonnegut said, "Science is magic that works…" I challenge us all to continue to make magic real.

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LIST OF ABBREVIATIONS

iAs   Inorganic Arsenic

WHO   World Health Organization

EPA   United States Environmental Protection Agency

FOAD   Fetal Origins of Adult Disease

LBW   Low Birthweight

MMAs   Monomethylated Arsenicals

DMAs   Dimethylated Arsenicals

Dw-iAs   Drinking Water Inorganic Arsenic

U-iAs   Total Urinary Inorganic Arsenic

U-MMAs   Monomethylated Arsenicals

U-DMAs   Dimethylated Arsenicals

OCM   One Carbon Metabolism

CHAPTER I: SPECIFIC AIMS

Arsenic (iAs) is a toxic metal of particular concern for human health risks is, as it is currently ranked the highest priority toxic agent by the Agency for Toxic Substances and Disease Registry (ATSDR).[1] Contamination of drinking water supplies from iAs is a critical global public health problem. Regulation of iAs for drinking water is based only on these levels of iAs in drinking water, where the EPA and WHO standard is set to not exceed 10ppb; however, differences in human metabolism of iAs may play a more significant role in disease etiology than just exposure to iAs alone. Additionally, certain populations may be more significantly impacted by exposure to iAs than others. Poor efficiency in metabolism of iAs has been associated with the development of several adverse health outcomes in humans, including urinary bladder cancer, non-melanoma skin cancers, carotid atherosclerosis, and chromosomal aberrations (as reviewed in [2]).

The prenatal period is a critical development window of susceptibility to iAs exposure is during the prenatal period. Prenatal exposure to iAs is associated with adverse birth outcomes and susceptibility to later in life diseases.[3] Specifically, prenatal iAs exposure has been associated with lower birth weight, preterm birth, reduced height and head circumference, increased susceptibility to infection, including inflammation and infectious disease, and cancers (later in life).[4] Additionally, inefficient maternal metabolism of iAs during pregnancy has been recently associated with decreases in birthweight and other birth outcomes.[5]

The inclusion of metabolism efficiency, and in particular in susceptible populations, would be a more informative approach to the assessments of risks and regulation for iAs- and thus prevention of iAs-associated diseases. In addition, metabolism of iAs and toxicity of iAs is influenced by many nutritional factors- where many vitamins are known to be important cofactors in both S-adenosyl methionine (SAM) synthesis and one-carbon metabolism, which play a key role in iAs methylation/biotransformation. Specifically, there is a relationship between folate, vitamins B12 and B6, cysteine and homocysteine choline, betaine, and methionine in the human methylation pathway.[6] This has been demonstrated in animal models where suboptimal intake of many vitamins and nutrients that are required for SAM synthesis, including methionine, choline, folate, and vitamin B12, or the knockout of the folate receptor impaired iAs methylation and increased susceptibility to both carcinogenic and non- carcinogenic effects from iAs exposure.[7-9] For example, Vitamin B12 plays an integral role in folate-dependent homocysteine metabolism as a rate-limiting co-factor in the conversion of homocysteine to methionine.[10] Studies have also suggested that metabolism of iAs can be altered via folate supplementation,[11] though this effect is less clear during pregnancy.[12] Additionally, removal of homocysteine is a prerequisite for adequate one-carbon metabolism and iAs methylation because the precursor to homocysteine, *S*-adenosylhomocysteine, is a strong feed-back inhibitor of the SAM-dependent methylation reactions, including the methylation of iAs.[6] This betaine-mediated remethylation of homocysteine is may be important in individuals with low folate/B12 intake.

The central hypothesis of this research is that nutrients involved in one-carbon metabolism (B12, folate, cysteine, and homocysteine) influences arsenic metabolism and birthweight. This hypothesis is based first upon our previous research that showed that the

metabolism of iAs, as measured by the levels and proportions of monomethylated arsenicals (MMAs) in maternal urine, was associated with significant decreases in birthweight.[5] Second, our preliminary data suggest that there are relationships between maternal nutritional biomarkers and iAs metabolism and birthweight.

In this proposed work I aim to investigate factors that may underlie the relationship between prenatal exposure to iAs and metabolism of iAs, maternal nutritional biomarkers, and birthweight in a cross-sectional study with the following three aims:

Aim 1: Examine whether levels of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine) as measured in maternal serum at delivery are associated with metabolism of arsenic.

Metabolism of arsenic is measured by the percentages of iAs, MMAs, and DMAs, as well as the two methylation steps (i.e., primary methylation, defined as the ratio between MMA and iAs, and secondary methylation step, defined as the ratio between DMA and MMA), in both maternal urine and fetal cord serum measured at delivery. This aim addresses the following hypothesis (Hyp1): *Maternal nutritional biomarkers are associated with a woman's metabolism of arsenic and the biomarkers of potential fetal exposure to arsenic and it's metabolites. Specifically, decreased levels of B12 or folate or Cys and increased levels of homocysteine are associated with a decrease in metabolism of arsenic; specifically, there will be an decrease in %MMAs and DMAs/MMAs (second methylation step), and an increase in the %DMAs.* Nutritional biomarkers will be modeled primarily as continuous variables, however nutritional deficiencies will be explored through categorical analyses. Other factors to consider in this aim are the correlations between the nutritional biomarkers as well as potential confounders.

Aim 2a: Estimate the total effect of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine) as measured in maternal serum at delivery on infant birthweight.

The total effect is any effect that is operating through the path of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine), iAs metabolism, and birthweight.

This aim addresses the hypothesis (*Hyp 2a*): *Maternal nutritional biomarkers are associated with infant birthweight.* Specifically, deficiencies in folate, B12, and cysteine are predicted to be associated with decreases in infant birthweight and elevated levels of homocysteine are predicted to be associated with decreases in infant birthweight.

Aim 2b. Estimate the indirect effect of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine) as measured in maternal serum at delivery on infant birthweight that operates through iAs metabolism.

The indirect effect is the effect of maternal iAs metabolism arsenic as measured by the percentages of iAs, MMAs, and DMAs, as well as two methylation steps (i.e., primary methylation, defined as the ratio between MMA and iAs, and secondary methylation step, defined as the ratio between DMA and MMA), in maternal urine on infant birthweight. Mediation analysis will be carried out using the Baron-Kenny approach and the mediator will be allowed to vary naturally. This aim addresses the hypothesis (*Hyp 2b*): *The effects of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine) on birthweight operate via the pathway of iAs metabolism that then effects infant birthweight.*

Aim 3: Investigate the total and indirect effects of maternal levels of nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine) on infant birthweight by different levels of drinking water iAs.

Exposure levels, characterized by the levels of iAs in drinking water, will be categorized as either above and below the WHO guideline level of 10 μg/L. This aim addresses the hypothesis (***Hyp 3***): *The levels of iAs in drinking water influence the effect of maternal nutritional biomarkers and the health of her newborn.*

CHAPTER II: OVERALL SIGNIFICANCE

Prenatal exposure to environmental contaminants, a global public health problem

Disease burdens associated with impaired fetal development, including disabilities, loss of human capital, and deaths (among others) are vastly impactful to many across the globe [13]. Underlying several of these burdens are preventable exposures to environmental contaminants. Exposures during the prenatal period are important, as it is a critical and sensitive time period for development. This period of exposure puts infants and children at risk for several adverse health outcomes. In fact, while an estimated 23% of all deaths globally are attributable to environmental factors in adults, this proportion significantly increases among children of 0–14 years of age, where the proportion of deaths attributable to environmental exposures is 36% [14]. Additionally, across the globe the per capita number of healthy life years lost to environmental risk factors is about 5-fold greater in children under 5 years of age than in the total population. Furthermore, estimated perinatal conditions, including low birth weight, prematurity, birth asphyxia and birth trauma that environmental causes account for is an average of 6% (range of: 2—10%) in developed countries, and an average of 11% (range of 3— 25%) in developing countries [14]. Importantly, many of these and other health impacts associated with prenatal exposures persist into childhood and later into adult life.

A particularly important modifiable exposure during the prenatal period is environmental toxicants. This is particularly true because the developing fetus is highly sensitive to the negative effects of exposures to toxicants, as this is a critical time of rapid

cell division, differentiation, and tissue growth. Prenatal exposure to environmental toxicants is associated with several adverse reproductive, pregnancy, and birth outcomes as well as later in life diseases. Susceptibility to later in life diseases from prenatal exposures is a theory that was put forth by Barker termed, "fetal origins of adult disease" (FOAD) [15]. The basis behind this theory is that there are specific developmental periods whereby an organism is "plastic" or "sensitive" to its environment and exposures can alter fetal programming-meaning when a stimuli or exposure occurs during early development, this may lead to permanent changes that persist throughout life [15]. One toxicant of particular concern for adverse birth, early life outcomes, and FOAD is iAs. The impacts of iAs on infant birth weight and FOAD are just beginning to be unraveled. Interestingly, as it relates to the research questions here, FOAD as a theory was originally founded based on the perinatal outcome of infant birthweight- a measurement that is still highly relevant and of great public health concern today.

The proposed work here is significant in that it addresses the highly toxic and widespread compound of iAs exposure in susceptible populations (*in utero*); in addition, this work is founded to better understanding mechanisms underlying iAs-associated alterations in infant birthweight-a risk factor for FOAD. Equally important is results from this study may be used to inform public health policies and potential interventions on exposure to iAs, in efforts to reduce disease burdens from iAs exposure. It is imperative these risks for iAs associated diseases from prenatal exposure are continually evaluated and that we work towards effective ways to eliminate this modifiable environmental exposure to prevent adverse birth outcomes and later in life diseases.

CHAPTER III: BACKGROUND

Birthweight, an important perinatal outcome

Birthweight, an easily measurable health outcome, is simply the weight of an infant at birth. Specifically, infants' weights are measured within the first hour of birth, to avoid any amount of postnatal loss of weight. On a population scale, the distribution of birthweights follow an almost normal frequency distribution with an extended lower tail [16].

Several terms are used to classify different categories and health descriptors of an infant's birthweight. An infant is classified as being born "low birthweight" (LBW) if it weighs 2500 grams or less, regardless of gestational age. LBW is further categorized based on gestational ages, where both "premature" or "preterm LBW" are terms to describe infants who weigh less than 2500 grams and are born before the completion of 37 weeks of gestation; "term LBW" is used to describe those that weigh less than 2500 grams and are born between 37 and 42 complete weeks of gestation; "postterm LBW", are those infants that weigh less than 2500 grams and are born after the completion of 42 weeks of gestation [17]. Two other descriptors used for infants' weight for both clinical and non-clinical purposes are, "small-for-gestational-age" (SGA) and "large-for-gestational-age" (LGA). These are statistical calculations based on a standard curve for the weight of infants by weeks of gestation, where those infants with weight less than the lower limit of the confidence interval of the normal curve are described as being SGA, and those infants that are greater than the upper limit of the confidence interval of the normal curve for weight are described as being LGA [17]. Additionally, the term "intra-uterine growth restriction" (IUGR) is used to explain

the condition of high mortality of LBW babies who are born at term and is based on any etiology that limits the potential for intra-uterine growth of the fetus that results in low birthweight [16].

The rate for LBW in the US is 8% of all live births [18]. Being born at a sub-optimal birthweight has many health consequences, starting at birth, through childhood, and later into adulthood [19]. It has been suggested that LBW in itself may not be a cause of poorer health outcomes, instead abnormal birthweight may actually be an indication of abnormal fetal growth due to an underlying problem [16]. Additionally, the relationship between birthweight an infant mortality is non-linear, in that there is an increase in risk for mortality at both lower and higher birthweights; this shape varies little among populations [17]. The lowest mortality of birthweight occurs at a weight just above the mean birthweight, a finding consistent across all known populations [20]. The relationship between birthweight and infant mortality should be interpreted with caution, for even though birthweight is highly predictive of infant mortality, it isn't necessarily deterministic of neonatal survival- as there are many paradoxes with this association [21].

Importantly, shifts in birthweight are also linked to fetal origins of adult disease (FOAD). For example, both epidemiological and animal studies have demonstrated that when LBW is followed by exponential childhood growth - a term referred to as "catch-up" growth- there are increases for risks for metabolic syndrome, obesity, insulin resistance, dyslipidemia, and hypertension [15, 22-25]. Additionally, infants born SGA are at increased risk for developing obesity, type two diabetes, coronary artery disease, hypertension, kidney disease, premature pubarche, Polycystic Syndrome, dyslipidemia, short stature, and osteoporosis (as reviewed in [19]).

Majority of perinatal studies focus on the association between LBW, SGA, and smaller babies, however, those with normal birthweights, and LGA may still be at risk for adverse health outcomes in early life and for FOAD [26-29]. For example, infants who are born bigger or LGA have an increase risk for diseases, including obesity and insulin resistance. This is likely based on changes in maternal behavior where LGA children are less likely to receive breast milk and may exhibit more weight gain in the first 6 months of life compared to those who were not LGA [30, 31]. Additionally, there may be exposure-specific biological mechanisms, for example disruption of molecular pathways that may regulate factors related to weight. Therefore, it is important to recognize this U-shaped relationship with both high birthweight and low birthweight for risks for later in life diseases.

Not only is birthweight an important outcome for public health purposes, it is also a widely available variable in reproductive/perinatal epidemiological studies as it is consistently recorded and measured well. The most direct measurement of this variable on a population level is to examine the entire distribution of birthweight, rather than classifying it into LBW, SGA, or LGA [16].

Up to 40% of fetal growth is influenced by genetic factors, and the remaining is thought to be due to environmental exposures [17]. Specifically, genetic and hereditary factors underlying neonatal growth include maternal and paternal birthweight, the sex of the infant, chromosomal anomalies, and alterations in the regulators of gene expression (i.e. epigenetics). Environmental factors (meaning anything outside of genetic influences) include socio-demographic risk factors, including maternal age, race and ethnicity, marital status, education, socio-economic level; maternal medical risks before and after pregnancy, including chronic hypertension, renal diseases, glucose metabolism disorders, chronic

cardiorespiratory disease and other disorders that involve hypoxemia, genitourinary

anomalies, autoimmune diseases and inherited or acquired thrombophilia, obstetrical history,

gestational hypertension and diabetes, pregnancy weight gain, parity and time in between

births, placental abnormalities, vaginal bleeding, increased α-fetoprotein, anemia, infections,

fetal congenital anomalies, prenatal care, maternal stress, including work and psychosocial

stress, maternal nutrition and the placenta's ability to supply nutrients, (amino acids, glucose,

fat, oxygen, and growth-stimulating hormones) maternal and second hand smoking, alcohol

consumption, caffeine consumption, maternal and paternal drug consumption, and

environmental toxicants (as reviewed in [17]).

Clearly there are several known associations between environmental exposures and

an infant's weight at birth, yet many of these remain generalities and there is much still

unknown about specific mechanisms, ways to prevent extremes in infant birthweights, as

well as FOAD associated with alterations in infant growth. Additionally, the role of

environmental toxicants in the etiology of these health outcomes is still an area of research

that is in need of further investigation. One toxicant of particular concern for adverse birth

outcomes, and in particular neonatal growth is exposure to iAs, as *in utero* exposure has been

associated with reduced growth, and several other adverse health outcomes, discussed in

detail below.

Sources of exposure to inorganic arsenic

Inorganic arsenic is a naturally occurring metalloid found in soil and rocks that can

become mobilized via natural processes, including from mineral weathering, biologically

aided mineralization, and volcanic emissions. These natural conditions can contribute to high

concentrations of iAs in ground water due to the strong effects of water–rock interactions and

the tendency for aquifers to have both the physical and geochemical conditions that support

iAs mobilization [32]. In addition to natural sources of iAs, human activity can introduce iAs into the environment from both commercial and industrial uses, with contamination from mining and smelting for the production and use of transistors, lasers and semiconductors, processing of glass, pigments, textiles, paper, metal adhesives, wood preservatives, coal, pesticides, feed additives, and pharmaceuticals [33].

People are exposed to iAs through a multitude of ways, including via ingestion from contaminated drinking water, using iAs-contaminated water in food preparation and bathing, and through the consumption of food sources irrigated with iAs-contaminated water or grown in contaminated soils, and through inhalation from smoking and occupational exposures. However, drinking iAs-contaminated water is the primary source of exposure to iAs for most individuals, where it is estimated that more than 200 million people are exposed to elevated levels of iAs from drinking water worldwide. In particular, iAs contaminated drinking water has been observed globally in China, including Taiwan, Bangladesh, Japan, Korea, Iran, India, Chile, Argentina, Mexico, Europe, and in the US [4].

The levels of iAs allowed in drinking water that are acceptable and enforced by regulatory agencies vary by region. The WHO and Australia, have a set guideline level of 10 µg/L for iAs in drinking water and the U.S. Environmental Protection Agency's (EPA) maximum contaminant level (MCL) is 10 µg/L. However, in many countries (e.g. Bangladesh) 50 µg/L is still the commonly adopted guideline, and in Mexico the MCL is set at 25 µg/L. It has been suggested that these differences in regulation may primarily be due to the difficulties in remediating iAs below the designated MCLs [33].

There is a wide range in severity of exposure to iAs from contaminated drinking sources by region. The most widely studied and large-scale area of exposure to iAs from

contaminated drinking water is in Bangladesh. When exposure in this region was first studied up to 94% of tube wells in certain regions and 35% of all wells in the country contained > 50 µg/L iAs [34]. A recent review has highlighted the levels of iAs in drinking water samples across populations globally, where the levels in Argentina range from less than 10 µg/L up to 7,550 50 µg/L, in Chile 600 to 800 µg/L, in China less than 50µg/L to 4,400 µg/L, in Ghana < 2 µg/L to 175 µg/L, in India < 10 µg/L to >800 µg/L, and in Taiwan < 1 µg/L to 3,000 µg/L [4].

While the U.S. has adopted the WHO's standard for municipally supplied water, there are still several areas in the U.S. that have exposure to iAs in drinking water through unregulated private wells supplied with groundwater. In the U.S. contaminated groundwater with elevated iAs is prevalent in the West, Midwest, parts of Texas and the Northeast and the South [35-37]. The exact number of households affected by contaminated drinking water is largely unknown; however, an EPA study in 2001 found that approximately 13 million U.S. residents are drinking water from private wells that exceed the federal drinking water standard for iAs [38]. Another nationwide survey by the U.S. Geological Survey (USGS), in 2009, found that 6.8% of the 1,774 wells tested exceeded the MCL [39]. State specific results also indicate several additional contaminated wells. For example, a USGS survey in Maine estimated that 24,000–44,000 households may be affected by levels greater than the U.S. EPA MCL [40]. Additionally, another study that used predictive modeling, estimates that 42.7% of the area of aquifers in the southwestern United States have iAs concentrations greater than10 µg/L [41]. Furthermore, our lab has demonstrated that there is a local concern for iAs contaminated wells, where out of 63,000 private drinking water wells tested in North

Carolina 1,436 had iAs concentrations greater than the MCL, and a maximum level of 806 μg/L was found [42].

While drinking water is the primary source of exposure for iAs there can be exposure to both organic and inorganic forms of arsenic from food sources. These sources include fish, shellfish, meat, poultry, dairy products, and cereals- notably, arsenic in seafood is mainly in the form of the organic arsenic, a less toxic organic form [33]. While majority of food exposure form iAs is thought to be low, a recent report on iAs in foods from the European Food Safety Agency (EFSA) identified rice, seafood, algae, wheat bread and rolls, and bottled water as the top food sources that contributed most to the intake of iAs by the European population (outside of drinking contaminated iAs water) [43]. The daily dietary exposures were within the range of reference values, however the authors concluded that the possibility of increased exposure to iAs from food can not be excluded [43].

Sources of iAs exposure via drinking water and diet may differ during various life stages. For example, infants have different dietary demands- where newborns' diet consists of primarily breast milk and/or formula. In general, studies have reported that arsenic in breast milk is low, regardless of exposure [44]; however, the species of arsenic most prevalent in breast milk is currently unclear [44] and may depend on the source or magnitude of maternal iAs exposure [45, 46]. Infant formula can be a source of iAs [47] as can the water that mixed with it [48]. Additionally, children's intake is relative to their body mass, which is higher than that of an adult, [49] therefore dietary exposure to iAs can be up to three times higher for infants and young children than iAs exposure for adults [43]. Also, because children consume a greater volume of rice products, it has been suggested that children less than 3 years of age may have the greatest exposures to iAs via their diet [43].

Properties of inorganic arsenic

The chemical properties and forms of arsenic are important in terms of biological fate of arsenic, and are essential for assessing associated risks in human populations. Arsenic has four oxidation states: +5, +3, 0, and −3, represented by arsenate, arsenite, elemental arsenic, and arsine, respectively. In living organisms arsenic is found mostly in the pentavalent (+5) and trivalent oxidation (+3/-3) states.

In considering arsenic exposure from water sources (e.g. natural ground water) arsenic is typically present in inorganic forms (iAs^{3+} and/or iAs^{5+}). Organic forms of arsenic are rare in water because they are a result of biological activity; however, organic arsenic is found in seafood, with arsenobetaine and different arsenosugars being the most common forms.

iAs can exist in either the trivalent form (iAs^{3+}) or pentavalent states (iAs^{5+}). iAs^{3+} is generally considered to be more toxic than iAs^{5+} based on its biological availability, as well as physiological and due to its increased toxicological effects [50, 51]. IAs^{5+} is rapidly reduced to iAs^{3+} when ingested and will exert toxicity to similar endpoints, however it has different toxicokinetics [51]. iAs^{3+} is considered about 60 times more toxic than the iAs^{5+}, because arsenite's ability to react with sulfydryl groups, whereas arsenate does not have this reaction in humans [52].

Once iAs is ingested by humans, it is metabolized in the body to produce monomethylated and dimethylated arsenicals (MMAs and DMAs, respectively), this process is often referred to as methylation of or metabolism of iAs. Six major arsenicals associated with iAs exposure and metabolism have been detected in human urine, namely arsenite (iAs^{III}), arsenate (iAs^{V}), monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^{V}), dimethylarsinous acid (DMA^{III}), and dimethylarsinic acid (DMA^{V})[53]. Most of this

metabolism of iAs is thought to take place in the liver, however, there is increasing evidence that the microbiome in the gut may play an important role in metabolism and impact absorption/excretion of iAs (60,86).

In humans the average half-life iAs in the body is about 10 days (79,80). Several different biomarkers are used to assess exposure to iAs. Specifically, in some studies, iAs concentrations are measured in urine, [54] blood, [55] and toenail samples [56]. Urinary iAs is used most often as a biomarker of exposure and is considered to be a reliable short-term measure of iAs exposure that remains consistent in adults, even during periods of pregnancy. In terms of metabolism of iAs, studies that have examined multiple biomarkers suggest strong a correlation between metabolites excreted and retained in the same individuals, with positive correlations between blood and urine metabolites as well as iAs measurements in hair and nail samples [57].

iAs has many toxicological properties. It is genotoxic and has multiple effects on cellular signaling, cellular proliferation, DNA structure, epigenetic regulation, and apoptosis [58-60]. These properties can be set even before birth from prenatal iAs exposure.

Prenatal exposure to iAs

In utero exposure to iAs increases the risk for both childhood diseases and for FOAD [3]. iAs is a transplacental toxicant, [61] and maternal exposure to iAs has adverse impacts during sensitive embryonic development and on fetal programming [62]. From epidemiological studies we know that prenatal exposure to iAs can increase oxidative stress [63] lipid peroxidation, interfere with hormonal activities, and perturb DNA methylation and gene expression-including those involved in deregulation of immune and inflammatory pathways [63, 64]. These mechanism influence fetal programming and may contribute to a wide range of adverse pregnancy and birth outcomes [65]. Specifically, prenatal exposure to iAs has been associated

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with adverse neurological outcomes [66, 67], increased cardiovascular disease, pulmonary diseases, susceptibility to infection, including inflammation and infectious disease, and later in life cancers (as reviewed in[4]).

Neurological impairments observed in children from prenatal and early life iAs exposure include a decrease in motor function [66], impairments in verbal and full-scale IQ in girls, [68] and cognitive impairments in children at 6 and 10 years of age [67]; these impairments may persist into adulthood and lead to a vast array of neurological based FOAD.

Cardiovascular impacts from prenatal iAs exposure have also been identified. A retrospective study of Chilean adults who were exposed to high levels of iAs prenatally and as very young children had a high standardized mortality ratio (SMR) from acute myocardial infarction compared to those who were not exposed to iAs [69]. Another study found an increase in blood pressure (both systolic and diastolic) at 4.5 years of age, with increases in iAs exposure, measured by maternal urinary markers [70]. The authors suggest that these changes, if sustained, may be damaging and long-term, particularly in genetically susceptible populations [70].

Pulmonary disease endpoints, both cancer and non-cancer, have been observed in prenatal cohorts who were exposed to iAs. A SMR of 50.1 from bronchiectasis was observed for those exposed to iAs contaminated drinking water prenatally and during early life [71]. Additionally, there is an increase in pulmonary tuberculosis from prenatal iAs exposure [72] A shocking reduction similar to that of smoking throughout adulthood in forced expiratory volume and forced vital capacity has been observed in adults who were exposed to iAs *in utero* [73]. Furthermore, a decrease in forced vital capacity and a restrictive spirometric pattern

in the lungs of children has been observed from exposure to iAs *in utero* and from early life exposure [74].

Exposure to iAs during pregnancy and childhood is also associated with increased incidence and mortality from various cancers both during childhood and later in life [69, 72, 73, 75]. One study found that childhood liver cancers were 9–14 times higher for those exposed to iAs as young children as compared with those who were not exposed [75]. The same trend was observed for childhood liver cancer, where SMRs were 14.1 times higher for individuals exposed to iAs *in utero* and during childhood as compared with individuals exposed to iAs during other periods of their lives [71] - indicating that the prenatal period is a sensitive time for the development of liver cancer.

There is also evidence that exposure to iAs *in utero* is associated with an increase in inflammation, risk for infection, immune dysfunction and diseases associated with these. Altered immune-related health outcomes, including an increased risk for lower respiratory infections and diarrhea has been observed in infants and children from prenatal iAs exposure[76]. Changes in immune-related gene expression and cytokine production in lymphocytes have also been observed in infants exposed to iAs [77, 78]. Additionally, studies have indicated that prenatal iAs exposure induces an inflammatory response where reduced numbers of T cells as well as altered cytokine profiles from cord blood were identified [63] as have changes to the number of specific CD4 + T cell populations present in cord blood, increased cord blood T cell proliferation, and greater IL1β expression in the placenta - indicating that this could potentially lead to immune dysregulation in the infant [79]. Furthermore, maternal urinary iAs during pregnancy is significantly associated with reduced

thymic function in infants, which is linked to reduced immune function in children and adults
[80].

In addition to the detrimental health consequences observed in children and adults
from exposure to iAs during the prenatal period, several adverse perinatal, pregnancy
outcomes, and birth outcomes have been observed [3]. Perinatal and birth outcomes of
increased infant mortality, increased spontaneous abortion and stillbirth, gestational age,
preterm birth [81-83], as well as changes in fetal growth factors, including birthweight [5, 84-87] and
height and head circumference have been associated with *in utero* iAs exposure [86]. A recent
meta-analysis examined iAs exposure and the risk of spontaneous abortion, stillbirth, preterm
delivery, birthweight, and neonatal/infant mortality [88]. The authors of this study concluded
that there was an excess risk of 102% for spontaneous abortion, 84% for stillbirth, 51% for
neonatal mortality, 35% for infant mortality, and a 53-g reduction in birthweight from
prenatal iAs exposure [88].

Alterations in fetal growth factors from iAs has immediate and later in life impacts. In
a large prospective analysis maternal urinary iAs was associated with a 0.05 mm lower
newborn head circumference [85]. There is also evidence of changes in head circumference
before the 3rd trimester from studies of ultrasound measurements- notably this was in a sex-
specific manner [89]. These negative associations between iAs and head circumference may
also be independent of birth size [86]. The impacts of iAs on fetal growth may persist
throughout life and lead to an increase in susceptibility to other diseases. For example,
decreases in head circumference closely correlate to brain volume [90] and head circumference
at birth is associated with later intellectual function [91]. However, a few studies have not
found any associations with fetal growth, including a cohort in Taiwan [92] and a small study

of maternal-child pairs from Tokyo who had low iAs exposure [93]. Other fetal growth implications have been suggested from iAs exposure where there was a negative association between iAs exposure and Ponderal Index in children that were overweight/obese and this thought to be driven by longer birth length in boys, but by lower birthweight in girls- to which the authors suggest this potential mechanistic differences in adiposity at birth could influence later growth trajectories [86]. Their findings are also supported by a study of mother-infant pairs where prenatal exposure to iAs was associated with an inverse relationship between children's weight, height, and growth velocity at age 5 [94].

The exact mechanism by which iAs might lead to fetal growth is largely unknown, however a few studies have suggested some potential mechanisms. iAs increases oxidative stress [95] and inflammatory processes [63, 64], and given that these factors are associated with growth restriction [86], this could be a potential underlying mechanism. iAs-associated alterations in fetal growth could also have molecular underpinnings, either through epigenetic modification, [96] and/or altered transcription [97]. One study found increased expression of the iAs transporter AQP9 in placental tissue, and the authors suggests that this could cause a decrease expression of the adipose tissue-derived ENPP2, [98] a gene that regulates adipose tissue growth [99]. In addition, iAs could influence fetal growth through other mechanisms such as endocrine disruption [100]. Furthermore, iAs exposure may worsen factors during pregnancy that can contribute to low birthweight, including gestational age and maternal health [101]. For instance, prenatal iAs exposure is linked to increases in both nausea and vomiting, [84] which this may influence maternal weight gain during pregnancy [101] and contribute to poor maternal nutritional status [102]. Two studies have indicated that iAs may be mediated by gestational age [5, 87]. In fact, a recent study demonstrated through structural

equation modeling that prenatal iAs exposure was associated with decreased birthweight in a dose-dependent manner, and the effect was mediated by both gestational and maternal weight gain during pregnancy- with greatest effect being gestational age [87]. Additionally, differences in metabolism may contribute to the underlying mechanism of iAs associated fetal growth patterns, as previous work from our lab found that maternal metabolism of iAs was associated with alterations in fetal growth and pregnancy outcomes. Specifically, maternal urinary MMAs were negatively associated with birthweight and gestational age, and iAs was negatively associated with birth length and gestational age [5].

In addition to these many early life adverse health outcomes, prenatal iAs exposure, as well as exposure throughout life, is also associated with diseases later into adulthood.

Exposure to iAs and later in life diseases

Diseases from iAs exposure are vast, and the disease burdens from exposure may be mostly observed later in life. The International Agency for Research on Cancer (IARC) classifies arsenic as a Group I known human carcinogen. Additionally, several non-cancer effects have been observed from iAs exposure [1, 33, 103]. Furthermore, iAs exposure from drinking water has also been associated with an overall high mortality rate [104]. In fact, no bodily system is free from potential damage as iAs affects the integumentary, cardiovascular, renal, nervous, hepatic, endocrine, respiratory, immune, endocrine, and hematological systems [105].

Interestingly, the relationship between iAs and cancer was first identified via epidemiological studies, as animal models were inconclusive for inducing cancer from iAs exposure. Ecological studies in Taiwan have demonstrated that there is an increase in mortality from iAs exposure and internal cancers, including cancer of the lung, liver, bladder, and kidney [106]. An increase in mortality from iAs associated bladder, lung, and

kidney cancers has also been observed in Argentina and Chile [71, 72, 75]. Case-control and cross-sectional studies have also identified an association with exposure to iAs <500 ug/L and cancers, including total and cancer mortality, [107] bladder [108] skin cancer [56, 109], and skin lesions [54, 110]. From these and other studies, we now know that the excess cancer risk from lifetime iAs exposure at iAs drinking water concentrations greater than the WHO/U.S. EPA limit of 10 µg/L is approximately 1 in 300. For comparison, this risk is much higher than cancer risks estimates for exposures to other known carcinogens in drinking water at concentrations equal to current U.S. drinking-water standards [34]. The estimated dose response relationship between iAs exposure to cause a 1 % increased risk of lung, skin and bladder cancer is 0.3 and 8 µg/kg bw/day [111].

One of the first clinical manifestations of chronic iAs exposure is cutaneous lesions and alterations of cutaneous texture, thickness, or color of the skin around them and can occur within months or after several years of exposure- even after exposure has ceased. Specific skin diseases from exposure to iAs include, hyperpigmentation (melanosis), hyperkeratosis (keratosis), squamous cell carcinoma *in situ* (Bowen's disease), invasive squamous cell carcinoma, and basal cell cancer [4]. Epidemiological studies of skin lesions indicate that lesions can occur at levels less than 50 ug/L, however most reports of lesions are from exposure greater than 100 µg/L [112, 113]. Even though lesions are one of the main clinical manifestations of exposure, the vast majority of iAs exposed individuals will not develop lesions, but are still at risk of iAs-related skin and internal cancers and other non-cancer diseases [4, 104, 109]

The association between exposure to iAs and non-cancer endpoints in adults has been thoroughly reviewed elsewhere[4]. Here I will broadly discuss a few of these non-cancer

endpoints, including neurological disorders, respiratory diseases, immune dysfunction, cardiovascular disorders, and endocrine related disorders.

There are a few studies that have found associations between exposure to iAs and cognitive dysfunction. This includes learning and memory deficits and mood disorders, where exposures to iAs may result in memory loss and emotional instability neurological and cognitive dysfunction[114]. Specifically, peripheral neuropathy has been observed from those exposed to iAs contaminated ground water in West Bengal, India[115]. Additionally, long-term exposure, even at low levels has been associated with the development of Alzheimer disease and its associated disorders [116]. Also, there have been links to scoring lower on tests of cognitive ability and lower education levels in adults from iAs exposure [117].

Studies have suggested that exposure to iAs has impacts on lung function, acute respiratory tract infections, respiratory symptoms, and non-malignant lung disease mortality- particularly at high levels of exposure, as identified by a recent review and meta analysis that examined associations between iAs and respiratory health[118]. Specifically, iAs exposure is associated with poorer lung function (particularly forced vital capacity) and increased reports of coughing and breathing problems. For example, in Bangladesh impaired lung function and tuberculosis was observed with both low and moderate exposure to iAs [119] and iAs exposure was associated with chronic cough, blood in the sputum, and other breathing problems[120].

Many epidemiological studies have suggested that there is an increase in risk of both cardiovascular disease and cardiovascular associated mortality from drinking water that has elevated iAs levels. Specific cardiovascular effects include carotid atherosclerosis, [121] and ischemic heart disease[122-124] and hypertension[122, 125] coronary heart disease, and peripheral arterial heart disease[126]. Cardiovascular effects may also be increased in women during

pregnancy as a recent study found an increase in blood pressure over the course of pregnancy from concurrent iAs exposure[127]. Mechanisms that may underlie these iAs-associated cardiovascular health endpoints are increased inflammation, disruption of lipid metabolism, endothelial dysfunction[123, 128].

Additionally, there are several endocrine effects from iAs exposure, this is evident from both animal and epidemiological studies. These include, but are not limited to, effects on the endocrine system via disruptions in hormone regulation via the retinoic acid, thyroid hormone, and estrogen receptors[100, 112, 129, 130].

Interestingly, differences in disease susceptibility is attributed to many factors included dosimetry of iAs- where low vs high exposures may have a difference in disease etiology, genetics of the person exposed to iAs, nutrition, as mentioned previously- timing of exposure to iAs-where there is increased susceptibility during critical time periods of growth and development, and differences in metabolism of iAs.

Metabolism of iAs and disease

There has been recent evidence that an individual's ability to metabolize iAs efficiently may play a role in the development of many of the diseases discussed previously- in fact this may play a larger role in disease etiology than any other factor. Again, efficient metabolism is one's ability to convert iAs into MMAs and DMAs and inefficient metabolism is characterized by the proportions of these in various biomarkers and tissues.

For example, differences in methylation of iAs has been associated with the development of several adverse outcomes in humans including urinary bladder cancer, non-melanoma skin cancers, carotid atherosclerosis, and chromosomal aberrations (reviewed in[2]).

The differences in metabolism may also differ between the two-methylation steps for iAs reduction in the body. A recent study found specifically that %MMAs (and thus efficient

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metabolism), may be more important at lower exposure levels where at higher levels of exposure the risk of developing skin lesions did not depend heavily on the efficiency of the first methylation step[131].

Furthermore, pregnancy can be a period where metabolism efficiency of iAs is particularly important[53]. Inefficient metabolism of iAs has recently been associated decreases in birth and placental weight[5].

Factors that influence metabolism of inorganic arsenic

There are many factors that may influence methylation/metabolism of iAs including, but not limited to, the level of exposure to iAs in drinking water or food, age, gender, pregnancy, nutritional status of folate, homocystein, and protein, creatine, and genotype for arsenic 3 methyltransferase (*AS3MT*). While many of these associations have been established, the exact mechanisms remain unclear, and there have been very few replications of these associations across multiple populations. Additionally, even fewer investigations have been focused on the mediation of exposure disease relationships by factors that regulate metabolism of iAs.

Methylation of iAs is conducted by the enzyme arsenic (+3 oxidation state) methyltransferase (*AS3MT*). The importance of this enzyme has been indicated by a reduction in iAs methylation from gene silencing in cultured cells and in knockout mice. Additionally, genome wide association studies (GWAS) have indicated that single nucleotide polymorphisms (SNPs) in the *AS3MT* gene influence arsenic methylation capabilities[132-135]. Previous research in our lab also supports this mechanism during pregnancy where maternal alleles for five SNPs of *AS3MT* were associated with maternal urinary concentrations of iAs metabolites, and alleles for one SNP were associated with birth outcomes/measures [136]. Moreover, these associations may be dependent upon the male sex of the fetus but

independent of fetal genotype for *AS3MT*[136]. Perhaps the most strong and intriguing evidence for *AS3MT*'s role in metabolism of iAs comes from indigenous populations in northern Chile[137] and northern Argentina[138] where individuals are exposed to high levels of iAs drinking water, yet even at levels known to impair iAs metabolism have efficient arsenic metabolism[139]- this is because they have a very functional genotype for *AS3MT*. Furthermore, a recent study has suggested that there may be differences in *AS3MT* SNPs for different steps in metabolism, where certain SNPs are more important in the second step of metabolism of iAs as *AS3MT* binding affinity differs between methylation steps[131]. While genotype for *AS3MT* is important for metabolism of iAs, comparisons across studies indicate that majority of populations do not carry the very functional genotype and therefore most efficiencies in metabolism of iAs would not be observed based on an individual's genotype[136].

Other influences to iAs metabolism have been observed in epidemiological studies. For example, there are differences in metabolism by gender, where women had decreased levels of %iAs compared to men[140]. The influence of age with metabolism of iAs is not clear, levels of %iAs decreases with increasing age[141-143] but associations have also been observed with %iAs increasing with age [144]. Increases in BMI have also been associated with %iAs[142, 145, 146]. For percentages of MMAs, lower levels have been observed among females compared to males[141, 143, 145, 147] among those who never smoked[147], and with an increase in age[141] and with an increase in BMI[143, 144, 148, 149] however, lower levels have also been observed with decreasing age[144]. Higher levels of %DMA have been observed among females compared to males[143, 147], with an increase in age[141, 144, 145], with an increase in BMI[143, 145, 146], and among those who never smoked[145].

The levels of iAs in drinking water also influence the levels of metabolites in urine. It has been suggested, that the ability to methylate MMA to DMA is reduced with increasing exposures- though this is not very clear. These levels of iAs in drinking water may influence the second methylation step. Previous studies have found that this step is inhibited at increased exposure levels (particularly elevated levels of iAs^{III} and MMA^{III}) both in the experimental setting and in human observational studies[131]. A recent study suggests that metabolism of iAs and the development of disease may depend on drinking water exposure levels where efficiency of the methylation steps of iAs was effected by lower vs higher levels of iAs [131].

It has been suggested that the efficiency of metabolism during pregnancy, may be increased[61, 83, 139], however this is unclear and may depend on the levels of exposure to iAs. A recent study found that in the second trimester at high iAs exposures (≥ 50 µg/L), methylation was inhibited, to which the authors suggested that this could lead to unmethylated iAs and MMAs in fetus blood plasma, and this could threaten fetal survival and growth[131].

Nutrient status has the ability to modify iAs diseases and metabolism of iAs

Diet and nutrition may have a large influence on iAs metabolism and/or iAs-associated diseases. This is intriguing from a public health perspective in that it may provide a way to mitigate the health effects from iAs exposure.

Previous epidemiological research has indicated that intake of fruit and canned goods has been associated with a reduced risk of iAs-related skin lesions [150]. However, intake of bean and betel nut was associated with an increased risk of skin lesions [150]. The mechanisms behind these dietary associations is not established, however the authors of the study suggest that the positive effect of fruit might be due to the carotenoids and other nutrients from fruits,

and betel nut could alter lipid and protein metabolisms, which would increase the toxic impacts from iAs[150].

Animal studies of vitamin supplementation have demonstrated that there are alterations in iAs-associated mechanisms of disease. For example, when rats were exposed to iAs with folic acid (vitamin B9) or vitamin B12, or both folic acid and vitamin B12 there was reduction in oxidative damage, apoptosis, and downstream changes in hepatic mitochondria[151]. Additionally, treatment of iAs exposed rats with Ascorbic acid (vitamin C) has shown to improve mitochondrial functions [152]. The benefits of vitamin supplementation may also occur during the prenatal period, where supplementation with zinc and vitamins C and E had an impact on the reduction of some of the deleterious effects (cholesterol levels, TBARS levels and catalase activity) from iAs exposure in rats and pups during gestation and lactation[153, 154]. In addition, increased intakes of pyridoxine (Vitamin B6), thiamin (Vitamin B1), and Vitamins A, C and E could lower the number of skin lesions in individuals from iAs exposure [155]. A reduction in risk of iAs-related urothelial carcinoma has been observed with supplementation of Alpha-tocopherol (Vitamin E)[156].

There are a few potential ways that vitamins may affect iAs metabolism and toxicity. One is that vitamins as antioxidant can act as free radical to reduce oxidative damage induced by iAs. Also, many vitamins are known to be important cofactors in both S-adenosyl methionine (SAM) synthesis and one-carbon metabolism, where both play a key role in iAs methylation. This has been demonstrated in animal models where suboptimal intake of many vitamins and nutrients that are required for SAM synthesis, including methionine, choline, folate, and vitamin B12, or the knockout of the folate receptor, impaired iAs methylation and increased susceptibility to both carcinogenic and non- carcinogenic effects from iAs

exposure[7-9]. For example, Vitamin B12 plays an integral role in folate-dependent homocysteine metabolism as a rate-limiting co-factor in the conversion of homocysteine to methionine[10]. More specifics of folate and those co-factors are discussed below.

Folate is a source for the generation of endogenous methionine to add methyl groups in the methylation process- this process of adding a methyl group to many chemical compounds is involved in hundreds of essential chemical/biological reactions in the body. As is pertinent to iAs exposure and metabolism of iAs this is important for the reduction of iAs to MMAs and DMAs. This methylation process is driven by an enzymatic response by methyltransferases that use S-adenosyl methionine (SAM) as a methyl group donor. Methionine, as a source of methyl group in the methylation cycle, transfers a methyl group but only when it is activated by ATP to form SAM. Folic acid can increase methionine to promote iAs methylation. Notably, iAs may cause a reduction in SAM[157]. SAM, the methyl donor for As methylation, relies on folate-dependent one-carbon metabolism[158]. By increasing methylation of iAs to DMAs, folate aides in the elimination of As in urine, thus this may be an indication of a lower amount of iAs in the body [158]. While this process is well understood, there are still many factors that regulate and can alter these biochemical reactions in the body and more *in vivo* and *in vitro* experiments, as well as epidemiological studies are needed to further demonstrate the influence of folate and other folate-dependent vitamins on iAs metabolism and toxicity.

Interests in folate's role in the metabolism of iAs have been a focus of a few intervention studies- not only because of its inherit biological properties, but it is also an easy and affordable supplement to acquire and distribute. The importance for the role of folate in iAs metabolism has been supported by a recent RCT that examined folate supplementation in

adults exposed to iAs[11]. Interestingly, the researchers found that after 24 weeks, people

taking a 800 µg dose of folic acid, including the ones who switched to placebo halfway

through, had an average decline in arsenic levels of 12%, compared with 2% for those taking

placebo[11]. Furthermore, these findings were also strong in people who already had adequate

blood levels of folate, where a higher dose of folate lowered the levels of iAs [11]. These

findings could significantly influence the amelioration of the numerous disease burdens from

iAs exposure.

The impact of folate to reduce the toxic impacts of iAs during pregnancy is less clear.

There has been an association found between the levels of folate and homocysteine and

metabolism of iAs [159]. However, in an RCT targeting the use of nutrition supplementation to

increase metabolism in in pregnant women the results indicate that there was only a marginal

effect from nutritional and folic acid supplementation [160]. In this study participants were

randomized by a 2x3 factorial design where all pregnant women received 400 ug of folic acid

and others received either food supplements during their 8th gestational week or received

food supplementation by their own choice- these two groups were further supplemented with

the either 30 mg of iron (Fe), 60 mg Fe or 30 mg Fe + 13 micronutrients. The researchers

measured change in metabolism of iAs by gestational week and identified there was no

change in metabolism with supplementation[6]. However, they noted that other measurements

of micronutrients as well as participation in food supplementation may have impacted their

findings[6]. Furthermore, the participants in this study were highly nutrient deficient, including

foliate deficient and may not have had adequate folic acid supplementation to impact iAs

metabolism- additionally there was no control group in this study.

Additionally, many of the other micronutrients essential for one-carbon metabolism and potentially iAs metabolism were not fully measured in these studies. Of course, these measurements and classification of these interactions in human studies remains quite difficult, costly, and require higher volumes of biological samples. In particular, there is a strong interaction between folate, vitamins B12 and B6, choline, betaine, and methionine in the human methylation pathways[161, 162] and it has been suggested that the betaine-mediated remethylation of homocysteine may be particularly important in individuals with low folate intake [6]. Therefore further classifications of diet and nutrition as it relates to iAs exposure in these and other populations are needed. Homocysteine must be removed because it can act as an inhibitor for this SAM dependent process (via hydrolyzation of SAH). This removal of homocysteine can occur via three ways, the folate-dependent pathway, betaine-dependent pathway, or via transsulfuration. Thus, lower folate and B12 are associated with higher homocysteine and this can cause a decrease in iAs methylation. However, homocysteine can also effect arsenic methylation independently. Previous studies suggest the potential positive association between homocysteine and %MMAs and negative association with %DMAs is likely to reflect inhibition of the second methylation step[11].

Notably, both of the previous mentioned RCTs were in highly exposed iAs populations and there was no inclusion of health endpoints that may be altered from changes in iAs metabolism and mediated by nutritional status. It has been suggested that nutritional factors such as folate[158, 159] and protein intake [163] may modify the associations between arsenic and fetal growth- therefore further investigation is of this relationship warranted [86]. As nutrient status relates to the aims of this study, the role of folate during pregnancy serves multiple purposes for birth outcomes, and in particular birthweight.

The role of one carbon metabolism indicators in pregnancy and birthweight

Not only is folate an important factor for the metabolism of iAs, it also plays an important role in pregnancy and birth outcomes. Clearly, the maternal diet during the peri-conceptional period and later in pregnancy is an important factor for the development and subsequent health outcomes of the developing fetus[164]. Interests in the role of folate during pregnancy and supplementation with folic acid has been a massive public health effort in the U.S. and elsewhere, as women with insufficient folate are at increased risk of giving birth to infants with neural tube defects [165]. Additionally, lower maternal folate status has also been associated with low infant birthweight, preterm delivery, and fetal growth retardation[166].

For clarity, folic acid refers to the oxidized form of the vitamin found in fortified foods and in supplements, whereas folate is the reduced form of the vitamin naturally in foods and in biological tissues. The bioavailability of folate can depend on the form, where there is a higher availability in supplementation of folic acid and lower bioavailability in foods[167]. The CDC recommends that women take 400 μg of folic acid a day, including for women that are of reproductive age and/or planning to get pregnant[167]. Additionally, it is recommended that supplementation with higher levels of folic acid, 600 μg of a day during pregnancy and 500 μg a day during breast feeding[167].

Folate deficiency status is measured by concentrations in the plasma, serum, or red blood cells, Serum and plasma reflect recent dietary intake, with serum folate levels less than 6.8 nmol/L (3 μg/L) suggest that there is a deficiency[167]. For long-term concentrations red blood cells are most representative of tissue levels, however these concentrations are affected by vitamin B12 deficiencies. Another measurement used to determine folate status/metabolism is elevated plasma or serum total homocysteine as it is considered a functional and sensitive marker of both folate and vitamin B12 deficiencies[168]. For

homocysteine, an "elevated value" is most often set at 16 micromoles/L, although slightly lower values of 12 to 14 micromoles/L have been used[165].

This measurement of homocysteine may be important as folate and vitamin B12 have a synergistic relationship and their role in the conversion of homocysteine to methionine along with the role of B6 in the conversion of cystathionine, and during early fetal development may be influenced directly by elevated total homocysteine or through other downstream effects of folate impairments[167].

This interplay between prenatal folate, vitamin B12 and homocysteine has been established as important factor for birthweight. Folate is important for cellular functioning, in particular in the synthesis and repair of DNA and in gene expression, through the process of DNA methylation [169]. Elevated total serum homocysteine have been associated with, placental vasculopathy[170, 171] which influences fetal growth [172]. Additionally, limited supply of vitamin B12 during pregnancy may have an impact on fetal growth. Specifically, vitamin B12 is known to be a significant predictor of tHcy levels in neonates, with relatively greater importance in the first few months of life, before folate assumes primacy as the predictor of tHcy[173].

Previous researchers have established the association between maternal folate status and infant birthweight. One study found a twofold greater risk for low birthweight for infants whose mother's had a lower mean daily folate intake (<240 μg/d) at 28 weeks gestation[166]. A positive association between maternal folate intake infant birthweight was found from maternal folate levels at weeks 18 and 30 gestation[174] Additionally, low serum folate levels at 30 weeks gestation and a an increase in fetal growth restriction has been observed[175]. Furthermore, maternal deficiencies in folate is also associated with intrauterine growth

restriction[166]. Such studies suggest a role for maternal folate in the determination of infant birth weight. Moreover, several studies have explored the relationship between tHcy and birth weight, with mixed findings [176-178]. It remains unclear whether tHcy is merely a biomarker of folate status or is deleterious in itself. Three intervention studies have shown a significant increase in birth weight associated with the use of prenatal multivitamin supplements, which included folate, whilst three other studies have failed to show an effect[179]. Clearly, the exact mechanism behind the relationship between maternal folate status and birthweight remains to be determined.

Current efforts in preventing exposure to iAs and altering metabolism of iAs

Reducing exposure to iAs from contaminated drinking water and preventing the associated health effects from iAs exposures is a multifactorial problem. Exposure levels, metabolism of, and susceptibility to iAs can be highly individualized. For example, there are many differences in exposure levels of iAs in drinking water across the globe, where the levels in drinking water may be based on proximity to naturally occurring iAs in bedrocks and sediments and/or proximity to hazardous waste sites. Therefore, drinking-water sources with both low and high iAs concentrations can exist in very close proximity to one another. Additionally, an individual's differences in susceptibilities to the toxic effects from differences in metabolism of iAs is highly individualized- as previously discussed. Furthermore, as stated previously, susceptible populations such as pregnant women and children may be more significantly impacted by exposure to iAs than others and have a higher risk for adverse health outcomes. Current regulation of iAs in drinking water is based only on the levels of iAs in drinking water, where the EPA and WHO standard is set to not exceed 10ppb. While top down regulatory action to reduce exposure to iAs can be effective

there are many obstacles that need to be assessed in risk assessment and future regulatory actions to prevent iAs-associated diseases.

Mitigation efforts in areas where there has been high exposure to iAs have included pond sand filters, home-based "3-pitcher" filters, rainwater harvesting, digging deeper wells, and switching drinking water sources[180-183]. In an area that has experienced and is still experiencing perhaps the most widespread exposure to iAs contaminated drinking in Bangladesh there have been many mitigation efforts to prevent exposure to iAs and iAs-associated diseases. For example, wells that contained more than 50 µg/L of iAs were painted red, and residents were encouraged to collect their drinking water from nearby green painted wells that had lower concentrations of iAs. Other strategies that have been put into place across the globe are suggesting that individuals switch to bottled water for drinking and cooking, installing treatment systems that focus on a single area of water use, such as a kitchen sink (commonly referred to as point-of-use or POU), and treatment systems that treat all the water entering the home (commonly referred to as point-of-entry or POE) [184]. Bottled water and POU treatments have been most commonly used in many areas, exemplified from a survey of central Maine residents with well water iAs levels above 10 µg/L, where more than 65% of respondents indicated they were using either bottled water or a POU treatment system to reduce exposure[185]. Interestingly, these two intervention strategies remained the most common methods even for households with higher iAs levels in drinking water (above 100 µg/L)[185].

However studies have revealed that many of the current strategies in prevention are not that effective in reducing exposure to or the levels of iAs found in human biomarkers[184]. This is due to the many limitations to completely removing exposure to iAs including, the

high cost of water filters and their monitoring and maintenance, the limited effectiveness of water filters in real-world settings (e.g adherence), and the limited availability of low iAs wells in some regions [11]. Additionally, long-term chronic exposure to iAs may lead to an increased body burden of As that persists long after exposure is removed.[11] For example, after a bottled water intervention in Arizona there was only a modest reduction (21%) in urinary iAs levels observed [186]. For bottled water and POU treatment strategies, effective iAs exposure reduction is also highly dependent on behavioral factors such as willingness to use only treated water or bottled water for beverage and food preparation, as well as for drinking. Of importance, even the occasional use of untreated water for beverage or food preparation after switching to bottled water or installing a POU treatment system could still lead to dangerous exposures to iAs, especially if water iAs levels are high- this exposure may also result from bathing-related contact with untreated water[187].

A recent cross-sectional study in the U.S. examined the effectiveness of various mitigation strategies for iAs exposure from private wells[184]. Shockingly, they found evidence of water-related exposure among families that relied on POU treatment systems or that supplemented water with bottled water, where even occasional consumption of untreated water was significantly associated with higher urinary iAs levels among private well owners who have elevated iAs in well water. This relationship was particularly true for young children, and to which the authors note that children's ability or willingness to refrain from ingestion of untreated water may be less consistent and enforceable. These findings support that compliance with using POU systems or with bottled water interventions is not effective for reducing iAs exposure[184].

Taken together, these studies indicate that the primary focus of removing the source of exposure to iAs (via drinking water) may present several issues and limit the ability to eradicate iAs-associated diseases. There is a need to investigate the potential for providing nutritional supplementation, as it may be a low-risk and affordable treatment option for those who are at risk for iAs-associated diseases including those currently and/or those who have had past exposure. The ability to investigate this relationship outside of expensive clinical trials has been a limitation, however the research suggested here could fill this limitation and research gap.

Application of mediation analysis

Knowing if an exposure or treatment causes an adverse health outcome within the same individual would be the ultimate study design for an epidemiologist, which is why the randomized trial is considered the gold standard for many epidemiological studies. However, with many exposures and specifically environmental exposures or treatments it is not possible to randomize or expose individuals based on feasibility, moral, costs, or other reasons. Additionally, knowing if an intervention could prevent the number of cases/diseases before the initiation of an intervention or trial would allow for the assessment of many more potential public health intervention scenarios before the initiation of costly RCTs or potential ineffective interventions. To this end, with the development of and continual updates in causal inference methods, causality can be better established (as long as certain conditions are met) and these potential interventions can be modeled. The idea behind an experiment is that when one is looking at an exposure disease relationship you can essentially ask the question, what if you could go back in time and change the exposure…would this alter the outcome?

Currently, many epidemiological studies measure associations of exposures/disease relationships and associations instead of assessing causality. To clarify, an "association" of an exposure/disease relationship is determined by a difference in an effect measure from two individuals in a population, where the individual's actual exposure/treatment is either that of exposed or unexposed, whereas causation is the difference in an effect measure in the entire population under two different treatment/exposure values[188]. While this is a good and appropriate approach for many disease/exposure relationships and epidemiological questions, there may be reasons to delve further into causality, and in particular to the topic here provide information that may allow for direct public health change across populations.

Recently, there has been much development in causal inference methods to address the potential of modeling interventions and applying these to environmental health studies. As this relates to mediation analysis, causal inference methods can greatly improve many of the current limitations by traditional mediation analyses. The question addressed for mediation is what if the effect of an exposure (A) on outcome (Y) is carried out through an intermediate variable (M) A $\rightarrow$ M $\rightarrow$ Y. The application of mediation analyses can serve to: (1) Understand the causal mechanism between an exposure, disease and an intermediate variable; (2) Confirm or refute a theory; (3) Refine interventions; (4) Intervene on the mediator when we cannot intervene on an exposure (perhaps due to costs) or target subgroups most at risk (in resource limited intervention); (5) Jointly test an effect can possible identify exposure/disease responses whereas testing an effect alone may fail to identify such a relationship. Specific approaches to determine mediation go beyond classic regression approaches, which are limited for application to nonlinear relationships and models of interactions; however, standard approaches are appropriate in certain

applications.[189] Instead, I suggest the use of causal/counterfactual framework. New advances in causal mediation analysis include using g-computation (the g-formula) and have been recently applied to mediation analyses.[189] These methods allow for the identification and decomposition both the total effect (i.e. the external exposure, mediator, and outcome relationship) direct (i.e. external exposure to outcome) and the indirect effects (i.e. the relationship between an exposure, a causal intermediate). Using a causal framework allows for nonlinear models and interactive effects, while addressing the potential bias arising from overly restrictive statistical analyses and suboptimal study design[190]. Furthermore, by examining the controlled direct effects, mediators can be set or fixed to levels of interest across the entire sample. However, the application of these methods for both environmental and reproductive epidemiological research is lacking.

Causal mediation analysis in detail has been described fully by Vanderweele and others[189]. Specific to this thesis work, methods include those described by Valeri and Vanderweele [191]. Where the following is denoted: a reference (baseline) level of exposure, a0 and a new level, a1; let m denote the mediator, for both a natural and a fixed level. Under this framework investigators can measure: the Natural direct effect (NDE), which can be interpreted as how much the outcome of would change if the exposure was set to a new level of exposure (a1) but the mediator (m) is kept at a level it would have taken in absence of the exposure; the Natural indirect effect (NIE) which can be interpreted as how much the outcome would change on average if the exposure were controlled at level a=1 but the mediator were changed from the level they would've taken if a=0 to the level it would've taken if a=1; the total effect (TE) which estimates how much the outcome would change overall for a change in the exposure from level a=0 to a=1; the controlled direct effect (CDE)

which estimates how much the outcome would change on average if the mediator were controlled at fixed level (assigned), and the percent proportion mediated (PM) can be calculated by the following formula: NIE/TE x 100[189].

Importantly, in the application of these methods there are strong assumptions for interpreting these findings within the causal framework. Specifically, the estimation of direct and indirect effects, and subsequent causal interpretations require that there be no unmeasured exposure-outcome, mediator-outcome, or exposure-mediator confounders[192]. Additionally, when modeling controlled direct effects if unmeasured or uncontrolled for confounders of the mediator- outcome relationship occur this can lead to biased estimates[193].

Overall study design and methods

Source of iAs in the study area

In Mexico, it is estimated that more than 450,000 people may be exposed to levels of iAs in drinking water that exceed the MCL of 25 µg/L[194]. Levels of iAs from central-eastern regions of Mexico have been found up to 1,504 µg As/L[195] and up to 215 µg/L[196] in Hidalgo and Zimapán, respectively. Reports of iAs-contaminated ground water and wells in the Lagunera region in the states of Durango and Coahuila, Mexico in the central part of North Mexico began as early as the 1970s. In the early 90's, Del Razo et al., found that in the Lagunera Region more than 50% of samples had iAs concentrations greater than 50 µg/L, which at the time was the MCL of the World Health Organization[197, 198]. Additionally, studies from the region of Lagunera found that individuals who were drinking well-water had an average exposure of 400 µg/L of iAs and had an average of 560 µg/L of U-tAs in their urine (range 320–1560) of iAs[198].

Previous researchers have concluded that drinking water is the main source of iAs exposure in this area[196, 198-200]. Additionally, a study in the region that investigated potential

iAs-exposure from food sources in this area found that the primary increase in iAs from foods was dependent on the amount of water used and cooking time, as most foods were boiled or cooked with water before consumption. Additionally, the authors suggested that iAs contamination from seafood is not a large problem in this area, as marine foods are not usually consumed in this area[201].

The geological and climatic properties of these areas make it prime for iAs contamination as they are mostly that of sedimentary basins and are inactive volcanic regions. For a long time the cause of iAs-contaminated drinking water was controversial, however, recent research has suggested that iAs present in groundwater in the Lagunera Region is ultimately a by product of water scarcity in the area and aquifer depletion. The depletion of aquifers is due to the damming and diversion of the area's rivers and a simultaneous overdraft of groundwater[202]. Additionally the area is plagued by a lack of rainfall, as Lagunera Region is also one of the driest in Mexico, with an average annual rainfall of 260mm,[202] and that most of the extracted ground water is used to grow crops for grazing cattle[202]. All of this combined can lead to a higher demand from other sources of water- and in this instance ones that may be contaminated by iAs.

Study population

This study uses data and samples from the Biomarkers of Exposure to ARsenic (BEAR), a prospective pregnancy cohort that was establish in Gómez Palacio, Mexico, to better understand the effects of iAs exposure on pregnant women and their children. BEAR participants, adult women, were recruited during the time frame of August 2011 to March 2012 at the General Hospital of Gómez Palacio. Recruitment took place prior to the time of delivery, which for most women was within 24 hours of birth. All procedures associated with this study were approved by the Institutional Review Boards of Universidad Juarez del

Estado de Durango (UJED), Gómez Palacio, Durango, Mexico, and the University of North Carolina at Chapel Hill (UNC), Chapel Hill, North Carolina, U.S.A. For each woman participation requirements at the time of recruitment included: (i) one year minimum residence in the Gómez Palacio region, which included urban locations of Gómez Palacio and surrounding rural locations, (ii) confirmation of a pregnancy without complications such as eclampsia or preeclampsia, and (iii) good overall health status (i.e. no signs of chronic or acute disease). A total of 221 women were approached for the study. Of those, 93% (n=206) consented to participate in the study. Six women were not included in the study as a result of confirmation of a twin pregnancy (n=1; 0.5%) or sample collection failure (n=5; 2.4%). The final cohort consists of 200 mom/baby pairs.

A social worker administered questionnaires to the study participants to collect the following information: age, education, occupation, time living at residence, smoking status and alcoholic beverage consumption during pregnancy (both defined as yes or no and frequency), daily prenatal supplement intake (yes or no), residence location (urban or rural), seafood consumption (yes or no), source and daily consumption of drinking and cooking water, and source of bathing water. In addition, information on previous pregnancy outcomes including number of pregnancies and number of previous pregnancy losses was gathered from questionnaires. Information on birth outcomes/measures including newborn birth weight, newborn length, gestational age, head circumference, placental weight, and 5-min Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score was gathered at time of delivery by the physician[203]. Data on adverse outcomes were collected, including preterm birth (gestational age < 37 weeks), low birth weight (LBW; < 2,500 g), small for gestational age (SGA; birth weight < 10th percentile), and large for gestational age (LGA; birth weight >

90th percentile). SGA and LGA categories were based on newborn data collected from northern regions of Mexico[204, 205].

Biospecimens collected around the time of delivery included: maternal spot urine samples, maternal serum, and cord serum, as well as blood samples from both where DNA and RNA was later extracted. All samples were immediately stored at -80°C. The concentrations of iAs in drinking water (micrograms As/L; DW-iAs) were measured at UJED, Mexico, using hydride generation–atomic absorption spectrometry (HG-AAS) supported by a FIAS-100. The Trace Elements in Water standard reference material (SRM 1643e) (National Institute of Standards and Technology, Gaithersburg, MD) was used for quality control. The limit of detection (LOD) for iAs in drinking water by HG-AAS was 0.46 µg As/L. All urine analyses were conducted at UNC-Chapel Hill. Concentrations of urinary biomarkers of exposure: U-iAs, U-MMAs, and U-DMAs were determined by HG-AAS with cryotrapping[206]. Five-point calibration curves were prepared using pentavalent iAs, MMAs, DMAs standards (> 98% pure) as described previously,[206] and the SRM 2669 Arsenic Species in Frozen Human Urine (National Institute of Standards and Technology) was used for quality control [207]. The LODs for U-iAs, U-MMAs, and U-DMAs were 0.2, 0.1, and 0.1 µg As/L, respectively. The specific gravity (SG) of each urine sample was measured using a handheld refractometer (Reichert TX 400 #13740000; Reichert Inc., Depew, NY). To account for differences in water intake/differential hydration, concentrations of U-iAs, U-MMAs, and U-DMAs in each urine sample were adjusted by the specific gravity. Maternal folate and homocysteine levels was measured in maternal serum.

Concentrations of iAs in drinking water (DW-iAs) ranged from below the LOD (0.46 µg As/L) to 236.0 µg As/L (mean, 24.6 µg As/L). More than half of the women (53%) had

DW-iAs that exceeded the World Health Organization's recommended guideline of 10 µg As/L. Maternal urinary concentrations of specific gravity adjusted total urinary arsenic (U-tAs) ranged from 4.3 to 319.7 µg As/L (mean, 37.5 µg As/L). These levels of U-tAs in the BEAR cohort are generally lower than levels observed in pregnant women in Bangladesh (SG-adjusted medians, 23.3 µg As/L vs. 80 µg As/L)[160] However, the levels of U-tAs in this cohort are higher than levels observed in U.S. women (SG-adjusted mean, 37.5 µg As/L vs. creatinine adjusted geometric mean, 7.3 µg As/L)[208].

Given that an individual's efficiency of iAs biotransformation/metabolism known to influence the susceptibility to iAs-associated diseases this was investigated. The mean urinary concentrations of individual SG-adjusted arsenicals were 2.1, 2.3, and 33.1 µg As/L for iAs, monomethylated arsenicals (MMAs), and dimethylated arsenicals (DMAs) respectively. This corresponds to the proportions of U-tAs comprising 6.1%, 6.4%, and 87.6% for U-iAs, U-MMAs, and U-DMAs respectively. DW-iAs was significantly associated with the sum of the urinary arsenicals (U-tAs) (r=0.51, p<0.0001). Maternal urinary concentrations of MMAs were negatively associated with newborn birth weight and gestational age. Maternal urinary concentrations of iAs were associated with lower mean gestational age and newborn length. The data support a relationship between iAs metabolism in pregnant women and adverse birth outcomes.

Previous findings in the BEAR cohort

A sub set of samples from the cohort were prioritized for molecular/mechanistic investigations, including assessment of iAs-associated microRNA expression[209], DNA methylation,[210] gene expression,[209, 210] and protein expression[211] in association with prenatal iAs exposure

MicroRNA (miRNA) expression changes associated with in utero arsenic exposure were assessed from cord blood samples (n = 40)[209]. Genome-wide miRNA expression analysis of cord blood revealed 12 miRNAs with increasing expression associated with U-tAs. Transcriptional targets of the miRNAs were computationally predicted and subsequently assessed using transcriptional profiling. Pathway analysis demonstrated that the U-tAs-associated miRNAs are involved in signaling pathways related to known health outcomes of iAs exposure including cancer and diabetes mellitus. Immune response-related mRNAs were also identified with decreased expression levels associated with U-tAs, and predicted to be mediated in part by the arsenic-responsive miRNAs. These results highlight miRNAs as novel responders to prenatal arsenic exposure that may contribute to associated immune response perturbations.

Epigenetic alterations via DNA methylation and alterations in gene expression were assessed from cord blood leukocyte samples (n=38)[210]. Changes in iAs-associated DNA 5-methyl cytosine methylation were assessed across 424,935 CpG sites representing 18,761 genes and were compared to corresponding expression levels and birth outcomes. In the context of arsenic exposure, a total of 2,705 genes were identified with iAs-associated differences in DNA methylation. Site-specific analyses identified DNA methylation changes that were most predictive of gene expression levels. Specifically, CpG methylation within CpG islands positioned within the first exon and 200bp upstream of the transcription start site yielded the most significant association with gene expression levels. A set of 16 genes was identified with correlated iAs-associated changes in DNA methylation and mRNA expression and all were highly enriched for binding sites of the early growth response (EGR) and CCCTC-binding factor (CTCF) transcription factors. Furthermore, DNA methylation levels

of seven of these genes were associated with differences in birth outcomes including gestational age, placental weight and head circumference. These data highlight the complex interplay between DNA methylation and functional changes in gene expression and health outcomes and underscore the need for functional analyses coupled to epigenetic assessments.

Additionally, protein expression was assessed from the cord blood from 50 of the newborns via a high throughput, antibody-based method[211]. A total of 111 proteins were identified that had a significant association between protein level in newborn cord blood and maternal U-tAs. Many of these proteins are regulated by tumor necrosis factor and are enriched in functionality related to immune/inflammatory response and cellular development/proliferation. Interindividual differences in proteomic response were observed in which 30 newborns were "activators," displaying a positive relationship between protein expression and maternal U-tAs. For 20 "repressor" newborns, a negative relationship between protein expression level and maternal U-tAs was observed. The activator/repressor status was significantly associated with maternal U-tAs and head circumference in newborn males. These results may provide a critical groundwork for understanding the diverse health effects associated with prenatal arsenic exposure and highlight interindividual responses to arsenic that likely influence differential susceptibility to adverse health outcomes.

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CHAPTER IV: MATERNAL ONE CARBON METABOLISM AND ARSENIC METHYLATION IN A PREGNANCY COHORT IN MEXICO[1]

Overview

The prenatal period represents a critical window of susceptibility to arsenic (As) exposure. Ingested inorganic arsenic (iAs) undergoes hepatic methylation generating mono- and di-methyl arsenicals (MMA and DMA, respectively) a process that facilitates urinary As elimination. Differences in pregnant women's metabolism of As (e.g. increases in %MMAs and decreases in %DMAs) are a risk factor for adverse birth outcomes. One carbon metabolism (OCM), the nutritionally-regulated pathway essential for supplying methyl groups in the body, plays a role in As metabolism and is understudied during the prenatal period. In this cross-sectional study from the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort in Gómez Palacio, Mexico, we assessed the relationships among OCM indicators (e.g. maternal biomarkers of serum B12, folate, and homocysteine (Hcys)), and levels of iAs and its metabolites in maternal urine and in neonatal serum. Interestingly, the prevalence of folate sufficiency (serum folate levels > 9 nmol/L) in the cohort was high 99%, and hyperhomocysteinemia (Hcys levels >10.4 μmol/L) was low (8%). However, 74% of the women displayed a deficiency in B12 (serum levels < 148 pmol/L). B12 deficiency was not associated with As methylation in pregnant women. Differences in lower B12 levels and higher Hcys were associated with increases in total arsenic levels in urine (U-tAs). In

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unadjusted comparisons, infants born to mothers in the lowest tertile of serum folate had significantly higher mean levels of C-%MMA relative to folate replete women. Furthermore, beta regression results demonstrated that maternal Hcys was positively associated with both C-tAs and %C-MMAs. The results from this study indicate that maternal OCM status may influence neonatal As metabolites.

Introduction

Inorganic arsenic (iAs) continues to contaminate the drinking water of millions of individuals world-wide, representing a critical global public health issue[1]. The main form of arsenic (As) present in drinking water is iAs, and it undergoes methylation to generate monomethyl- arsenic (MMAs) and dimethyl arsenicals (DMAs). The toxicity of iAs in humans is influenced by an individual's methylation capacity. Increases in the proportions of MMAs in urine have been associated with adverse health outcomes, including urinary bladder cancer, non-melanoma skin cancers, carotid atherosclerosis, and chromosomal aberrations (reviewed in [2]). The role of metabolism of As in disease etiology may be particularly important during the prenatal period, as it is a critical window of susceptibility to iAs exposure. Prenatal iAs exposure has been associated with lower birth weight, preterm birth, reduced height and head circumference, increased susceptibility to infection, and later in life cancers[3]. Additionally, inefficient maternal metabolism of As during pregnancy has been recently associated with decreases in birthweight and other birth outcomes[4-6].

There are many factors that may influence methylation/metabolism of As including, but not limited to, the level of exposure to iAs via drinking water and/or food[7], age[8], gender[9], pregnancy[10], genotype for arsenic (+3 oxidation state) methyltransferase (*AS3MT*)[7, 11], and nutritional status of an individual[12, 13]. The methyl group for methylation of As is derived from S-adenosylmethionine (SAM). The synthesis of SAM is influenced by micronutrients

involved in OCM, such as such as folate, cobalamin (vitamin B12), vitamin B6, betaine and choline[14]. When OCM is compromised, Hcys levels become elevated. This can be reversed through a folate-dependent mechanism where 5-methyltetrahydrofolate donates its methyl group, using B12 as a cofactor, for the remethylation of homocysteine (Hcys) to generate methionine[15]. Various biomarkers are used to measure OCM including, but not limited to folate, B12, and Hcys in human samples[14].

There is support for the supplementation of OCM nutrients and/or vitamins in iAs exposed populations to increase methylation of As. For example, folic acid supplementation to improve As methylation in adults exposed to iAs in contaminated drinking water is supported by a recent randomized control trial (RCT)[13]. However, the role of OCM nutrients in improving As metabolism during pregnancy is less clear. In a cross-sectional study, levels of folate and Hcys assessed in pregnancy did influence metabolism of As[16]; however, in another study focused on general nutritional supplementation to increase As metabolism there was a significant, but marginal, effect on the changes in metabolism of iAs over the course of pregnancy in relation to folate levels[17]. Furthermore, as these previous studies were conducted in nutrient poor populations, the role of indicators for OCM in other populations sufficient in both OCM and other nutrients is warranted. This may be particularly important for pregnant women with higher folic acid intake from prenatal vitamins and fortification of foods. Additionally, the interactions between OCM nutrients with each other during pregnancy may be important risk factors for adverse birth and later in life health outcomes. Specifically, imbalances in maternal vitamin B-12 status as it relates to sufficient and/or higher folate status during pregnancy may have detrimental health impacts for both the mother and her infant (as reviewed in [18]). Additionally, in folate sufficient populations, low

B12 becomes a stronger predictor of higher Hcys, a known independent risk factor for numerous adverse pregnancy and health outcomes[19]. Taken together, there is a need to further characterize the relationship between nutrients involved in OCM and As metabolism during pregnancy.

The primary aim of this cross-sectional study within the Biomarkers of Exposure to ARsenic (BEAR) cohort in Gómez Palacio, Mexico was to examine the levels of maternal serum indicators of OCM (B12, folate, Hcys) in association with biomarkers of iAs exposure and metabolism of iAs during the prenatal period. We hypothesized that OCM indicators of higher B12 and folate, and lower Hcys would be associated with decreases in %iAs and %MMAs, and increases in %DMAs in both maternal and neonatal samples.

Materials and methods

Cohort selection

The BEAR cohort was established to investigate the underlying etiologies of prenatal iAs exposure. This cohort has been previously described.[4] Briefly, women were recruited prior to the time of delivery from the time frame of August 2011 to March 2012 at the General Hospital of Gómez Palacio. All procedures associated with this study were approved by the Institutional Review Boards of Universidad Juárez del Estado de Durango (UJED), Gómez Palacio, Durango, Mexico, and the University of North Carolina at Chapel Hill (UNC), Chapel Hill, North Carolina, U.S.A.

A total of 221 women were originally approached for the study. Of those, 93% ($n = 206$) provided informed consent for participation in the study. Of these, two women were not included based on a confirmation of a twin pregnancy ($n = 1$; 0.5%), or sample collection failure ($n = 1$; 0.5%). Additionally, for this cross-sectional analysis there were six women who were excluded because there was insufficient serum to measure the OCM indicators.

Therefore in the present study to investigate the nutritional biomarkers involved in one carbon metabolism as it relates to maternal metabolism of iAs there are a total of 197 women. For the assessment of neonatal exposure to iAs there are 188 mom/baby pairs, for there was not enough cord serum to measure the levels of As in samples from nine subjects.

Determination of exposure to iAs

Maternal spot urine samples were collected at the hospital before birth, immediately transferred to cryovials, and placed in liquid nitrogen. Aliquots of urine samples were shipped on dry ice to UNC-Chapel Hill and immediately stored at $-80°C$. Cord blood was collected immediately after newborn delivery using an anticoagulant-free vacutainer tube. Following clot formation, the tube was centrifuged at 177.1 g-force and the serum was collected and stored at -80°C until aliquots were shipped on dry ice to UNC-Chapel Hill, NC, and stored at -80ºC.

Arsenical levels in maternal urine have been used as indicators of prenatal iAs exposure. Here, concentrations of urinary iAs (U-iAs), urinary monomethyl arsenic (U-MMAs) and dimethyl arsenical (U-DMAs) were determined by HG-AAS with cryotrapping (CT) as described previously[20-22]. The LODs for U-iAs, U-MMAs, and U-DMAs were 0.2, 0.1, and 0.1 μg As/L, respectively. U-tAs was determined by summing U-iAs, U-MMAs and U-DMAs. To account for differences in water intake/differential hydration, concentrations of U-iAs, U-MMAs, and U-DMAs in each urine sample were adjusted as previously described[23]. The efficiency of iAs biotransformation was determined by calculating the proportions of the individual arsenicals iAs, MMAs, and DMAs relative to total urinary As for each study subject. Metabolism efficiency for iAs in maternal urine was determined by calculating the percentage of the individual metabolites out of the total (%U-iAs, (%U-MMAs, and (%U-DMAs).

Levels of arsenic in whole cord blood have been used before as a measurement of potential neonatal exposure[24]; however, here we use cord serum values to measure potential neonatal exposure to iAs and As metabolites. Concentrations of neonatal cord serum arsenic included the measurement of cord serum-analyzed iAs (C-iAs), MMAs (C-MMAs), and DMAs (C-DMAs) and were determined using HG-CT-ICP-MS as described previously[21, 25]. The LODs for C-iAs, C-MMAs, and C-DMAs were 1.45, 0.06, and 0.12 pg As/mL, respectively. A certified standard reference material, Arsenic Species in Frozen Human Urine (SRM 2669; National Institute of Standards and Technology) was used to assure accuracy of arsenic (As) speciation analysis. Here, aliquots of SRM were diluted in urine or serum from an unexposed subject and analyzed by HG-CT-AAS and HG-CT-ICP-MS, respectively. The concentrations of As species in SRM in urine (after deduction of background As) ranged from 88% to 105% of the certified values (105% for iAs, 88% for MMAs, 99% for DMAs); the concentrations of As species in SRM in plasma ranged from 85% to 90% of the certified value (85% for iAs, 90% for MAs, and 87% for DMAs. The sum of C-iAs, C-MMAs and C-DMAs was calculated to determine C-tAs. Neonatal cord exposure was also calculated based on the percentage of the individual metabolites in neonatal cord serum out of the total As in neonatal cord serum (%C-iAs, %C-MMAs, and %C-DMAs).

Determination of one-carbon metabolism indicators

Measurements of the indicators for OCM were carried out on banked maternal serum stored at -80°, and shipped on dry ice to Columbia University, New York, NY. Maternal serum folate and B12 were analyzed by radio protein-binding assay (SimulTRAC-S; MP Biomedicals, Orangeburg, NY, USA). To determine folate concentrations, folic acid as pteroylglutamic acid was used for calibration, and its 125 I-labeled analog as the tracer. All samples were run on a Gamma counter (Perkin Elmer). Cys and Hcys were analyzed via a

HPLC - fluorescent detector with 385nm excitation and 515nm emission[26]. OCM indicators deficiencies and/or sufficiency's were determined for each woman as has previously been described[27], and as in other iAs-exposed populations[28]. B12 deficiency was set at <150 pmol and ≥ 150 pmol for B12 sufficiency. Folate deficiency was set at <9 nmol and ≥ 9 nmol for folate sufficiency. Hyperhomocysteinemia (high Hcys) was defined by Hcys ≥ 10.4 nmol and normal Hcys was set at < 10.4 nmol.

Statistical analyses

Spearman rank correlations (coefficients reported as r_s) were calculated between the levels of arsenicals in both maternal urine and in neonatal cord serum. Spearman rank correlations were also calculated between the levels of arsenicals in both maternal urine and in neonatal cord serum and the OCM indicators, as well as for the individual OCM indicators to each other.

Differences in the levels of arsenicals based on nutritional categories were calculated to compare those that were B12 sufficient (≥150 pmol) and deficient (<150 pmol), who had higher Hcys (hyperhomocysteinemia as defined in non-pregnant populations) (>10.4 nmol) and normal (<10.4 nmol) levels of Hcys using Wilcoxon Rank Sum tests, and between tertiles of folate for those who were in the lowest tertile (< 29.0 nmol), middle tertile (≥ 29.0 nmol and < 47.0 nmol) and the upper tertile (≥ 47.0 nmol) using an ANVOA. The same comparisons for the individual OCM indicators by nutritional categories were calculated. These comparisons were not made for those who were deficient/sufficient in folate as there were not enough women who were deficient in folate for categorical comparisons.

The relationship between the maternal biomarkers of B12, folate, Cys, and Hcys and maternal U-tAs and/or neonatal C-tAs was investigated via linear regression models. To improve model fit the independent variables (OCM indicators) and dependent variables (U-

tAs, C-tAs) were log transformed, model fit was assessed by inspecting the normality of residuals. Regression assumptions of linearity and the homogeneity of residuals were evaluated by examination of appropriate residual plots. Beta regression models were used to examine the relationship between the percentages of iAs, MMAs, and DMAs and OCM indicators using a previously establish SAS macro[29] These models were chosen because percentages of As metabolites are used an indication of metabolism efficiency, yet percentages can be problematic as dependent variables in traditional linear regression models because the outcome is only observed over the unit interval (i.e., 0,1). Beta regression modeling has been previously used for percentage-scaled dependent variables due as it is flexible for capturing various skewed unimodal and bimodal distributions, especially when normalizing and other transformations do not work well[29]. Beta regression models were also fitted to investigate the relationship between the arsenicals and nutritional categories based on B12 deficiency, folate tertiles, and for categories of Hcys. Regression coefficients were converted to a percentage and results are presented as the estimated percent difference in the log odds of %iAs, %MMA, and/or %DMAs with a one unit difference in B-12, folate, and/or Hcys. Potential confounders for all models were identified *a priori* based on their known or potential association with both the exposure (the individual OCM indicators) and the outcome (As or its methylated metabolites). To determine if maternal OCM indicators predicated maternal U-tAs and/or metabolism and neonatal levels of As all models were adjusted for maternal age (linear continuous), maternal education (categorical), maternal parity (categorical) and gestational age (linear continuous).

Results

Demographic characteristics

Selected characteristics of the BEAR cohort are presented in Table 4.1. The cohort has been previously described[4]. Briefly, all women were Hispanic and were an average age of 24 years at the time of delivery. Most of the women in this cohort had an educational level of high school or greater (74%). Almost all the women reported to take prenatal vitamins during pregnancy (96%), and for the majority of the women this was not their first pregnancy (65%).

Exposure to iAs

Levels of the arsenicals measured in drinking water, maternal urine, and neonatal cord serum are presented in Table 4.2. The mean level of DW-iAs was 24.7 μg/iAsL (range: 0.33-235.6) and mean level of U-tAs was 37.5 μg/L (range: 4.3 to 319.7). DW-iAs was significantly positively correlated with U-tAs (r_s= 0.60; p <0.001) (Table 4.3). The average levels of maternal metabolism arsenicals were 6.1% (range: 0.77% to 45.1%) for U-%iAs, 6.4% (range: 1.3% to 24.9%) for U-%MMAs, 87.6% (range: 32.7% to 96.7%) for U-%DMAs. For comparison to other studies, the ratios of U-MMAs/iAs and U-DMAs/MMAs are presented.

The average level of C-tAs was μg/L (range: 0.0042 to 3.0). The mean levels for the neonatal cord serum metabolites were determined to be 0.038 μg/L (range: 0.00085 to 0.50) for C-iAs, 0.062 μg/L (range: 0.000042 to 0.56) for C-MMAs and 0.26 μg/L (range: 0.0031 to 2.7) for C-DMAs. This corresponded to an average of 10.9% (range: 0.088 to 69.5) for %C-iAs, 16.7% (range: 0.032 to 51.8) for %C-MMAs and 72.5% (range: 21.6 to 97.8) for %C-DMAs. For comparison to other studies, the ratios of C-MMAs/iAs and C-DMAs/MMAs are presented.

The totals and proportions of urinary arsenicals were compared to neonatal serum arsenicals using spearman rank correlation calculations and are presented in Table 4.3. Maternal total urinary arsenic was significantly positively correlated with neonatal total serum arsenic (r_s= 0.60; p <0.001). The percentages and ratios of metabolites %U-MMAs and %C-MMAs (r_s= 0.34; p <0.001) were significantly correlated as were %U-DMAs and %C-DMAs (r_s= 0.17; p = 0.02). However, %U-iAs was not significantly correlated with %C-iAs, (r_s= 0.075; p = 0.3). While the proportions of metabolites were correlated in maternal and neonatal samples, the proportions of iAs and MMAs were higher in fetal cord serum in comparison to maternal urine. The levels of DMAs were lower in fetal cord serum in comparison to maternal urine (Table 4.2).

One carbon metabolism indicators

The levels of maternal serum nutritional biomarkers are presented in Table 4.4. The average maternal serum folate level was 40.6 nmol/L (range: 7.1 to 171.5). The average maternal serum B12 level was 127.4 pmol/L (range: 48.0 to 284.1). The average maternal serum Hcys level was 6.9 μmol/L (range: 4.1 to 19.4).

B12 was not significantly correlated with folate (r_s= -0.021; p= 0.7), or Hcys (r_s= -0.037; p= 0.6). Folate was significantly negatively correlated with Hcys (r_s= -0.25; p= 0.0005).

The majority of women (n=195, 99%) were sufficient in folate, and only 2 (1%) women were deficient in folate (data not shown). A large proportion (n=145, 74%) of the pregnant women were deficient in B12 (Table 4.4). Only 15 women (8%) had hyperhomocysteinemia (Table 4.4). Levels of individual OCM indicators by nutritional categories are presented in Table 4.4. There were significant differences in the levels of Cys by Hcys status, where those who had higher Hcys also had higher mean levels of Cys.

Furthermore, those in the lowest tertile of folate had the highest mean level of Hcys. There were no other statistically significant differences by categories of nutritional OCM indicators.

Relationship among OCM indicators and the methylated iAs arsenicals

Maternal urinary iAs was significantly negatively correlated with B12 (r_s= -0.22; p= 0.002), and positively correlated with Hcys (r_s= 0.19; p= 0.19) (Table 4.5). There were no significant correlations between urinary markers of maternal metabolism (%U-iAs, %U-MMAs, and %U-DMAs) and OCM indicators. However, the percentages of cord serum iAs were significantly positively correlated with maternal B12 (r_s= 0.16; p= 0.02), and the percentages of cord serum MMAs were significantly negatively associated with folate (r_s= -0.15; p= 0.04) and positively correlated with Hcys (r_s= 0.17; p= 0.01) (Table 4.5).

Differences in the levels of maternal urinary or neonatal cord serum levels of arsenicals based on levels of maternal serum nutritional levels were calculated using Wilcoxon Rank Sum tests and are presented in Table 4.6. U-tAs was significantly ($p<0.05$) higher in those who were deficient in B12. There were no significant ($p>0.05$) differences in any of the other arsenicals and categories of B12. %U-iAs and %U-MMAs were higher in those that had a normal level of Hcys. %C-MMAs were significantly lower in the middle and highest tertile compared to the lowest tertile of folate. However, there were no other statistical differences in any of the other arsenicals by tertiles of folate. As higher levels of Hcys are associated with folate/B12 deficiencies, differences of hcys by B12 deficiencies and/or tertiles of folate were explored. There was no significant difference in Hcys levels by B12 deficiency status, however there was a significant difference ($p<0.05$) by tertiles of folate. Specifically, individuals in the lowest folate tertile had the highest levels of Hcys.

Multivariable linear regression was used to determine if there was a relationship in the levels of maternal one carbon metabolism indicators and maternal and/or neonatal levels

of total iAs (tAs) (Table 4.7). Because the both the independent variables (OCM indicators) and dependent variables (U-tAs, C-tAs) were log transformed the betas are interpreted as a difference in the % of U-tAs or C-tAs based on a one percent difference of the OCM indicators. B-12 was significantly negatively associated with U-tAs where a one-unit difference in %B-12 pmol was associated with a -0.56% (95% CI: -0.31, -0.062) decrease in U-tAs. Hcys was also significantly associated with U-tAs where a one-unit difference in %Hcys was associated with a 0.44% (95% CI: 0.043, 0.83) increase in U-tAs. Furthermore, Hcys was significantly associated with C-tAs where a one-unit difference in %Hcys was associated with a 0.48% (95% CI: 0.054, 0.91) increase in C-tAs. There were no significant relationships in U-tAs or C-TAs and folate. Beta regression models were used to estimate the log difference in arsenicals based on OCM indicators. There were no significant relationships identified between maternal serum B12, folate. Interestingly, Hcys was significantly positively associated with neonatal serum %MMAs, where a 4.4 (95% CI: 0.89, 7.9) % increase in MMAs with for a one-unit difference in log Hcys. Even though the other relationships were not significant, B12 was positively associated with %U-iAs, %U-MMAs, %U-DMAs, %C-iAs, and %C-MMAs, and negatively associated with %C-DMAS. The relationship was positive with both folate and %MMAs, and %DMAs and negative relationships with %iAs although not statistically significant. Beta regression models were also performed to investigate the relationship between the arsenicals and nutritional categories based on B12 deficiency, tertiles of folate, and higher/lower Hcys (data not shown).

Discussion

Exposure to inorganic arsenic (iAs) during pregnancy is associated with detrimental birth outcomes, and has been previously linked to maternal arsenic (As) metabolism capacity[4-6]. Metabolism capacity of As is influenced by nutrients and vitamins involved in one carbon metabolism (OCM). As the relationship between indicators of OCM and As metabolism in pregnant women is understudied, we set out to determine if levels of OCM indicators are associated with alterations in the levels of total As and metabolism of As in the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort in Gómez Palacio, Mexico. In this region, iAs contaminates the drinking water, with levels up to 236 parts per billion[4]. In the present study we examined the levels of maternal serum nutritional biomarkers of OCM indicators (B12, folate, homocysteine) and contrasted these to maternal urinary markers and metabolism of iAs exposure and levels of neonatal cord serum iAs metabolites. We demonstrated that maternal and fetal cord serum levels of total As and As metabolites are significantly correlated. Almost all the pregnant women (99%) were folate sufficient yet, surprisingly, 74% of the women were deficient in B12. Additionally, a few (8%) of the women had hyperhomocysteinemia, as indicated by higher Hcys levels. Given that B12 is known to influence Hcys and methionine levels,[30] we hypothesized such deficiencies would influence metabolism of iAs in pregnant women and the proportions of arsenic in neonatal cord serum. There were four major findings of this study. First, in contrast to our *a priori* hypothesis, we found that lower levels of serum B12 were not associated with differences in the metabolism of As as measured in maternal urine. However, elevated levels of serum B12 were associated with lower levels of total arsenic in maternal urine (U-tAs). Second, elevated levels of Hcys were positively associated with total As in both maternal urine and neonatal cord serum. Third, maternal serum Hcys was positively associated with neonatal cord

%MMAs. Last, despite few of the women having folate deficiency, infants born to mothers in the lowest tertile of serum folate had significantly higher mean levels of %C-MMA as compared to those born to folate replete women. These data support that nutritional biomarkers involved in OCM are associated with biomarkers of total arsenic for both maternal and neonatal samples (urine and serum, respectively) and %MMAs in neonatal cord serum.

We demonstrated a strong association between biomarkers of total As in maternal urine and both total As and metabolites in cord samples. The finding that maternal and fetal cord serum metabolites are significantly correlated is an indication that maternal-fetal transport of iAs and its metabolites across the placenta does indeed occur. Specifically, maternal total urinary arsenic was significantly positively correlated with neonatal total serum arsenic. Additionally, percentages of the As metabolites of MMAs and DMAs between maternal urine and neonatal cord serum were significantly positively correlated. Maternal-fetal correlations between As in maternal blood and neonatal samples have been supported by others[16, 31, 32]. Furthermore, the proportions of iAs, and MMAs were higher while DMAs were lower in fetal cord serum in comparison to maternal urine. Placental transfer of As is well established, however it is not known whether fetal metabolism of As occurs *in utero*[16]. Together, these findings suggest that the fetus is exposed to all of arsenic's metabolites.

Pregnant women in the BEAR cohort were folate sufficient, yet B12 deficient, and a small proportion of women had high Hcys levels (hyperhomocysteinemia). This relationship between B12 and folate is supported in several other studies with high rates of vitamin B-12 insufficiency during pregnancy, where folate deficiency is estimated to be less than 10% (as

reviewed in[33]). The sufficiency of folate is more than likely due to adequate dietary intake of folate and/or prenatal supplementation of folic acid[33]. This result is supported by the large number (96%) of women who reported taking prenatal vitamins. The causes of B12 deficiencies during pregnancy are multifactorial, where deficiencies can occur due to dietary reasons, such as adhering to a vegetarian and specifically a lactovegetarian diet. Lower levels of vitamin B-12 during pregnancy could also be due to biological changes related to pregnancy including, hemodilution, active transport to the fetus, and changes in binding proteins[34]. Of concern, impaired vitamin B12 status alone during pregnancy has been associated with numerous adverse pregnancy outcomes, including intrauterine growth restriction, preterm delivery, neural tube defects (NTD)[35, 36]. B12 deficiency has also been linked to later in life health outcomes, including immune function impairment and neurological and cognitive functions in children[37]. Notably, a few women (8%) had clinically high levels of Hcys (hyperhomocysteinemia), however, the mean observed level of serum Hcys was 6.9 μmol/L, is in line with other populations with high rates of vitamin B-12 insufficiency[16, 38, 39]. This is concerning as imbalances in Hcys is a well-established risk factor several adverse birth outcomes including, placental abruption, preeclampsia, and adverse pregnancy outcomes[19]. Taken together there is a concern for the levels and interactions of B12, folate, and Hcys in this cohort as they relate to birth outcomes, and later in life health.

We demonstrated that higher levels of serum B12 are associated with lower levels of maternal U-tAs. In support of our findings, this inverse association between maternal levels of B12 has previously been observed, where the As+5 was in cord blood[16]. Given that maternal urinary arsenic has been used as an indicator of iAs exposure, these elevated levels

could suggest differences in the underlying exposure to iAs by those who have B12 deficiencies. In contrast to the B12 results, we demonstrate that elevated levels of Hcys were associated with elevated levels of total As in women's urine and also with total As in neonatal cord serum. A similar relationship between Hcys and total As+5 in blood has been demonstrated in another cross-sectional pregnancy cohort.[16] Given that As metabolism has been influenced by folate supplementation in adults,[13] we hypothesized similar findings in this pregnancy cohort. Contrary to our hypothesis, we did not find any significant relationships between the B12, folate and the metabolites in maternal urine. Still, there were differences in %C-MMAs in association with levels of maternal folate and Hcys. Specifically, infants born to mothers in the lowest tertile of serum folate had significantly higher mean levels of %C-MMA as compared to those born to folate replete women. Additionally, higher maternal serum levels of Hcys were associated with higher neonatal serum levels of %MMAs. These results have not been previously identified for fetal cord serum. Conversely, in an adult population in Bangladesh plasma Hcys levels were positively associated with urinary %MMAs.[40] The authors suggest that this positive association may be due to inhibition of the second methylation step by SAH, as OCM is a SAM-dependent methylation reaction producing SAH. SAH is hydrolyzed to homocysteine, a reversible process. Our results highlight that in folate sufficient, B12 deficient pregnant women maternal metabolism of As was not influenced by OCM, however maternal folate and Hcys levels may be an important factor for As metabolites in neonates.

Several factors should be considered in the interpretation of the study results. Given the cross-sectional design, the maternal biomarkers of both serum OCM indicators markers and metabolism of As were measured once during pregnancy. However, changes in As

metabolism that occur differentially between trimesters of pregnancy would not influence interindividual shifts, as all samples represented urinary collection during the end of pregnancy immediately before delivery.[41] Furthermore, a strength of our outcome assessment is the use individual markers of exposure, and in particular sensitive biomarkers of *in utero* exposure to As and its metabolites in both urine and neonatal cord serum. Serum levels of B12 decrease over the course of pregnancy, therefore the deficiency of B12 in this population as it relates to levels throughout should be interpreted with caution.[33] There is currently no standard for determining differences of B12 over the course of pregnancy; however, measurements of serum B12 levels are currently the clinical standard to determine deficiencies, despite such variations[42]. Furthermore, the prevalence of vitamin B12 deficiency observed here is potentially representative of the area, as data from the recent National Health and Nutrition Survey (Ensanut) demonstrate that B12 deficiency is at 40% and rising country-wide in Mexico for women of reproductive age, particularly in rural areas[43]. Additionally, any potential misclassification of either the exposure or the outcome would more than likely be non-differential, therefore would not influence the interpretation of our findings. Furthermore, other nutrients were not measured in this study that may not be required for OCM indicators but can contribute to the availability of methyl groups ultimately used in SAM biosynthesis including, betaine, choline, riboflavin, and serine. Additionally, we cannot rule out any potential unmeasured confounding between OCM indicators and the iAs biomarkers. While we did not find that any of the nutrients are associated with maternal metabolism of iAs in this cohort in Mexico, deficiencies in folate and/or B12 could still potentially influence methylation of As in other populations as they are

necessary in converting Hcys into methionine, and methionine is needed for the synthesis of SAM,[14] therefore replication of this work is needed.

In conclusion, this is among the first studies to assess B12, folate, and Hcys together on As metabolism in a folate sufficient pregnant population in Mexico. Our data highlight that pregnant women and their infants are exposed to iAs and that may necessitate further monitoring for B12 deficiencies. The findings from this study elucidate the need to investigate these relationships as they relate to disease etiologies associated with prenatal iAs exposure.

Table 4.1. Selected demographic characteristics of participants of the Biomarkers of Exposure to ARsenic (BEAR).

Characteristic	Total (n=197)
	N (N%)
Maternal age at delivery, years[a]	24 (5.5)
Race/ethnicity (Hispanic)	197 (100)
Educational level	
< High School	51 (25.9)
High School	95 (48.2)
> High School	51 (25.9)
Smoking Status	
Non-smokers	184 (93.4)
Current smokers	13 (6.6)
Alcohol Consumption	
None	156 (79.2)
Some	41 (20.8)
Daily Prenatal Vitamin intake	
No	8 (4.1)
Yes	189 (95.9)
Seafood Consumption	
None	154 (78.2)
Some	43 (21.8)
Parity	
1st pregnancy	68 (34.5)
More than 1 pregnancy	129 (65.5)
Gestational Age, weeks[a]	39.3 (1.2)
Newborn sex	
Male	103 (52.3)
Female	94 (47.7)
Birth weight (g)	3338.3 (481.7)

[a] mean (standard deviation)

Table 4.2. Levels of arsenicals and one carbon metabolism indicators from participants+ of the Biomarkers of Exposure to ARsenic (BEAR) cohort.

	Mean	Median	Range
DW-iAs (µg As/L)	24.7	14.0	0.33-235.6
Maternal Urinary arsenicals			
U-tAs (µg/L)	37.5	23.4	4.3-319.7
U-iAs (µg/L)	2.1	1.3	0.14-23.0
U-MMAs (µg/L)	2.3	1.3	0.082-18.2
U-DMAs (µg/L)	33.1	20.8	1.4-292.5
U-iAs (%)	6.1	5.3	0.77- 45.1
U-MMAs (%)	6.4	5.9	1.3-24.9
U-DMAs (%)	87.6	88.5	32.7- 96.7
U-MMAs/iAs	1.2	1.2	0.13-5.5
U-DMAs/MMAs	17.3	14.7	1.5-75.2
Neonatal Cord Serum Arsenicals			
C-tAs (µg/L)	0.36	0.24	0.0042-3.0
C -iAs (µg/L)	0.038	0.011	0.00085-0.50
C -MMAs (µg/L)	0.062	0.044	0.000042-0.56
C -DMAs (µg/L)	0.26	0.17	0.0031-2.7
C -iAs (%)	10.9	4.5	0.088- 69.5
C -MMAs (%)	16.7	16.7	0.032- 51.8
C -DMAs (%)	72.5	74.9	21.6- 97.8
C –MMAs/iAs	21.0	3.9	0.00075-249.4
C -DMAs/MMAs	23.3	4.5	0.92-1768.1
Maternal Serum One Carbon Metabolism Indicators			
Folate (nmol/L)	40.6	37.9	7.1-171.5
B12 (pmol/L)	127.4	116.5	48.0-284.1
Hcys (µmol/L)	6.9	6.4	4.1-19.4

[#]Reported results are based upon a sample of 197 and 188, for measured values for urinary and neonatal cord serum arsenicals, respectively and 197 samples of maternal OCM indicators.

Table 4.3. Spearman rank correlation coefficients (rs) for the levels of in organic arsenic (iAs) in drinking water, maternal urinary arsenicals, and neonatal serum arsenicals.

	C-tAs (µg/L) r_s (*p*-value)	C -%iAs r_s (*p*-value)	C -%MMAs r_s (*p*-value)	C -%DMAs r_s (*p*-value)
Drinking water iAs				
DW-iAs (µg As/L)	0.60 (<0.001)	-0.093 (0.20)	0.26 (0.0004)*	-0.14 (0.06)
Maternal urinary arsenicals				
U-tAs (µg/L)	0.60 (<0.001)	-0.27 (0.0002)*	0.19 (0.010)*	0.11 0.1286
U-%iAs	-0.049 (0.50)	0.075 (0.3)	0.17 (0.018)*	-0.11 (0.12)
U-%MMAs	-0.0068 (0.92)	0.011 (0.87)	0.34 (<0.0001)*	-0.18 (0.012)*
U-%DMAs	0.0012 (0.98)	-0.025 (0.72)	-0.32 (<0.0001)*	0.17 (0.02)*

*p-value<0.05

Table 4.4. Levels of one carbon metabolism indicators by nutritional categories of carbon metabolism indicators from participants of the Biomarkers of Exposure to ARsenic (BEAR) cohort.

	Maternal Serum One Carbon Metabolism Categories						
	B12		Homocysteine		Folate		
One Carbon Metabolism Indicators	Deficient (N=145, 74%)	Sufficient (N=52, 26%)	Normal (N=182, 92%)	High (N=52, 8%)	Lower Tertile (n=49)	Middle Tertile (N=98)	Upper Tertile (N=50)
	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)
B12	104.4 (23.6)	192.0 (38.2)	127.3 (46.1)	129.1 (65.1)	134.0 (53.1)	123.5 (44.6)	128.7 (48.0)
Folate	40.6 (18.9)	40.8 (26.0)	41.4 (21.1)	31.9 (14.6)	20.8 (5.4)	38.0 (4.9)	65.2 (25.2)
Homocysteine	6.9 (2.3)	6.9 (2.0)	6.4 (1.5)	12.5 (2.2)	8.1* (2.9)	6.5 (1.7)	6.6 (2.0)

*p-*value*<05, Wilcoxon Rank Sums Non parametric test for comparisons for B12 and Hcys and ANOVA for tertiles of folate.

Table 4.5. Spearman rank correlation coefficients (rs) for the nutritional biomarkers (B12, folate, and homocysteine) and exposure indicators of iAs in the BEAR cohort.

	B12 r_s (*p*-value)	Folate r_s (*p*-value)	Cys r_s (*p*-value)	Hcys r_s (*p*-value)
Drinking water iAs				
DW-iAs (µg As/L)	-0.12 (0.1)	-0.020 (0.7)	-0.016 (0.8)	0.22 (0.002)*
Maternal urinary arsenicals				
U-tAs (µg/L)	-0.22 (0.002)*	0.045 (0.5)	0.046 (0.5)	0.19 (0.006)*
U-iAs (µg/L)	-0.22 (0.001)*	0.036 (0.6)	-0.044 (0.5)	0.11 (0.1)
U-MMAs (µg/L)	-0.17 (0.01)*	0.056 (0.4)	0.095 (0.1)	0.18 (0.01)
U-DMAs (µg/L)	-0.21 (0.002)*	0.044 (0.5)	0.054 (0.4)	0.20 (0.005)*
U-%iAs	0.047 (0.5)	-0.031 (0.6)	-0.17 (0.01)	-0.11 (0.1)
U-%MMAs	0.047 (0.5)	0.055 (0.4)	0.075 (0.2)	0.00049 (0.9)
U-%DMAs	-0.038 (0.5)	-0.0081 (0.9)	0.066 (0.3)	0.057 (0.4)
U-MMAs/iAs	-0.0093 (0.8)	0.12 (0.1)	0.18 (0.01)*	0.071 (0.3)
U-DMAs/MMAs	-0.049 (0.4)	-0.043 (0.5)	-0.063 (0.3)	0.0014 (0.9)
Fetal cord serum arsenicals				
C-tAs (µg/L)	-0.0011 (0.9)	0.067 (0.3)	0.14 (0.06)	0.24 (0.001)*
C-iAs (µg/L)	0.15 (0.03)*	-0.037 (0.6)	-0.031 (0.6)	-0.030 (0.6)
C-MMAs (µg/L)	0.073 (0.3)	-0.021 (0.7)	0.11 (0.1)	0.30 (<0.0001)*

C-DMAs (µg/L)	-0.024 (0.7)	0.097 (0.1)	0.17 (0.02)*	0.25 (0.0005)*
C-%iAs	0.16 (0.02)*	-0.048 (0.5)	-0.11 (0.1)	-0.15 (0.03)*
C-%MMAs	-0.095 (0.1)	-0.15 (0.04)*	0.019 (0.7)*	0.17 (0.01)*
C-%DMAs	-0.073 (0.3)	0.11 (0.1)	0.10 (0.1)	0.0093 (0.8)
C-MMAs/iAs	-0.16 (0.03)*	0.026 (0.7)	0.086 (0.2)	0.16412 (0.02)*
C-DMAs/MMAs	0.034 (0.6)	0.19 (0.008)*	-0.0024 (0.9)	-0.16 (0.02)*
B12	1	-0.021 (0.7)	0.16 0.02*	-0.037 (0.6)
Folate	-0.021 (0.7)	1	-0.022 (0.7)	-0.25 (0.0005)*
Hcys	-0.037 (0.6)	-0.25 (0.0005)*	0.51 (<0.0001)*	1

*p-value<0.05

Table 4.6. Levels of arsenicals by nutritional categories of one carbon metabolism indicators from participants of the Biomarkers of Exposure to ARsenic (BEAR) cohort.

	Maternal Serum One Carbon Metabolism Categories						
	B12	Homocysteine			Folate		
Arsenicals	Deficient (N=145)	Sufficient (N=52)	Normal (N=182)	High (N=52)	Lower Tertile (n=49)	Middle Tertile (N=98)	Upper Tertile (N=50)
	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)
Maternal Urinary Arsenic							
U-tAs (µg/L)	40.8* (45.9)	37.7 (25.1)	37.5 (44.2)	28.5 (32.5)	38.4 (40.3)	33.5 (39.9)	44.7 (50.8)
%U-iAs	6.0 (3.0)	5.1 (2.2)	6.2* (4.0)	6.4 (5.8)	6.0 (2.5)	5.9 (2.8)	6.7 (6.2)
%U-MMAs	6.3 (2.9)	5.9 (2.9)	6.4* (3.0)	6.6 (3.2)	6.1 (2.4)	6.1 (2.4)	7.2 (4.2)
%U-DMAs	87.7 (5.0)	88.9 (4.4)	87.5 (6.2)	87.3 (8.4)	87.9 (3.6)	88.1 (4.6)	86.2 (9.5)
Neonatal Cord Serum Arsenic							
C-tAs (µg/L)	0.37 (0.43)	0.33 (0.21)	0.36 (0.42)	0.33 (0.33)	0.36 (0.39)	0.34 (0.43)	0.40 (0.40)
%C-iAs	10.2 (14.0)	4.9 (11.2)	11.3 (14.4)	12.8 (15.1)	11.8 (15.6)	10.8 (14.4)	10.2 (13.0)
%C-MMAs	16.8 (6.7)	19.8 (5.1)	16.4 (6.6)	16.2 (5.9)	18.8* (6.4)	15.9 (7.1)	16.1 (5.0)
%C-DMAs	73.0 (12.8)	75.3 (9.8)	72.3 (13.5)	70.9 (14.8)	69.4 (14.2)	73.3 (13.3)	73.7 (12.4)

*p-*value*<05, Wilcoxon Rank Sums Non parametric test for comparisons for B12 and Hcys and ANOVA for tertiles of folate.

Table 4.7. Multivariableτ relationship of arsenicals and one-carbon metabolism indicators from participants of the Biomarkers of Exposure to ARsenic (BEAR) cohort.

	B-12	Folate	Hcys
	β (95% CI) [*p*-value]	β (95% CI) [*p*-value]	β (95% CI) [*p*-value]
U-tAs⁺	-0.56 (-0.86, -0.26) [0.0002]*	0.027 (-0.22, 0.27) [0.82]	0.44 (0.043, 0.83) [0.029]*
%U-iAs⁺⁺	0.037 (-0.11, 0.18) [0.6]	-0.098 (-0.44, 0.25) [0.57]	2.1 (-5.5, 1.2) [0.21]
%U-MMAs⁺⁺	0.088 (-0.041, 0.22) [0.18]	0.097 (-0.20, 0.39) [0.51]	-0.29 (-3.3, 2.7) [0.84]
%U-DMAs⁺⁺	1.1 (-1.9, 4.2) [0.48]	0.017 (-0.30, 0.33) [0.91]	1.1 (-2.0, 4.1) [0.48]
C-tAs⁺	-0.095 (-0.43, 0.24) [0.57]	0.096 (-0.18, 0.37) [0.49]	0.48 (0.054, 0.91) [0.027]*
%C-iAs⁺⁺	0.21 (-0.076, 0.49) [0.15]	-0.025 (-0.77, 0.72) [0.94]	-5.0 (-11.4, 1.5) [0.13]
%C-MMAs⁺⁺	0.0562 (-0.11, .22) [0.51]	-0.17 (-0.59, 0.25) [0.43]	4.4 (0.89, 7.9) [0.014]*
%U-DMAs⁺⁺	-0.090 (-0.27, 0.092) [0.32]	0.062 (-0.39, 0.51) [0.78]	0.32 (-3.8, 4.4) [0.87]

#Reported results are based upon a sample of 197 and 188, for measured values for urinary and neonatal cord serum arsenicals, respectively.

τ All models adjusted for maternal age, gestational age, parity, and maternal education.

⁺Beta values represent the estimated percent difference the arsenicals with a one percent difference in OCM indicators.

⁺⁺ Beta values represent the estimated percent difference in the log odds of the arsenicals with a one-unit difference in the log of OCM indicators.

*$p<0.05$; 95% CI does not include the null of 0.

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CHAPTER V: MATERNAL FOLATE LEVELS MODIFY THE RELATIONSHIP BETWEEN MATERNAL B12 LEVELS AND INTERACTIONS WITH MATERNAL ARSENIC METABOLISM ON INFANT BIRTHWEIGHT.

Overview

Inorganic arsenic (iAs) is metabolized in the body into mono- and di-methyl arsenicals (MMAs and DMAs, respectively). Metabolism of iAs can be influenced by nutrients involved in one carbon metabolism (OCM), and in fact, supplementation of OCM nutrients and/or vitamins in iAs exposed populations has been demonstrated to increase methylation of iAs. However, the role of OCM on arsenic metabolism during pregnancy and as it relates to birth outcomes has not been fully elucidated. In the present study, we estimate the relationship between OCM indicators of folate, B12, and homocysteine (hcys), iAs metabolism, and infant birthweight in a cross-sectional study from the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort in Gómez Palacio, Mexico.

We show that infant birthweight z-scores are negatively associated with B12 deficiency, but only in the context of a lower folate status. Maternal metabolism of iAs was determined to not represent a mediator of the relationship between OCM indicators and infant birthweight, but instead may influence the proportion of the effect of OCM and birthweight via an interaction with OCM. These results indicate that maternal nutritional status of serum B12 and folate and maternal metabolism of arsenic may play an interactive role in infant's birthweight. Such findings can inform future interventions to reduce the impacts of combined nutritional deficiencies in iAs exposed populations and associated adverse birth outcomes.

Introduction

Elucidating mechanisms that underlie adverse birth outcomes in relation to environmental factors is challenging due to the complex biological interactions from exposures during the prenatal period, as well as the practical and ethical limitations of studying pregnant women. This complexity is apparent in the interplay between maternal nutritional factors and exposure to environmental toxicants, as it is increasingly evident that both play a critical role in healthy fetal growth and development.

A toxicant of particular concern for fetal growth is inorganic arsenic (iAs). Millions of people around the world are exposed to iAs from contaminated drinking water, and maternal exposure to iAs is associated with altered fetal growth. Specifically, *in utero* exposure to iAs is associated with reduced birthweight,[1-5] height, and head circumference[4]. In fact, a recent meta-analysis identified an expected 53g reduction in birthweight from increasing prenatal iAs exposure across multiple studies[6]. Furthermore, maternal metabolism of iAs may have a greater impact on birthweight and other health outcomes than just exposure alone[2, 4, 5]. Drinking water iAs is metabolized in the body via a two-step methylation process, where it is methylated to monomethylated and dimethylated arsenicals (MMAs and DMAs, respectively). Efficiency of iAs metabolism, is characterized by the percentages of arsenicals in human tissues (i.e. %iAs, %MMAs, %DMAs).

Metabolism of iAs can be influenced by nutrients involved in one carbon metabolism (OCM). This process occurs because the methyl group for the methylation of iAs is derived from S-adenosylmethionine (SAM), and synthesis of SAM is influenced by micronutrients involved in OCM, such as such as folate, cobalamin (vitamin B12), homocysteine (Hcys) vitamin B6, betaine and choline[7]. Interestingly, supplementation of OCM nutrients and/or vitamins in iAs exposed populations has been demonstrated to increase methylation of iAs.

For example, folic acid supplementation was used to improve arsenic metabolism in adults exposed to iAs in contaminated drinking water in a recent randomized control trial (RCT)[8]. However, the role of OCM in iAs metabolism during pregnancy and as it relates to birth outcomes has not been fully elucidated.

Adequate levels of nutrients involved in OCM for pregnant populations are also important for healthy fetal development. For example, folic acid supplementation during the prenatal period reduces neural tube defects and is required for healthy fetal growth. Specifically, folate can activate cell growth and biosynthetic processes that are essential over the course of pregnancy, and is critical for protein, DNA, and lipid synthesis, and is essential for epigenetic mechanisms[9]. This has led to a large public health effort to increase prenatal folic acid intake through the fortification of foods and prenatal vitamins. Vitamin B12 is also essential during pregnancy due to its role in folate dependent reactions, mitochondrial energy and lipid metabolic pathways, and normal cell growth[10]. Additionally, there is evidence that lower levels of maternal B12 is associated with reduced birthweight and increased susceptibility for metabolic diseases (as reviewed by [10]). Furthermore, deficiencies in either B12 or folate can influence OCM, where the regeneration of methionine is inhibited, resulting in increases in Hcys; such imbalances in Hcys levels are inversely associated with lower birthweight[11]. Despite these well-known impacts of deficiencies in OCM indicators, there is still little known about their interactions with one another. However, recent evidence suggests that imbalances in B12 and folate during pregnancy may influence birth outcomes more than one nutrient independently[10, 12, 13]. Investigations of interactions of various nutrients involved in OCM and arsenic metabolism during pregnancy as they relate to birth outcomes has been unexplored.

We recently demonstrated in the Biomarkers of Exposure to ARsenic (BEAR) cohort in Gómez Palacio, Mexico that 74% of the pregnant women displayed a B12 deficiency, yet were folate sufficient. Our work demonstrated that maternal serum markers of B12 and Hcys influenced total iAs in maternal urine (U-tAs), and folate and Hcys influenced the proportions of neonatal serum metabolites of %MMAs. Furthermore, we have also observed that inefficient maternal metabolism of iAs was negatively associated with infant birthweight[2]. In the present study, we assess the relationship between OCM indicators, maternal metabolism of iAs, and infant birthweight through mediation and interaction analyses. Specifically, we use a counterfactual approach to estimate the natural direct effects (NDE) of OCM indicators on infant birthweight, the natural indirect effects (NIE) through the mediated pathway of maternal metabolism of iAs and the total effects (TE) and controlled direct effects (CDE) of both OCM indicators and maternal metabolism of iAs on infant birthweight. This approach to investigate mediation allows for the exploration of non-linear models and potential important exposure-mediator interactions[14]. By estimating the natural direct and indirect effects, we can potentially clarify the underlying mechanisms[15] of the relationship between OCM indicators and iAs metabolism as they relate to infant birthweight. Furthermore, by estimating the controlled direct effects, we can model how much birthweight would change on average when the mediator of arsenic metabolism is set at a predetermined level. This is important in understanding the underlying mechanisms of OCM indicators and arsenic metabolism, as it seems the interactions of OCM and iAs are most evident in nutrient poor environments[8]—a factor that we cannot ethically assign in human pregnant populations.

Materials and methods

Study population

The study population has been fully described previously[2]. Briefly, women were recruited at the time of the admission affiliated with their delivery from the General Hospital of Gómez Palacio. For women to be eligible for the study, participants must have met the following criteria: One-year minimum residence in the Gómez Palacio region, (urban locations of Gómez Palacio and surrounding rural locations), confirmation of a pregnancy without complications such as eclampsia or preeclampsia, and good overall health status (i.e., no signs of chronic or acute disease). The present study designed to analyze maternal serum levels of OCM indicators, iAs metabolism, and infant birthweight included a total of 197 women. Demographic data was gathered from a clinical worker and included information on maternal age at delivery, education, occupation, time living at residence, smoking status and alcoholic beverage consumption during pregnancy, daily prenatal supplement intake, seafood consumption, source and daily consumption of drinking and cooking water, and participants previous pregnancies (number of pregnancies and number of previous pregnancy losses). Information on birth measures, including newborn birth weight, newborn length, gestational age, head circumference, placental weight, and 5-min Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score was gathered at time of delivery by the physician and clinical staff. The outcome of infant birthweight was standardized by deriving z- scores that were calculated from the international infant growth charts of birthweight for gestational age at delivery (measured by last menstrual period)[16]. All procedures associated with this study were approved by the Institutional Review Boards of Universidad Juárez del Estado de Durango (UJED), Gómez Palacio, Durango, Mexico, and the University of North Carolina at Chapel Hill (UNC), Chapel Hill, North Carolina, U.S.A.

Determination of exposure to iAs

To determine maternal exposure to iAs, maternal spot urine samples were collected at the hospital during the delivery admission for all participants, and immediately transferred to cryovials and placed in liquid nitrogen. Aliquots of urine samples were shipped on dry ice to UNC-Chapel Hill where concentrations of U-iAs, U-MMAs, and U-DMAs were determined by HG-AAS as described previously[17-19]. Maternal total urinary arsenic (U-tAs) was determined by summing U-iAs, U-MMAs and U-DMAs. Concentrations of U-iAs, U-MMAs, and U-DMAs in each urine sample were adjusted for specific gravity as previously described[20]. Maternal metabolism efficiency of iAs was determined by calculating the proportions of the individual arsenicals iAs (%U-iAs), MMAs (%U-MMAs), and DMAs (%U-DMAs) relative to U-tAs.

Determination of OCM indicators

Banked maternal serum samples that were collected during the time of delivery admission for all participants and stored at -80°C were shipped on dry ice to Columbia University, New York, NY to measure OCM indicators. Maternal serum folate and B12 were analyzed by radio protein-binding assay (SimulTRAC-S; MP Biomedicals, Orangeburg, NY, USA), where folic acid as pteroylglutamic acid was used for calibration, and its 125 I-labeled analog as the tracer. All samples were run on a Gamma counter (Perkin Elmer). Hcys levels were analyzed via a HPLC- fluorescent detector with 385nm excitation and 515nm emission[21]. Deficiencies and/or sufficiency's of the OCM indicators were determined for each woman as has previously been described[22], and as in other iAs-exposed populations[23]. Because there were relatively few women (n=2) who had serum levels that indicated they were deficient in folate (<9 nmol), binary cutoffs for folate were created, where lower folate is defined as those women who had serum levels below the median level (37.9 nmol/L) and

higher folate is defined as those women who had serum levels of folate above or equal to the median (37.9 nmol/L) levels of the cohort. B12 deficiency was set at <148 pmol and ≥ 148 pmol for B12 sufficiency. Hyperhomocysteinemia (high Hcys) was defined by Hcys ≥ 10.4 nmol and normal Hcys was set at < 10.4 nmol. Four categories of combined B12 and folate status (B12/folate) were created based on the cutoffs of B12 deficiency or sufficiency and above or below the median levels of folate. Specifically, category I is defined as those women whose serum B12 levels were < 148 pmol and folate levels < 37.9 nmol; category II is defined as those women whose serum B12 levels were < 148 pmol and folate levels ≥ 37.9 nmol; category III is defined as women whose serum B12 levels were ≥ 148 pmol and folate levels < 37.9 nmol, and category IV is defined as those women whose serum B12 levels were ≥ 148 pmol and folate levels ≥ 37.9 nmol.

Statistical analyses of the relationship of one carbon metabolism indicators with birthweight z-score

All statistical comparisons and analysis were carried out using SAS v9.3. Differences in mean birth weight z-scores were determined based on the nutritional categories of OCM indicators using t-tests for two categorical comparisons of B12 deficiency and sufficiency, normal and high Hcys, and lower and higher folate levels. To compare mean levels of birthweight z-scores for the four categories of B12/folate (I, II, III, IV), ANOVA was used. Multivariable linear regression was used to determine the relationship between OCM indicators (continuous and based on nutritional categories) and birthweight z-score (continuous). All models were adjusted for a set of potential confounding variables selected *a priori*, including: maternal age (continuous), maternal education (categorized as below high school, high school, and above high school), and parity (dichotomized as one pregnancy or more than one previous pregnancy). Assumptions of linearity were assessed by examining

indicator variables for all modeled continuous variables (i.e. OCM indicators and maternal age) for the their linear relationship to infant birthweight z-score. Additionally, because of the potential interactions between folate and B12 during pregnancy on adverse birth outcomes, we were interested in determining if there was modification between the B12 deficiency and folate levels on birthweight z-scores. To test the hypothesis that the estimates of B12 deficiency on birthweight z-score are modified by lower or higher folate levels, we modeled an interaction term of binary B12 deficient/sufficient (with sufficient B12 as the referent) and binary folate median levels (with higher than the median folate as the referent) as a full model and compared these results to a reduced model with no interaction term using a Likelihood Ratio test to assess statistical significance. We also presented these results in stratum specific effect estimates from a stratified multivariable regression analyses of the relationship of B12 deficiency on infant birthweight z-scores. Statistical significance was defined using p-values under 0.05.

Mediation analysis

Mediation analysis was carried out using methods and SAS code previously described by Valeri and Vanderweele[24]. In all models the variables are denoted as the following: A =exposure (OCM indicators), M =mediator (%U-iAs, %U-MMAs, %U-DMAs), Y =outcome (birthweight z-score), and C=covariates. The derivation of the specific regression models used to determine the estimates have been previously fully described elsewhere[24]. In this study, the following regression equation was used to estimate the expected value of the potential mediator of arsenicals, given the exposure of OCM indicators and covariates: $E[M|a,c]=\beta_0+\beta_1 a+\beta'_2 c$. The following regression equation was used to estimate the expected value of the outcome of infant birth weight z-scores, given the exposure of OCM indicators, the potential mediators of arsenicals, and covariates: E

$[Y|a,m,c]=\theta_0+\theta_1a+\theta_2m+ \theta_3am + \theta^{'}_4c$. Covariates were selected *a priori* and all models were adjusted for a set of potential confounders of the main exposure to outcome (OCM indicators to birthweight z-score), the mediator to outcome (birthweight z-score to the arsenicals), and the mediator to the main exposure (arsenicals to OCM indicators). Final models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized). For specific parameters estimated for the potential effects of OCM indicators and iAs metabolism on infant birthweight, denote a reference (baseline) level of maternal serum OCM indicators, a0, and a new level, a1; and M as the mediator (e.g %U-iAs, %U-MMAs, and %U-DMAs) for both a natural and a set level to estimate all of the following: the natural direct effect (NDE), which can be interpreted as how much the outcome of birthweight z-score would change if the exposure of OCM indicators were set to a new level of exposure (a1) but the mediator of arsenicals (M) is kept at a level it would have taken at level a0 of exposure; the natural indirect effect (NIE), which can be interpreted as how much the outcome of birthweight z-score would change on average if the exposure (OCM indicators) were controlled at level a1 but the mediator (arsenicals) were changed from the level they would have taken if a0 to the level it would have taken if a1; the total effect (TE), which estimates how much the outcome of birthweight z-score would change overall for a change in the exposure of OCM indicators from level a0 to a1; the controlled direct effect (CDE), which estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at a set level (assigned), and the percent proportion mediated (PM)[25]. These parameters are represented by the following equations:

CDE=$(\theta_1 - \theta_3 m)$ (a-a*)

NDE= $\{\theta_0 + \theta_3 (\beta_0 + \beta_1 a^* + \beta'_2 c)\}$ (a-a*)

NIE= $(\theta_2 \beta_1 + \theta_3 \beta_1 a)$ (a-a*)

PM= NIE/TE x 100

To estimate the relationship of OCM indicators on infant birthweight, we modeled the OCM indicators (a) as a population that would be in an adverse nutritional state (indicated by the previously mentioned serum cutoffs) by setting (a0) to a positive nutritional status (i.e. sufficient for B12, higher folate median levels, and lower Hcys levels) and setting (a1) to a poorer nutrient status (i.e. deficient for B12, lower folate median levels, and higher Hcys levels). The coding of variables for the specific model parameters is displayed in Table 5.1. Specifically, estimates for the relationship between OCM indicators on the outcome of infant birthweight z-score were assessed using the following models for categories of OCM indicators: Model 1 B12 sufficiency/deficiency was assessed, Model 2 examined folate as lower or higher than the median level; Model 3 examined Hcys categorized as normal or higher. Models were run with and without an interaction term of iAs metabolism and OCM indicators to measure OCM indicators on infant birthweight z-score through the path of the potential mediator of iAs metabolism. The levels that the mediators of each of the indicators for iAs metabolism were set to are presented in Table 5.1. Specifically, the population was set at the median levels of the arsenicals previously found in the cohort where %U-iAs were set at 5.3%, %U-MMAs were set at 5.9%, and %U-DMAs were set to 88.7% (models 1a, 2a, 3a, and 4a). We were also interested in examining these associations when the population arsenicals were set at the poorest metabolism of iAs found in the cohort, therefore we also modeled maternal metabolism of iAs to the population's respective 90th percentile for %U-

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iAs at 9.5% and %U-MMAs at 9.6%, and the 10th percentile for %U-DMAs at 88.1% (models 1b, 2b, 3b, and 4b). To assess whether the arsenical proportions (e.g. %U-iAs, %U-MMAs, %U-DMAs) may mediate or interact with B12 in the context of modification by folate levels of the relationship between B12 and infant birthweight, models 1a and 1b were carried out but stratified by binary folate median levels (represented by models 4a and 4b).

Results

The demographic characteristics for the study subjects are presented in Table 5.2. Briefly, all women were of Hispanic decent, and most had at least a high school education or greater (48% and 26%, respectively). Nearly all (96%) women reported that they took prenatal vitamins during pregnancy. The mean birthweight adjusted for gestational age z-score was -0.29 with a range of -2.6, 2.8. Nutritional levels of OCM indicators, as measured in maternal serum and biomarkers of As exposure, are presented Table 5.2 and have been previously described.

Differences in unadjusted mean birthweight z-scores are presented in Table 5.3. There were no significant differences in mean birthweight z-score between those that were B12 deficient compared to those who were sufficient in B12. There were also no significant differences in mean birthweight z-scores for newborns born to women who had above or below median serum folate levels. Similarly, there were no differences in mean birthweight z-scores between newborns born to mothers who had high or normal serum Hcys levels. Conversely, when analyzed in the context of the B12/folate categories, there were significant differences in the comparisons of mean birthweight z-score (p=0.0037) for the unadjusted models. Specifically, newborns born to mothers in category III (sufficient B12/lower folate) displayed higher mean levels birthweight z-score, while those born to women in categories I and II of deficient B12/lower or higher folate were similarly low (-0.43, -0.31, respectively).

However, newborns born to mothers in category IV (sufficient B12/higher folate) had the lowest mean birthweight z-score of -0.52 (Table 5.3).

The adjusted multivariable linear regression models quantifying relationships between each individual OCM indicators and birthweight z-score are presented in Table 5.4. There were no significant linear relationships in birthweight z-score from examining the multivariable regression analyses for levels of maternal B12, folate, or Hcys. Specifically, infants born to those women who were B12 deficient had a mean difference of birthweight z-score of -0.26 (95%CI of -0.57, 0.047) compared to those infants who were born to mothers who were B12 sufficient. Additionally, infants born to those women who had serum levels of folate below the median value of 37.9 pmol had a mean difference of birthweight z-score of -0.11 (95%CI of -0.38, 0.17) compared to infants born to mothers who had serum folate levels above the median level. Furthermore, infants born to those women who had high Hcys levels had a mean difference of birthweight z-score of -0.24 (95%CI of -0.75, 0.28) compared to those born to mothers with low Hcys levels.

We next assessed whether there was a relationship between B12 status and birthweight stratified by folate status, as there are known interactions between these two in relation to infant birth outcomes[14]. Modification of the relationship between B12 status and birthweight z-score by folate levels was assessed using the likelihood ratio test, where models were fit with (e.g. the full model) and without an interaction term (reduced model). The likelihood ratio test statistic *p-value* of $p < 0.05$ indicates the presence of effect measure modification by median folate levels (Table 5.5). Stratified estimates of the relationship of B12 deficiency and folate are presented in Table 5.5. When maternal serum folate is lower, the beta estimate was -0.74 (95% CI: -1.2, -0.31; *p*=0.008). This indicates average

birthweight z-score is -0.74 lower for newborns born to women who are deficient in B12 and with lower folate relative to those sufficient in B12. In the higher folate strata, the estimate was 0.29 (95% CI: -0.70, 0.13; p=0.17) (Table 5.5).

We next set out to describe potential mediation of the relationships between all OCM indicators and birthweight, by maternal arsenic metabolism. Results of the mediation analysis assessing the categories of OCM indicators, with iAs metabolism on infant birthweight for categories of B12 are presented in Table 5.6, for categories of folate are presented in Table 5.7, and for Hcys are presented in Table 5.8. When allowing the exposure of B12 and mediator of %U-DMAs to interact, a significant estimate was observed for the controlled direct effect (CDE) of B12 deficiency on infant birth weight z-scores when %U-DMAs was set at the median level of (88.7%) (Table 5.6). Specifically, when the population was modeled to change their exposures from B12 sufficiency to B12 deficiency and all have %U-DMAs set at 88.7%, the difference between B12 deficiency to birthweight z-score to is -0.43 (95% CI: -0.83, -0.018; p-value= 0.040) (Table 5.6). There were no statistically significant natural direct or indirect effects for any of the arsenicals on the relationships between folate and birthweight z-score (Table 5.7). When contrasting higher Hcys to lower Hcys when %U-iAs was set at the 90th (higher %iAs) percentile, the controlled direct effect (CDE) was estimated to be a -1.3 (95% CI: -2.6, -0.049; p=0.041) difference in infant birth weight z-score (Table 5.8).

Folate levels modified the association between B12 deficiency and infant birth weight z-scores in the stratified regression analysis, therefore we sought to modification of folate in the estimations of mediation by maternal metabolism of iAs of the relationship of B12 deficiency on infant birthweights in strata of median folate, allowing for an interaction

between B12 and the mediator of maternal iAs metabolism (Table 5.9) and for non-interaction of B12 and iAs metabolism (Table 5.9b). The estimates for the natural direct effect (NDE), natural indirect effect (NIE), and total effect (TE) for the linear effects of B12 deficiency on birthweight z-score for all mediation analyses of maternal metabolism of iAs, were negative when stratified by lower folate levels, and positive when stratified by higher folate levels (Table 5.9, 5.10). When folate was lower the percent mediated (PM) was small, yet differed by mediator interaction/non-interaction models with increases in the percent mediated when allowing for the mediator of maternal metabolism of arsenic and B12 interaction. Furthermore, the estimated NIEs were not statistically significant; however, they did increase when modeling the mediators with interaction with B12 (Table 5.9). Additionally, when modeling the mediators of maternal metabolism of iAs as an interaction with B12, the CDEs changed based on the levels that the percentages of maternal arsenical were set when folate was low and/or high. Specifically, the controlled direct effects (CDE) of the estimated effect of B12 deficiency on birthweight z-scores were significant in the stratum of lower folate when setting the mediator of %U-iAs, %U-MMAs, and %U-DMAs to their median values, and to percentiles to simulate a poorer iAs metabolism (%U-iAs and %U-MMAs to their 90th percentile and %U-DMAs to its 10th percentile). Specifically, in this stratum when the arsenicals were set at the median level, the controlled direct effect for B12 deficiency on infant birthweight %iAs was estimated to be -0.61 (95% CI: -1.07, -0.51), %U-MMAs was estimated to be -0.72 (95% CI: -1.2, -0.27), and for %U-DMAs the estimated effect was -0.71 (95% CI: -1.2, -0.27). Similarly, in the lower folate stratum when %iAs and %U-MMAs were set at 90th percentile, the estimated controlled direct effect was -1.4 (95% CI: -2.2, -0.56) and -0.65 (95% CI: -1.5, -0.20), respectively, and when %DMAs were set at

90th percentile, the estimated effect was -1.04 (95% CI: -2.0, -0.13). However, when folate was high, the controlled direct effect estimates of the relationship between B12 deficiency with the mediators of %U-iAs, %U-MMAs, and %U-DMAs were all positive (Table 5.9).

Discussion

We previously demonstrated that differences in maternal metabolism of iAs, as indicated by %U-iAs, %U-MMAs and %U-DMAs in urine, was associated with lower infant birth weight in the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort[2]. Since metabolism of iAs can be influenced from nutritional supplementation of nutrients and vitamins involved in one carbon metabolism (OCM), we analyzed the relationships between OCM indicators and metabolism of iAs, and found associations between OCM indicators and iAs metabolites in fetal cord serum (Chapter IV of this thesis). Given the potential for OCM indicators to influence iAs metabolism and fetal growth, and inefficient metabolism of iAs to influence neonatal growth, we sought to clarify these potential biological mechanisms using a causal mediation approach. Here we show that maternal imbalances in B12 and folate together influence birthweight adjusted for gestational age z-scores, whereas independently there is no effect. In the causal mediation analysis, maternal iAs metabolism was not found to mediate the relationships between OCM indicators and birthweight z-score. However, there is evidence of interaction between iAs metabolism and B12 on the estimates of the relationship between levels of B12 and changes in infant birthweight. These results suggest that both folate levels and maternal arsenic metabolism interact in the relationship between B12 deficiency during pregnancy and infant birthweight. These complex biological interactions have not been previously identified in iAs exposed pregnant populations.

Stratified estimates of B12 deficiency displayed that when pregnant women have lower folate serum levels, there is a negative association with birthweight z-score and for

those that have higher folate serum levels, there is a positive association with birthweight z-score. These data suggest that independent of iAs exposure, maternal folate serum levels modify the impacts of B12 deficiency on infant birthweight z-score in this population. Previous studies have also indicated that imbalances in folate/vitamin B12 ratios identified in blood during and at the end of pregnancy have been associated with lower birth weight, spontaneous abortion, placental abruption and congenital malformations[13, 26, 27]. However, it also important to note that when mean levels of birthweight z-scores were compared across four subcategories of B12/folate, the women in the higher B12 and higher folate levels had newborns with the lowest mean birthweight z-score. These results are supported from another study that demonstrated lower fetal growth due to a higher folate/vitamin B12 ratio from serum levels in the third trimester[28]. While the precise mechanisms of these B12/folate imbalances on fetal growth are unknown, this effect could potentially occur because both folate and B12 deficiencies can influence maternal-fetal metabolism, and effect methyl transfers in the mother and the fetus[29], which can lead to vascular compromise to the fetus and insufficient placental development in the early embryonic period[30]. Taken together, these results suggest that the effect of B12 on infant birthweight is different across levels of folate and is imperative to investigate this modification rather than levels of B12 and folate independent of one another.

Given that we found significant modification by folate for the relationship of B12 deficiency on birthweight z-score, we focused part of our mediation analyses on these relationships. Specifically, we estimated the effects of potential mediation and/or interaction by maternal arsenic metabolism on B12 deficiency stratified by lower and higher folate serum levels. Interestingly, these results indicate that the natural direct effect, which

represents the change in birthweight z-score while allowing %U-iAs, %U-MMAs, and %U-DMAs to be kept at a level that it would have taken in absence of the B12 deficiency, was negative for %U-iAs, %U-MMAs, and %U-DMAs, and positive in the lower folate categories. These data support the direct relationship between B12 deficiency and birthweight, where birthweight is lower when pregnant women have lower levels of serum folate. To quantify the proportion of mediation explained by maternal metabolism of iAs for the relationship of B12 deficiency to infant birth weight z-score, we estimated both the natural indirect effect (NIE) of the arsenicals on infant birthweight z scores and calculated the proportion mediated (PM) for the potential mediators %U-iAs, %U-MMAs, and %U-DMAs. We found that the proportion mediated by maternal metabolism was rather small, and none of the natural indirect effects were significant. These results suggest that the effect of B12 deficiency on birthweight z-score is not mediated by arsenical urinary proportions.

Conversely, there was evidence of potential interactive effects (estimated by the controlled direct effects), indicating that the relationship of B12 on infant birthweight changes depending on the levels of the modeled maternal arsenicals. Whereby, when the levels of maternal arsenicals were set to levels of either the median and/or the 90th percentile (for %U-iAs, and %U-MMAs) and 10th percentile for %U-DMAs to simulate altered maternal metabolism of iAs, we find that there is evidence of potential interaction of %U-iAs, %U-MMAs, and %U-DMAs with B12 deficiency on infant birthweight. In particular, among those with lower folate levels, the controlled direct effect for the B12-birthweight association becomes significantly more negative when %iAs is higher (90th percentile) than when allowed to naturally vary or set to the median level. This models a condition of poorer metabolism during pregnancy where one would accumulate higher levels of %U-iAs.

Similarly, when %U-DMAs were set to the 10th percentile, thus simulating a potential poorer metabolism of arsenic, the controlled direct effect for B12-birthweight association becomes significantly more negative than when allowing %U-DMAs to naturally vary. While the effect on B12 associated changes in infant birthweight with a modeled high proportion of U-MMAs was not statistically significant, a similar trend for birthweight was observed. When folate was higher, there were no statistically significant controlled direct effects of the relationship between B12 deficiency and infant birthweight z-scores for any of the modeled arsenicals; however, the estimates between B12 deficiency and infant birthweight z-scores remained positive, regardless of the levels of arsenicals. These simulated findings from the present study are interesting as previous research from a clinical trial indicates that folate can increase arsenic metabolism[8]. However, the comparison of our results to this previous study is not fully comparable as the supplementation in the previous study was carried in adults of both gender (i.e. not pregnant women) and excluded those who were deficient in B12. Together, these data suggest the relationship between B12 deficiency and infant birthweight z-score is not only modified by folate, but there is the potential for an interaction between OCM and metabolism of iAs on infant birthweight.

There are several limitations to this study that should be kept in mind when interpreting these findings. First, both OCM indicators and maternal arsenicals were measured at the time of delivery, thus we do not have longitudinal exposure information. This is important for the measurement of B12 deficiency, as serum levels decrease over the course of pregnancy[10]. However, despite such variations, measurements of biomarkers for B12, Hcys, and folate are currently the standard for estimating deficiencies, as biomarker data can be more accurate and precise than questionnaire data, and daily B12 and folic acid

intakes are positively correlated with predictors of serum B12[31]. Second, interpretations of the change in birthweight z-scores do not represent a low birthweight phenotype. Therefore, the outcome interpretation represents a population shift in average birthweight z-score, instead of low infant birthweight. We did not categorize birthweights to explore typical cutoffs, such as small for gestational age or large for gestational age phenotypes, as this reduces the power to detect small associations between fetal exposures and birth weight[32]. In support of the methodology employed in the present study, a continuous measurement of birthweight-z score to examine the entire distribution of birthweights is beneficial to detect variation within the majority of newborns that are average for gestational age and may be more generalizable to many populations[32]. Third, causal interpretations from these analyses require strong assumptions. In particular, for causal interpretation of mediation analyses, we need to assume that there is no unmeasured confounding between the relationships of the main exposure to outcome (OCM indicators to birthweight z-score), the mediator to outcome (birthweight z-score to the arsenicals), and the mediator to the main exposure (arsenicals to OCM indicators). While we cannot rule out unmeasured confounding, we were careful in the selection of confounders based on *a priori* knowledge. Furthermore, the study population (i.e., women in a single town in Mexico) is fairly homogenous compared to national or international studies of birth weight, thus reducing the potential for residual confounding by restriction to women residing in a single town. However, sensitivity analysis will need to be further explored[33]. Lastly, because we found modification of the main exposures of B12 and folate, we targeted the mediation analysis to describe effects of iAs metabolism within these different strata. This further reduced our sample size and limited the ability to detect statistical mediation. This study is limited by only being able to explore dichotomous

categorization of folate levels, as further categorization reduced our sample size. Therefore, it will be important to analyze the relationship of B12 deficiency on infant birthweight across other cutpoints of folate. Replication of these findings in other populations will be important, as there is still potential that the relationship between OCM indicators and infant birthweight could be mediated through maternal iAs metabolism. This is particularly important in regard to the estimation of the total effects, as the association with the exposure in a given population depends on the population prevalence of the mediator,[34] and despite the effort to simulate poorer metabolism, we were limited in our ability to do such, as most women in this population were efficient at metabolizing iAs. One major strength of this study is the estimation of potential causal effects, especially since we found evidence of potential interaction between the OCM indicators and iAs metabolism on infant birthweight. Another major strength of this work lies in the modeling of interactions between the exposure and mediator. In many mediation analysis that are carried out not using causal methods (e.g. the classical product method for estimating indirect effects), there is the assumption that the exposure and mediator do not interact, and this corresponds to misleading assumptions of effect homogeneity and that causal effects are constant across all individuals[25].

In conclusion, in the study of the cohort in Gomez Palacio Mexico who are exposed to iAs from drinking water, we observe the population is B12 deficient, yet folate sufficient.

Across the cohort, the newborn birthweight z-scores were negatively associated with B12 deficiency when analyzed in the context of folate status. Additionally, there is evidence of potential interaction between iAs metabolism and B12 on the estimates of the relationship between levels of B12 and shifts in infant birthweight. These findings may inform future interventions to reduce the impacts of both nutritional imbalances and exposures to toxic substances.

Table 5.1. Coding of variables used for mediation models to estimate the relationships between one carbon metabolism indicators, arsenicals, and infant birthweight.

Model: OCM indicator	Baseline exposure	New exposure	Mediator		
	a0	a1	%U-iAs	%U-MMAs	%U-DMAs
Model 1: B12					
1a	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	Median 5.3	Median 5.9	Median 88.5
1b	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7
Model 2: Folate					
2a	Higher ≥ 37.9 nmol/L	Lower <37.9 nmol/L	Median 5.3	Median 5.9	Median 88.5
2b	Higher ≥ 37.9 nmol/L	Lower <37.9 nmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7
Model 3: Homocysteine					
3a	Lower <6.4 μmol/L	Higher ≥6.4 μmol/L	Median 5.3	Median 5.9	Median 88.5
3b	Lower <6.4 μmol/L	Higher ≥6.4 μmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7
Model 4: B12[+]					
4a	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	Median 5.3	Median 5.9	Median 88.5
4b	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7

[+]Model estimates are presented as stratified below and above median folate levels.

Table 5.2. Selected demographic characteristics, levels of arsenicals, and one carbon metabolism indicators of participants of the Biomarkers of Exposure to Arsenic (BEAR).

	N (N%) or Mean, Median [range]
Maternal age at delivery, years	24.0, 23 [18-41]
Race/ethnicity (Hispanic)	197 (100)
Educational level	
< High School	51 (25.9)
High School	95 (48.2)
> High School	51 (25.9)
Daily Prenatal Vitamin intake	
No	8 (4.1)
Yes	189 (95.9)
Parity	
1st pregnancy	68 (34.5)
More than 1 pregnancy	129 (65.5)
Newborn sex	
Male	103 (52.3)
Female	94 (47.7)
Birth weight (g)	3338.7, 3340.0 [1800-5120.00]
Gestational Age, weeks	39.3, 40 (34-42)
Birth weight (z-score)	-0.29, -0.37 (-2.6, 2.7)
Maternal Urinary arsenicals	197 (100%)
U-tAs (µg/L)	37.5 23.4 [4.3-319.7]
U-iAs (µg/L)	2.1, 1.3 [0.14-23.0]
U-MMAs (µg/L)	2.3, 1.3 [0.082-18.2]
U-DMAs (µg/L)	33.1, 20.8 [1.4-292.5]
U-iAs (%)	6.1, 5.3 [0.77- 45.1]
U-MMAs (%)	6.4, 5.9 [1.3-24.9]

U-DMAs (%)	87.6, 88.5 [32.7- 96.7]
One Carbon Metabolism Indicators	197 (100%)
B12 (pmol/L)	127.4, 116.5 [48.0-284.1]
Deficient (< 148)	145 (74%)
Sufficient (≥ 148)	52 (26%)
Folate (nmol/L)	40.6, 37.9 [7.1-171.5]
< Median (37.9)	98 (50%)
≥ Median (37.9)	99 (50%)
Homocysteine (μmol/L)	6.9, 6.4 [4.1-19.4]
Normal (<10.4)	182 (92%)
High (≥ 10.4)	15 (8%)
B12/Folate Interaction categories	
I: B12 deficient /<median folate	70 (36%)
II: B12 deficient /≥ median folate	75 (38%)
III: B12 sufficient /<median folate	28 (13%)
IV: B12 sufficient /≥ median folate	24 (12%)

Table 5.3. Mean birth weight-for-gestational age z-score by categories of maternal serum one carbon metabolism indicators (OCM indicators).

	B12		Folate		Hcys		B12/Folate categories			
Categories[+]	Deficient	Sufficient	<Median	≥Median	Normal	High	I	II	III	IV
N	(145)	(52)	(98)	(98)	(182)	(15)	(70)	(75)	(28)	(24)
	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)
birthweight z-score	-0.36 (0.99)	-0.064 (1.01)	-0.21 (1.1)	-0.36 (0.94)	-0.27 (1.01)	-0.51 (0.90)	-0.43 (0.95)	-0.31 (1.02)	0.33 (1.1)	-0.52 (0.61)
***p*-value**		0.067		0.29		0.33				0.0037

+B12 categories are set at deficient <148 pmol and sufficient ≥ 148 pmol for maternal serum B12 levels.
Folate categories are set at above or below the median of 37.5 nmol for maternal serum folate levels.
Normal homocysteine (Hcys) was set at < 10.4 nmol and high hcys was was set ≥ 10.4 nmol for maternal serum hcys levels.
B12/folate categories were created based on deficient/sufficient B12 status and median levels of folate.
    I: Deficient B12 (<148 pmol)/higher folate (≥37.9 nmol).
    II: Deficient B12 (<148 pmol)/lower folate (< 37.9 nmol).
    III: Sufficient B12 (≥148 pmol)/lower folate (< 37.9 nmol).
    IV: Sufficient B12 (≥148 pmol)/higher folate (≥ 37.9 nmol).

Table 5.4. Multivariable# linear regression models testing relationships between each individual OCM indicators and birthweight z-score.

	B12	Folate	Hcys
Categories of OCM+	Beta estimate [95 % CI] (*p-value*)	Beta estimate [95 % CI] (*p-value*)	Beta estimate [95 % CI] (*p-value*)
Birthweight z-score	-0.26 [-0.56, 0.047] (0.096)	-0.10 [-0.38, 0.18] (0.47)	-0.24 [-0.75, 0.28] (0.37)

#All models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized).
^Referent for B12 is sufficient, for folate >the median level, and for homocysteine <normal level

Table 5.5. Multivariable# linear regression to determine effect measure modification of B12 to birth weight-for-gestational age z-score by categories of folate.

B12 deficiency to birth weight z-score	Beta Estimate for B12 deficient ^	[95% CI] (*p-value*)	Test for modification+
Reduced model	-0.26	[-0.56, 0.051] (0.10)	
Full model with an interaction term of folate and B12	-0.72	[-1.1, -0.31] (0.006)	
Stratified estimates			
Estimate for B12 deficiency with Lower Folate	-0.74	[-1.2, -0.31] (0.0008)	
Estimate for B12 deficiency with Higher Folate	0.29	[0.70, -0.13] (0.17)	10.1 (0.0015)

#All models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized).
^reference of B12 sufficient for all models; for interaction model reference is the lower folate level
+ LRT test statistic and p-value

Table 5.6. Mediation analysis of Model 1#, to estimate the relationship between B12 deficiency with and without interaction between the potential mediators of maternal iAs metabolism and B12 (%U-iAs, %U-MMAs, %U-DMAs) on infant birthweight z-scores.

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
Model 1[+]	B12 with interaction	B12 without interaction	B12 with interaction	B12 without interaction	B12 with interaction	B12 without interaction
NDE	-0.29 [-0.61, 0.033] (0.078)	-0.27 [-0.58, 0.044] (0.093)	-0.27 [-0.58, 0.044] (0.091)	-0.27 [-0.58, 0.042] (0.091)	-0.28 [-0.59, 0.039] (0.086)	-0.27 [-0.58, 0.043] (0.090)
NIE	0.024 [-0.046, 0.093 (0.50)	0.0075 [0.58, -0.019] (0.033)[*]	0.0074 [-0.029, 0.044] (.69)	0.0080 [-0.031,0.046] (0.68)	0.015 [-0.045,0.076] (0.62)	0.0085 [-0.026, 0.043] (0.63)
TE	-0.26 [-0.58, 0.050] (0.099)	-0.26 [-0.57, 0.052] (0.10)	-0.26 [-0.58, 0.054] (0.10)	-0.26 [-0.57, 0.053] (0.10)	-0.26 [-0.57, 0.052] (0.10)	-0.26 [-0.57, 0.053] (0.10)
PM	-2.9%	-8.9%	-2.8%	-3.1%	-2.8	-3.1
CDE						
Model 1a[++]	-0.20 [-0.52, 0.13] (0.23)	N/A	-0.27 [-0.59, 0.043] (0.090)	N/A	-0.43 [-0.83 -0.018] (0.040)[*]	N/A
Model 1b [+++]	-0.20 [-0.52, 0.13] (0.23)	N/A	-0.24 [-0.71, 0.24] (0.32)	N/A	-0.24 [-0.56, 0.073] (0.13)	N/A

[#]All models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized).

[+] B12 a0 set to sufficient <148 pmol/L, B12 a1 set to deficient ≥148 pmol/L and modeled with an without an interaction for B12 and arsenicals

NDE: Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of B12 was changed from sufficient to deficient and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.

NIE: Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure of B12 was changed from sufficient to deficient but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.

TE: total effect, estimates how much birthweight z-score would change overall for a change in the exposure of B12 sufficient to deficient

PM: proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.

CDE: the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at the set level (assigned).

[++]Mediator set at the 50th percentile (median value), set at 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .

[+++] Mediator set at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7).

Table 5.7. Mediation analysis of Model 2#, to estimate the relationship between folate levels with and without interaction between the potential mediators of maternal iAs metabolism and folate (%U-iAs, %U-MMAs, %U-DMAs) on infant birthweight z-scores.

Model 2[+]	%U-iAs Folate with interaction	%U-iAs Folate without interaction	%U-MMAs Folate with interaction	%U-MMAs Folate without interaction	%U-DMAs Folate with interaction	%U-DMAs Folate without interaction
NDE	0.11 [-0.18, 0.39] (0.48)	0.10 [-0.18, 0.39] (0.48)	0.076 [-0.21, 0.36] (0.60)	0.079 [-0.20, 0.36] (0.58)	0.087 [-0.20, 0.37] (0.54)	0.093 [-0.19, 0.37] (0.52)
NIE	0.00010 [-0.028, 0.028] (0.99)	0.00006 [-0.017, 0.017] (0.99)	0.026 [-0.037, 0.088] (0.41)	0.023 [-0.020, 0.065] (0.29)	0.015 [-0.037,0.068] (0.56)	0.0092 [-0.021,0.040] (0.55)
TE	0.10 [-0.18, 0.38] (0.48)	0.10 [-0.18, 0.38] (0.48)	0.10 [-0.18, 0.38] (0.48)	0.10 [-0.18, 0.38] (0.48)	0.10 [-0.18, 0.38] (0.48)	0.10 [-0.18, 0.38] (0.48)
PM	0.10%	0.06%	26%	23%	15%	9%
CDE						
Model 2a[++]	0.12 [-0.18, 0.42] (0.44)	N/A	0.082 [-0.20, 0.37] (0.57)	N/A	0.0094 [-0.42, 0.43] (0.96)	N/A
Model 2b [+++]	0.049 [-0.37, 0.47] (0.81)	N/A	0.052 [-0.42, 0.52] (0.82)	N/A	0.10 [-0.18, 0.39] (0.47)	N/A

[#]All models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized).

[+]Folate a0 set to lower than the median folate level of <37 nmol/L, Folate a1 was set to higher than the median folate level of ≥37 nmol/L and modeled with an without an interaction for folate and arsenicals.

NDE Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of B12 was changed from sufficient to deficient and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.

NIE Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure of B12 was changed from sufficient to deficient but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.

TE total effect, estimates how much birthweight z-score would change overall for a change in the exposure of B12 sufficient to deficient

PM proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.

CDE the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at the set level (assigned).

[++]Mediator set at the 50th percentile (median value), set at 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .

[+++] Mediator set at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7).

Table 5.8. Mediation analysis of Model 3#, to estimate the relationship between Homocysteine (Hcys) status (higher vs. normal) with and without interaction between the potential mediators of maternal iAs metabolism and Hcys (%U-iAs, %U-MMAs, %U-DMAs) on infant birthweight z-scores.

Model 3[+]	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
	Hcys with interaction	Hcys without interaction	Hcys with interaction	Hcys without interaction	Hcys with interaction	Hcys without interaction
NDE	-0.48 [-1.1, 0.11] (0.11)	-0.25 [-0.78, 0.28] (0.35)	-0.31 [-0.84, 0.22] (0.25)	-0.26 [-0.78,0.27] (0.33)	-0.41 [-0.96, 0.15] (0.14)	-0.26 [-0.78,0.27] (0.33)
NIE	0.22 [-0.31, 0.75] (0.40)	0.016 [0.033,0.064] (0.53)	0.071 [-0.17, 0.32] (0.57)	0.022 [-0.040, 0.084] (0.49)	0.16 [-0.26,0.58] (0.45)	0.022 [-0.040,0.084] (0.48)
TE	-0.25 [-0.95, 0.44] (0.47)	-0.24 [-0.76, 0.29] (0.38)	-0.24 [-0.81, 0.33] (0.40)	-0.24 [-0.76, 0.29] (0.38)	-0.2 [-0.89, 0.40] (0.44)	-0.24 [-0.76, 0.29] (0.38)
PM	-88.4%	-6.7	-29.5	-9.4%	-64.7	-9.4%
CDE[+]						
Model 3a[++]	-0.22 [-0.74, 0.31] (0.42)	N/A	-0.25 [-0.77, 0.27] (0.34)	N/A	-1.04 [-2.04, -0.035] (0.042)[*]	N/A
Model 3b [+++]	-1.3 [-2.6, -0.049] (0.041)[*]	N/A	-0.71 [-1.6, 0.20] (0.12)	N/A	-0.30 [-0.82, 0.22] (0.26)[*]	N/A

Hcys a0 set to normal < 10.4 nmol, Hcys a1 was set to higher ≥10.4 nmol and modeled with an without an interaction for Hcys and arsenicals
NDE: Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of Hcys was changed from normal to higher and the mediator of arsenicals is kept at a level it would've taken in absence of Hcys exposure.
NIE: Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure exposure of Hcys was changed from below the median to above the median value (6.4) but the mediator (arsenicals) were changed from the level it would've taken in absence of higher Hcys.
TE: total effect, estimates how much birthweight z-score would change overall for a change in the exposure of exposure of Hcys was changed from normal to higher.
PM: proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.
CDE: the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at a set level (assigned).
++Mediator set at the 50th percentile (median value), set at 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .
+++ Mediator set at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7).

Table 5.9. Mediation analysis to model the effects of B12 deficiency in strata of above or below the median levels of maternal serum folate, allowing for an interaction between the potential mediators of maternal iAs metabolism and B12 (%U-iAs, %U-MMAs, %U-DMAs).

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
Est[#]	Folate Low	Folate High	Folate Low	Folate High	Folate Low	Folate High
NDE	-0.74 [-1.2, -0.26] (0.0027)[*]	0.22 [-0.24, 0.67] (0.34)	-0.73 [-1.2, -0.27] (0.0016)[*]	0.25 [-0.19, 0.68] (0.27)	-0.72 [-1.2, -0.27] (0.0018)[*]	0.23 [-0.22, 0.67] (0.32)
NIE	-0.010 [-0.076, 0.056] (0.76)	0.070 [-0.082, 0.22] (0.37)	-0.011 [-0.060, 0.038] (0.65)	0.045 [-0.052, 0.14] (0.36)	-0.020 [-0.090, 0.048] 0.56	0.064 [-0.070, 0.20] (0.34)
TE	-0.75 [-1.2, -0.29] (0.0016)[*]	0.29 [0.19, -0.14] (0.71)	-0.74 [-1.2, -0.29] (0.0013)[*]	0.29 [-0.14, 0.72] (0.18)	-0.74 [-1.2, -0.29] (0.0012)[*]	0.29 [-0.14, 0.72] (0.18)
PM	1.3%	24%	1.5%	16%	2.7%	22%
INT	-0.19 (0.072)	-0.065 (0.14)	0.021 (0.83)	-0.052 (0.87)	0.048 (0.74)	0.036 (0.16)
CDE[+]						
50[th++]	-0.61 [-1.07, -0.15] (0.0091)[*]	0.33 [-0.10, 0.77] (0.13)	-0.72 [-1.2, -0.27] (0.0016)[*]	0.33 [-0.13, 0.78] (0.16)	-0.71 [-1.2, -0.27] (0.0018)[*]	0.33 [-0.11, 0.76] (0.14)
90th or 10[th +++]	-1.4 [-2.2, -0.56] (0.0016)[*]	0.063 [-0.47, 0.58] (0.81)	-0.65 [-1.5, 0.20] (0.13)	0.13 [-0.38, 0.64] (0.60)	-1.04 [-2.0, -0.13] (0.025)[*]	0.079 [-0.43, 0.58] (0.75)

Table 5.10. Mediation analysis to model the effects of B12 deficiency in strata of above or below the median levels of maternal serum folate, without interaction between the potential mediators of maternal iAs metabolism and B12 (%U-iAs, %U-MMAs, %U-DMAs).

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
	Folate Low	Folate High	Folate Low	Folate High	Folate Low	Folate High
NDE	-0.73 [-0.79, -0.035] (0.0012)*	0.28 [-0.15, 0.71] (0.20)	-0.72 [-1.2, -0.27] (0.0015)*	0.26 [-0.17, 0.68] (0.24)	-0.72 [-1.2, -0.27] (0.0015)*	0.27 [-0.16, 0.70] (0.22)
NIE	-0.0043 [-0.035, 0.026] (0.78)	0.0066 [-0.047,0.060] (0.80)	-0.013 [-0.062, 0.036] (0.60)	0.029 [-0.043, 0.10] (0.42)	-0.014 [-0.066, 0.037] 0.59	0.018 [-0.043, 0.079] (0.55)
TE	-0.74 [-1.2, -0.29] (0.0012)*	0.29 [-0.14, 0.72] (0.19)	-0.74 [-1.2, -0.28] (0.0012)*	0.29 [-0.14, 0.72] (0.19)	-0.74 [-1.2, -0.29] (0.0012)*	0.29 [-0.14, 0.72] (0.19)
PM	0.58%	2.3%	1.7%	10%	1.9%	6.4%

#All models were adjusted for maternal age (continuous), maternal education (coded as an indicator variable), and parity (dichotomized).
+B12 a0 set to sufficient <148 pmol/L, B12 a1 set to deficient ≥148 pmol/L and modeled with an without an interaction for B12 and arsenicals
NDE: Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of B12 was changed from sufficient to deficient and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.
NIE: Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure of B12 was changed from sufficient to deficient but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.
TE: total effect, estimates how much birthweight z-score would change overall for a change in the exposure of B12 sufficient to deficient
PM: proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.
INT: the interaction coefficient and p-value from the interaction of B12 and arsenicals to birthweight.
CDE: the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at the set level (assigned).
++Mediator set at the 50th percentile (median value), set at for 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .
+++ Mediator set at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7).

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CHAPTER VI: LOW LEVELS OF EXPOSURE TO INORGANIC ARSENIC IN DRINKING WATER MAY MODIFY THE RELATIONSHIP BETWEEN MATERNAL SERUM LEVELS OF HOMOCYSTEINE, AND INFANT BIRTHWEIGHT.

Overview

Exposure to inorganic arsenic (iAs) from contaminated drinking water is a widespread global public health problem, and is particularly harmful during sensitive windows of exposure such as the prenatal period. Nutrients involved in one carbon metabolism (OCM) may play an important role in metabolism of iAs and is associated with changes in birthweight. Additionally, the levels of exposure to iAs may modify the associations between OCM, iAs metabolism, and infant birthweight. This work focuses on the impacts of the levels of drinking water iAs below, or at and above the World Health Organization (WHO) guideline level of 10ppb on OCM metabolism, iAs metabolism, and infant birthweight. Mediation analysis was carried out to estimate the effects of indicators of OCM, namely maternal serum B12, folate, and Homocysteine (Hcys) on infant birthweight and potential mediation by maternal arsenic metabolism (percentages of iAs, mono and di methylated arsenicals) stratified by iAs drinking water levels. We find that drinking water levels are marginally correlated with maternal metabolism of iAs, and that maternal serum Hcys is correlated with drinking water levels. The levels of drinking water did not modify the association between maternal B12, folate and infant birthweight. However, there was evidence of potential modification between higher Hcys exposure and infant birthweight when exposure to iAs in drinking water was below the WHO guideline level. There was no indication of mediation by iAs metabolism of the relationship between OCM and birthweight

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regardless of drinking water levels. These data indicate that lower levels of iAs in drinking water might play a role in the relationship between maternal one carbon metabolism and iAs associated alterations in birthweight.

Introduction

Exposure to inorganic arsenic (iAs) from contaminated drinking water is a widespread global public health problem, and particularly for populations that may be most sensitive and susceptible to exposure such as the developing fetus. *In utero* exposure to iAs is associated with several adverse birth outcomes, including lower birth weight, preterm birth, reduced height and head circumference, increased susceptibility to infection, including inflammation and infectious disease, and later in life cancers[1]. There are many factors that influence risks of iAs-associated birth outcomes. Of particular importance are differences in pregnant women's ability to metabolize iAs, as this may influence risks for adverse birth outcomes, such as alterations in fetal growth and increased risks for later in life disease[2-5]. Inorganic arsenic is metabolized in the body into mono- and di-methyl arsenicals (MMAs and DMAs, respectively).

Recently, there is interest in reducing iAs-associated diseases by increasing individuals' capacity to metabolize iAs[6]. Two modifiable factors that may be of importance for influencing iAs metabolism from a potential intervention approach are nutrition and the levels of exposure to iAs from drinking water. Specifically, metabolism of iAs may be influenced by nutrients involved in one carbon metabolism (OCM), and in fact, supplementation of OCM nutrients and/or vitamins in iAs exposed populations has been demonstrated to increase methylation of iAs[6, 7]. Another factor that may contribute to metabolism of iAs, and therefore increases in risks for iAs-associated diseases, are the levels of exposure in drinking water. This has been demonstrated in previous studies where, with

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increasing exposure to levels of iAs in drinking water, the urinary proportions of iAs and MMAs in increased and the proportions of DMAs decreased[8, 9]. However, in other studies the amount of iAs levels in drinking water were not associated with metabolism of iAs[8]. Furthermore, it is unclear whether individuals who are exposed above and below the regulatory guidelines, e.g. the World Health Organization (WHO) recommended guideline, display differences in metabolism of iAs. For example, in pregnant populations exposed to iAs, there is evidence of increases in %MMA (an indicator of inefficient iAs metabolism) at even very low levels of iAs[10].

Knowing how this modifiable factor of exposure to levels of iAs from drinking water influences metabolism of iAs may be important for environmental regulation of iAs. This could identify populations at a higher risk for iAs-associated diseases. This is particularly important since regulations vary by region and are continually being reexamined. For example, WHO and Australia have a set guideline level of 10 ppb (µg/L) for iAs in drinking water, and the U.S. Environmental Protection Agency's (EPA) maximum contaminant level (MCL) is 10 ppb. Additionally, some states have set limits of exposure to lower levels, such as 5ppb. However, in many countries (e.g. Bangladesh) 50 µg/L is still the commonly adopted guideline, and in Mexico, the MCL is set at 25 µg/L. It has been suggested that these differences in regulation may primarily be due to the difficulties in remediating iAs below the designated MCLs[11]. Furthermore, despite guidelines, there is still widespread exposure from iAs in drinking water. For example, there are several areas in the U.S. that have exposure to iAs in drinking water from unregulated private wells supplied with groundwater. Specifically, contaminated groundwater with elevated iAs is prevalent in the West, Midwest, parts of Texas, the Northeast and the South [12-14].

The impacts of the levels of iAs in drinking water on maternal metabolism of iAs in relation to birth outcomes have not been fully elucidated. Furthermore, whether exposure levels of iAs in drinking water modify the relationship between metabolism of nutrients involved in OCM, related to iAs methylation, and associated birth outcomes has not been previously studied. The aim of this work was to investigate the direct effects of maternal levels indicators of one carbon metabolism (B12, folate, cysteine, homocysteine) on infant birthweight stratified by different levels of drinking water iAs, and the indirect effects of this relationship in the context of potential mediation by maternal metabolism of iAs in the Biomarkers of exposure to arsenic (BEAR) cohort. We hypothesized that the levels of iAs in drinking water influence the effect of maternal nutritional biomarkers and infant birthweight, as iAs in drinking water is the primary source of exposure to iAs in this cohort.[3] Knowing how OCM indicators influence birthweight across different levels of exposure could inform important biological mechanisms of *in utero* exposure.

Materials and methods

Study population

The study population has been fully described previously[3]. Briefly, women were recruited at the time of the admission affiliated with their delivery from at the General Hospital of Gómez Palacio. The present study designed to analyze maternal serum levels of OCM indicators, iAs metabolism, and infant birthweight by levels of exposure to iAs via drinking water included a total of 197 women. Demographic data was gathered by a clinical worker and included information on maternal age at delivery, education, occupation, time living at residence, smoking status and alcoholic beverage consumption during pregnancy, daily prenatal supplement intake, seafood consumption, source and daily consumption of drinking and cooking water, and participants previous pregnancies (number of pregnancies

and number of previous pregnancy losses). Information on birth measures including newborn birth weight, newborn length, gestational age, head circumference, placental weight, and 5-min Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score was gathered at time of delivery by the physician and clinical staff. The outcome of infant birthweight was standardized by deriving z- scores that were calculated from the international infant growth charts of birthweight for gestational age at delivery (measured by last menstrual period)[15]. All procedures associated with this study were approved by the Institutional Review Boards of Universidad Juárez del Estado de Durango (UJED), Gómez Palacio, Durango, Mexico, and the University of North Carolina at Chapel Hill (UNC), Chapel Hill, North Carolina, U.S.A.

Assessing the levels of inorganic arsenic in drinking water

The methods for drinking water sampling have been described previously[3]. Briefly, within 4 weeks of newborn delivery, a drinking-water sample was collected by the research team at the homes of each of the study participants. The concentrations of iAs in drinking water (micrograms As/L; DW-iAs) were measured at UJED, Mexico, using hydride generation–atomic absorption spectrometry (HG-AAS) supported by a FIAS-100 flow injection accessory system as described previously[16]. The Trace Elements in Water standard reference material (SRM 1643e) (National Institute of Standards and Technology, Gaithersburg, MD) was used for quality control. The limit of detection (LOD) for iAs in drinking water by HG-AAS was 0.46 µg As/L. For this present study, drinking water iAs (Dw-iAs) levels were categorized into binary values of participants' drinking water levels being either $<$ or $\geq$ WHO guideline level of 10ppb. This included 92 (47%) women who had Dw-iAs below the WHO guideline level of 10 ppb and 105 (53%) women who had Dw-iAs equal to and/or above 10ppb (Table 6.2).

Determination of inorganic arsenic metabolism

Maternal metabolism of inorganic arsenic was determined from spot urinary samples collected at the hospital during the delivery admission for all participants. These samples were immediately aliquoted, transferred to cryovials, and placed in liquid nitrogen. Aliquots of urine samples were shipped on dry ice to UNC-Chapel Hill where concentrations of U-iAs, U-MMAs, and U-DMAs were determined by HG-AAS as described previously[16-18]. Maternal total urinary arsenic (U-tAs) was determined by summing U-iAs, U-MMAs and U-DMAs. Concentrations of U-iAs, U-MMAs, and U-DMAs in each urine sample were adjusted for specific gravity as previously described[19]. Metabolism efficiency was determined by calculating the proportions of the individual arsenicals iAs (%U-iAs), MMAs (%U-MMAs), and DMAs (%U-DMAs) relative to U-tAs.

Determination of OCM indicators

Banked maternal serum samples that were collected during the time of delivery admission for all participants and stored at -80° were shipped on dry ice to Columbia University, New York, NY to measure OCM indicators. Maternal serum folate and B12 were analyzed by radio protein-binding assay (SimulTRAC-S; MP Biomedicals, Orangeburg, NY, USA), where folic acid as pteroylglutamic acid was used for calibration, and its 125 I-labeled analog as the tracer. All samples were run on a Gamma counter (Perkin Elmer). Hcys levels were analyzed via a HPLC- fluorescent detector with 385nm excitation and 515nm emission[20]. OCM indicators deficiencies and/or sufficiencies were determined for each woman as has previously been described[21], and as in other iAs-exposed populations[22]. Because there were relatively few women (n=2) who had serum levels that indicated they were deficient in folate (<9 nmol), binary cutoffs for folate were created, where lower folate is defined as those women who had serum levels below the median level (37.9 nmol/L) and

higher folate is defined as those women who had serum levels of folate above or equal to the

median (37.9 nmol/L) in the cohort. B12 deficiency was set at <148 pmol and ≥ 148 pmol for

B12 sufficiency. Hyperhomocysteinemia (high Hcys) was defined by Hcys ≥ 10.4 nmol and

normal Hcys was set at < 10.4 nmol.

Statistical analyses of the relationship of the drinking water levels of inorganic arsenic (iAs) to metabolism of iAs and one carbon metabolism indicators.

All statistical comparisons and analysis were carried out using SAS v9.3. Spearman

rank correlations coefficients (r_s) were calculated between the levels of arsenicals in maternal

urine including total maternal urinary arsenic (U-tAs), %U-iAs, %U-MMAs, and %U-

DMAs, and maternal one carbon metabolism (OCM) indicators of B12, folate, and Hcys,

across all women and by stratified levels of Dw-iAs (< or ≥ WHO guideline level of 10ppb).

Differences in mean levels of maternal urinary arsenicals, maternal serum OCM indicators,

and differences in infant birthweight z-scores were determined based on Dw-iAs levels

below or above the WHO guideline level of 10 ppb using a two sided t-test.

Mediation methods

Mediation analysis for OCM indicators, iAs metabolism, and infant birthweight were

carried out using mediation methods and SAS code previously described by Valeri and

Vanderweele[23]. Specifically, these mediation analysis were carried out for each strata of

drinking water iAs levels, where the women were stratified by their drinking water iAs levels

of < or ≥ WHO guideline level of 10ppb. This analysis aimed to address whether the

arsenical proportions (e.g. %U-iAs, %U-MMAs, %U-DMAs) are mediators of the

relationship between B12, folate or Hcys and birthweight z-score. In all models, the variables

are denoted as the following: *A* =exposure (OCM indicators), *M* =mediator (%U-iAs, %U-

MMAs, %U-DMAs), *Y* =outcome (birthweight z-score), and *C*=covariates. The derivation of

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the specific regression models used to determine the estimates have been previously fully

described elsewhere[23]. Specifically for this current study, the following regression equation

was used to estimate the expected value of the potential mediator of arsenicals, given the

exposure of OCM indicators and covariates: $E[M|a,c] = \beta_0 + \beta_1 a + \beta'_2 c$. The following

regression equation was used to estimate the expected value of the outcome of infant birth

weight z-scores, given the exposure of OCM indicators, the potential mediators of arsencials,

and covariates: $E[Y|a,m,c] = \theta_0 + \theta_1 a + \theta_2 m + \theta_3 am + \theta'_4 c$. Covariates were selected *a priori,* and

all models were adjusted for a set of potential confounders of the main exposure to outcome

(OCM indicators to birthweight z-score), the mediator to outcome (birthweight z-score to the

arsenicals), and the mediator to the main exposure (arsenicals to OCM indicators). Final

models were adjusted for maternal age (continuous), maternal education (coded as an

indicator variable), and parity (dichotomized). For the specific parameters estimated for the

potential effects of OCM indicators, iAs metabolism and infant birthweight, denote a

reference (baseline) level of maternal serum OCM indicators, a0, and a new level, a1; and the

mediator or maternal arsenicals (e.g %U-iAs, %U-MMAs, and %U-DMAs) for both a natural

and a set level to estimate: the natural direct effect (NDE), which can be interpreted as how

much the outcome of birthweight z-score would change if the exposure of OCM indicators

were set to a new level of exposure (a1) but the mediator of arsenicals (M) is kept at a level it

would have taken at level a0 of exposure; the natural indirect effect (NIE), which can be

interpreted as how much the outcome of birthweight z-score would change on average if the

exposure (OCM indicators) were controlled at level a1, but the mediator (arsenicals) were

changed from the level they would have taken if a1; the total effect (TE), which estimates

how much the outcome of birthweight z-score would change overall for a change in the

exposure of OCM indicators from level a0 to a1; the controlled direct effect (CDE), which estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at a set level (assigned), and the percent proportion mediated (PM)[24]. These parameters are represented by the following equations:

$$CDE=(\theta_1-\theta_3 m)\ (a-a^*)$$

$$NDE=\theta_0+\theta_3\ (\beta_0+\beta_1 a^*+\beta'_2 c)\ (a-a^*)$$

$$NIE=(\theta_2\beta_1+\theta_3\beta_1 a)\ (a-a^*)$$

$$PM=NIE/TE \times 100$$

To estimate the relationship of OCM indicators on infant birthweight, we modeled the OCM indicators (a) as a population that would be in an adverse nutritional state (indicated by the previously mentioned serum cutoffs) by setting (a0) to a positive nutritional status (i.e., sufficient for B12, higher folate median levels, and lower Hcys levels) and setting (a1) to a poorer nutrient status (i.e., deficient for B12, lower folate median levels, and higher Hcys levels). Coding of variables for the specific model parameters are displayed in Table 6.1. Specifically, estimates for the relationship between OCM indicators on the outcome of infant birthweight z-score were assessed using the following models for categories of OCM indicators where, Model 1 B12 sufficiency/deficiency was assessed, Model 2 examined folate as lower or higher than the median level; and Model 3 examined Hcys categorized as normal or higher. Models were run with and without an interaction term of the iAs metabolism and the OCM indicators to estimate the effect of OCM indicators on infant birthweight z-score through the path of iAs metabolism. The levels that the individual arsenicals were set to are presented in Table 6.1. Specifically, the population was set at the median levels of the arsenicals previously found in the cohort where %U-iAs were set at

5.3%, %U-MMAs were set at 5.9%, and %U-DMAs were set to 88.7% (models 1a, 2a, 3a).

We were also interested in examining these associations when the population arsenicals were

set at the poorest metabolism of iAs found in the cohort; therefore, we also modeled maternal

metabolism of iAs to the population's respective 90th percentile for %U-iAs at 9.5% and %U-

MMAs at 9.6%, and the 10th percentile for %U-DMAs at 88.1% (models 1b, 2b, 3b).

Results

The demographic characteristics for the study subjects are presented in Table 6.2.

Briefly, all women identified as being Hispanic, and most had at least a high school

education or greater (48% and 26%, respectively). Nearly all (96%) women reported that

they took prenatal vitamins during pregnancy. The mean birthweight adjusted for gestational

age z-score was -0.29 with a range of -2.6 to 2.8. The mean level of iAs in drinking water

samples was 24.6ppb with a range of 0.46-236.0 ppb. Nutritional levels of OCM indicators,

as measured in maternal serum and biomarkers of As exposure as measured in maternal

urine, are presented Table 6.2.

Correlations of maternal urinary arsenicals and maternal OCM indicators across the

entire the cohort and stratified by below or at/above the World Health Organization's (WHO)

guideline level are presented in Table 6.3. Across the entire cohort, Dw-iAs was significantly

correlated with U-tAs (r_s= 0.60, p <0.001), and this was the same for the correlation of U-tAs

and Dw-iAs in the context of the stratum where Dw-iAs was above the WHO guideline level

(r_s= 0.39, p <0.001). However, U-tAs was not correlated with Dw-iAs in the context of the

stratum where Dw-iAs was below the WHO guideline level. Hcys was significantly

correlated with drinking water levels among all women (r_s =0.22, p= 0.0025), but not when

stratified by Dw-iAs WHO guideline levels. There were no other statistically significant

correlations between OCM indicators and Dw-iAs (Table 6.3).

Differences in mean maternal urinary arsenicals and maternal OCM indicators stratified by below or above the WHO guideline level are presented in Table 6.3. U-tAs was significantly higher for women whose drinking water levels of iAs was above the WHO guideline level ($p < 0.001$). There were no statistically significant differences in the mean levels of %U-iAs, %U-MMAs, or %U-DMAs between the women who were categorized as below or at/above the Dw-iAs WHO guideline. Mean birthweight adjusted for z-score for infants whose mothers had drinking water levels of iAs below the WHO guideline was -0.41 with a standard deviation of 0.94, and for those infants who mother's had drinking water levels of iAs at or above the WHO guideline, mean birthweight z-score was -0.18 with a standard deviation of 1.04. The difference between these means was not statistically significant ($p > 0.05$) (data not shown).

We next set out to describe potential mediation of the relationships between all OCM indicators on birthweight, through the path of maternal arsenic metabolism stratified by iAs drinking water levels (< or $\geq$ WHO guideline level of 10ppb). Results of the mediation analysis assessing the categories of OCM indicators, with iAs metabolism on infant birthweight for categories of B12 are presented in Table 6.3, for categories of folate in Table 6.4, and for Hcys in Table 6.5.

Beta estimates for the natural direct effect (NDE) and total effect (TE) that represent a one unit difference in birthweight z-scores based on B12 deficiency compared to sufficiency were negative and similar in magnitude of effect, regardless of the stratification by drinking water levels (Table 6.3a, 6.3b). When Dw-iAs was lower, the percent mediated (PM) by all arsenicals was small, yet differed by mediator interaction/non-interaction models for the relationship between B12 and infant birthweight. Furthermore, the estimated natural indirect

effects (NIEs) were not statistically significant, and were close to the null value of 0. When modeling the mediator of all %U-iAs as an interaction with B12, the controlled direct effect (CDE) estimate became more negative for the relationship of B12 on infant birthweight for %U-iAs when the mediator was fixed at both the median and 90th percentile, when levels were below the recommended guideline than the natural direct effect. However, such relationships were not observed for %U-MMAs or %U-DMAs when they were fixed at their respective median and/or 90th percentile (Table 6.3a).

All estimated effects for the comparison of the estimated change in infant birthweight with the exposure of below the median serum levels of folate to above the serum folate levels in strata below or at/above the WHO guideline level were not significant. This observation was true when allowing for an interaction between the mediator of maternal iAs metabolism with folate and for non mediator interaction models (Table 6.4, 6.5).

We modeled the effects of being below the median serum levels of Hcys in strata of above or below the WHO guideline level, allowing for an interaction between the mediator of maternal iAs metabolism and for non mediator interaction. The estimates for the natural direct effect (NDE), natural indirect effect (NIE), and total effect (TE) for the linear effects of higher Hcys on birthweight z-score for all mediation analyses of maternal metabolism of iAs were negative and similar in the magnitude of effect when stratified by levels below the WHO guideline level, and positive when stratified by levels above the WHO guideline level (Table 6.6, 6.7). When Dw-iAs was lower, then the WHO guideline level percent mediated (PM) was small, yet differed by mediator interaction/non-interaction models with increases in the percent mediated when allowing for mediator Hcys interaction; however, these estimates were very imprecise. Furthermore, the estimated natural indirect effects (NIE) were

not statistically significant (Table 6.7). When modeling %iAs, allowing for interaction with Hcys, the CDE weakened, -0.41 [-0.82, -0.0049] in the strata below the WHO guideline level of 10ppb when %iAs was set at the median. However, when modeling %MMAs to represent a poorer state of iAs metabolism (i.e. setting them to the 90th percentile) and allowing for interaction with Hcys, the estimate for the controlled direct effect (CDE) for of Hcys on infant birthweight becomes more negative than when allowing %U- MMAs to naturally vary (i.e. the natural direct effect)

Discussion

In previous work, differences in maternal metabolism of iAs as indicated by %U-iAs, %U-MMAs and %U-DMAs in urine was associated with lower infant birth weight in the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort[3]. Given that metabolism of iAs can be influenced from nutritional supplementation of nutrients and vitamins involved in one carbon metabolism (OCM)[6, 25], we analyzed the relationships between OCM indicators, maternal metabolism of iAs, and impacts on infant birthweight. Specifically, we sought to clarify these potential biological mechanisms using a causal mediation approach and stratified our analysis by drinking water levels below or at/above the World Health Organization (WHO) guideline level of 10ppb-, as the relationship between OCM and iAs metabolism on infant birthweight may differ by exposure levels to iAs in drinking water. Maternal iAs metabolism was not found to mediate the relationships between OCM indicators and birthweight z-score when stratified by levels of iAs in drinking water by the WHO standard of below or at/above 10ppb. Interestingly, homocysteine (Hcys) was significantly correlated with levels of iAs in drinking water, and in mediation analysis, higher Hcys levels had a negative association with infant birthweight z-scores for those women with drinking water levels of iAs below the WHO guideline level of 10ppb, but not for those that

who had drinking water levels of iAs higher than the WHO guideline level. Additionally, the controlled direct effect of Hcys on infant birthweight, when allowed to interact with %MMAs indicated that there was a negative relationship with infant birthweight. These data suggest the interaction of Hcys and metabolism of iAs may be particularly important at lower levels of exposure to iAs from drinking water. These complex biological interactions in the context of a dose response to iAs exposure have not been previously identified in iAs exposed pregnant populations.

We found that Dw-iAs was significantly correlated with U-tAs across the entire cohort 0.60 (<0.001) (as previously reported), suggesting that drinking water is the primary source of exposure to iAs in this population[3] . This positive correlation remained strong for those women whose iAs levels in drinking water were above the WHO guideline level, however this was not observed for those with exposure to Dw-iAs below the WHO guideline level. Urinary proportions of metabolites were not significantly associated with Dw-iAs water across the cohort. Although not statistically significant ($p=0.07$), %U-MMAs were negatively correlated with drinking water levels in all women, and %iAs was marginally significantly (i.e. *p-value* slightly greater than 0.05) negatively correlated for those women with Dw-iAs above the WHO guideline level. A marginal association between Dw-iAs and maternal metabolism of iAs has been previously reported – where the effect of the dose of iAs in drinking water on the methylation efficiency seems to be small across a multitude of studies[8]. The reasons for such variation include that there are many influences to metabolism of iAs, including but not limited to, the level of exposure to iAs in drinking water or food, age, gender, pregnancy, nutritional status of folate, homocysteine, and protein, creatine, and genotype for arsenic 3 methyltransferase (*AS3MT*)[8, 9]. This was emphasized by a recent study

that biological and behavioral factors were more significant predictors of absolute and relative levels of iAs, MMAs, and DMAs in urine[8]. However, the role that drinking water iAs levels have on metabolism of iAs is debated. Together, these results indicate that drinking water levels may be an important predictor for some of proportions of the metabolites and in particular for %iAs when drinking water levels are above the WHO guideline level.

We estimated the effects of potential mediation and/or interaction by maternal arsenic metabolism on the relationship of maternal B12, folate, and Hcys levels stratified by $<$ or $\geq$ the WHO guideline level of 10ppb. The relationship of B12 deficiency on infant birthweight did not differ by levels of iAs in drinking water when iAs was allowed to naturally vary (natural direct effect). There was an observed increase in the estimated controlled direct effect for the estimate of B12 deficiency on differences in infant birthweight z-scores as compared to the natural direct effect when metabolism of %iAs was set at 90th percentile and when %U-MMAs was set to the median level in the lower strata of iAs drinking water levels. This finding is interesting in that there is evidence that lower levels of maternal B12 is associated with reduced birthweight and increased susceptibility for metabolic diseases (as reviewed by [26]). These results indicate that there could be potential shifts in infant birthweights for those that are B12 deficient even at low levels of exposure to Dw-iAs, but only in the context of when maternal metabolism is also poorer.

A surprising finding was that there were interactions between Hcys and maternal metabolism of arsenic on infant birthweight when women had lower levels of drinking water exposure. Specifically, the natural direct effects for a change in Hcys to a higher level was negatively associated with infant birthweights when women had drinking water levels below

the WHO guideline level. Additionally, this effect may be worsened by an interaction with maternal metabolism of iAs. This is exemplified when %U-MMAs were set to represent poorer metabolism of iAs and the estimate (controlled direct effect) for a difference in birthweight with exposure to Hcys became more negative than when %U-MMAs were allowed to vary naturally (the natural direct effect) in the strata of drinking water iAs levels below the WHO guideline level. Previous work has indicated that the linear dose response between increases of serum Hcys and decrease in birthweight occur with increases in Hcys of around 31g for 1-SD increase in maternal total homocysteine[27]. Potential biological mechanisms of this relationship include that Hcys may directly cause impaired fetal growth or via its effect on placental growth and function via its vascular effects; however, this has not been elucidated in studies in human[28]. These results indicate that Hcys levels may influence infant birthweights, and are modified by levels of drinking water iAs.

This study is not without limitations that may influence the interpretations of these findings. The first limitation of this work is in the context of the statistical estimation of the relationship between drinking water levels of iAs and the proportions of metabolites in maternal urine. Specifically, we did not find that all the proportions of maternal urinary arsenicals were associated with drinking water levels from correlation tests. This lack of correlation should be interpreted with caution as there are many other factors that may confound this relationship and here these were modeled in a bivariate assessment without the inclusion of other key predictors. Future studies will need to model these relationships in a manner that would account for potential confounders of drinking water exposure and metabolism of iAs that were not included here and examine the potential for linear and/or non-linear trends. The second limitation of this work is that both OCM indicators and

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maternal arsenicals were measured at the time of delivery, thus we do not have longitudinal exposure information, limiting the causal interpretations. Additionally, causal interpretations from mediation analyses require strong assumptions. In particular, we need to assume that there is no unmeasured confounding between the relationships of the main exposure to outcome (OCM indicators to birthweight z-score), the mediator to outcome (birthweight z-score to the arsenicals), and the mediator to the main exposure (arsenicals to OCM indicators). While we cannot rule out unmeasured confounding, we were careful in the selection of confounders based on *a priori* knowledge. Sensitivity analysis will need to be further explored[29]. Lastly, the targeted mediation analysis to describe effects of iAs metabolism within different strata of exposure to iAs in drinking water further reduced our sample size and limited the ability to detect statistical mediation and this led to imprecise estimates, particularly for the estimates within drinking water levels of iAs above the WHO guideline. Replication of these findings in other populations will be important to extrapolate findings for potential regulatory purposes.

Despite these limitations, there are many strengths of this study, including a strong correlation between exposure to iAs in drinking water and the maternal biomarker of urinary total arsenic. Therefore, attempts to elucidate dose–response still remain important as these relationships can influence policies regarding exposure, as exposure to iAs is widespread. The exact number of households affected by contaminated drinking water is largely unknown; however, an EPA study in 2001 found that approximately 13 million U.S. residents are drinking water from private wells that exceed the federal drinking water standard for iAs [30]. Additionally, this is of local concern as demonstrated previously by our

lab. Out of 63,000 private drinking water wells tested, 1,436 wells had iAs concentrations greater than the WHO guideline level, and a maximum level of 806 µg/L was found[31].

In conclusion, we find that drinking water levels of iAs are correlated with maternal metabolism of iAs, specifically %U-iAs. There could be potential shifts in infant birthweights for those that are B12 deficient, even at low levels of exposure to Dw-iAs. This relationship may be even more important in the context of poorer maternal metabolism of iAs (e.g. where %U-MMAs are elevated). Additionally, there was modification between higher Hcys exposure and differences in infant birthweight when drinking water levels were below the WHO recommended guideline level. These results indicate that the levels of exposure to iAs may need to be considered in the relationship of B12 and Hcys on infant birthweight.

Table 6.1. Coding of variables used for mediation models to estimate the relationships between one carbon metabolism indicators, arsenicals, and infant birthweight.

Model[+] OCM indicator	Baseline exposure	New exposure	Mediator		
	a0	a1	%U-iAs	%U-MMAs	%U-DMAs
Model 1: B12					
1a	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	Median 5.3	Median 5.9	Median 88.5
1b	Sufficient <148 pmol/L	Deficient ≥148 pmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7
Model 2: Folate					
2a	Higher ≥ 37.9 nmol/L	Lower <37.9 nmol/L	Median 5.3	Median 5.9	Median 88.5
2b	Higher ≥ 37.9 nmol/L	Lower <37.9 nmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7
Model 3: Homocysteine					
3a	Lower <6.4 μmol/L	Higher ≥6.4 μmol/L	Median 5.3	Median 5.9	Median 88.5
3b	Lower <6.4 μmol/L	Higher ≥6.4 μmol/L	90th percentile 9.3	90th percentile 9.5	10th percentile 81.7

[+]All model estimates are presented as stratified as < or ≥ WHO guideline level of 10ppb

Table 6.2. Selected demographic characteristics, levels of arsenicals, and one carbon metabolism indicators of 197 participants of the Biomarkers of Exposure to Arsenic (BEAR) cohort study from 2011 to 2012.

	N (N%) or Mean, Median [range]
Maternal age at delivery, years	24.0, 23 [18-41]
Race/ethnicity (Hispanic)	197 (100)
Educational level	
< High School	51 (25.9)
High School	95 (48.2)
> High School	51 (25.9)
Daily Prenatal Vitamin intake	
No	8 (4.1)
Yes	189 (95.9)
Parity	
1st pregnancy	68 (34.5)
More than 1 pregnancy	129 (65.5)
Newborn sex	
Male	103 (52.3)
Female	94 (47.7)
Birth weight (g)	3338.7, 3340.0 [1800-5120.0]
Gestational Age, weeks	39.3, 40 (34-42)
Birth weight (z-score)	-0.29, -0.37 (-2.6, 2.7)
Drinking water levels (µg/L)	24.6, 13.0 [0.46-236.0]
Maternal Urinary arsenicals	197 (100%)
U-tAs (µg/L)	37.5 23.4 [4.3-319.7]
U-iAs (µg/L)	2.1, 1.3 [0.14-23.0]
U-MMAs (µg/L)	2.3, 1.3 [0.082-18.2]
U-DMAs (µg/L)	33.1, 20.8 [1.4-292.5]

U-iAs (%)	6.1, 5.3 [0.77- 45.1]
U-MMAs (%)	6.4, 5.9 [1.3-24.9]
U-DMAs (%)	87.6, 88.5 [32.7- 96.7]
One Carbon Metabolism Indicators	197 (100%)
B12 (pmol/L)	127.4, 116.5 [48.0-284.1]
Deficient (< 148)	145 (74%)
Sufficient (≥ 148)	52 (26%)
Folate (nmol/L)	40.6, 37.9 [7.1-171.5]
< Median (37.9)	98 (50%)
≥ Median (37.9)	99 (50%)
Homocysteine (μmol/L)	6.9, 6.4 [4.1-19.4]
Normal (<10.4)	182 (92%)
High (≥ 10.4)	15 (8%)

Table 6.3. Spearman rank (rs) and mean level comparison of drinking water inorganic arsenic (iAs) levels to maternal urinary arsenicals and maternal one carbon metabolism indicators (OCM) in the entire Biomarkers of Exposure to Arsenic (BEAR) cohort study and stratified by below or equal to/above the World Health Organization (WHO) recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb).

	Drinking water-iAs Entire Cohort n=197		Below WHO n=92		Above WHO n=105	
	r_s (*p*-value)	Mean Stdv	r_s (*p*-value)	Mean Stdv	r_s (*p*-value)	Mean Stdv
Drinking Water (Dw-iAs)	-	24.7 (41.7)	-	1.7 (2.4)	-	44.0 (48.8)
Maternal Urinary arsenicals						
U-tAs (µg/L)	0.60 (<0.001)*	37.5 (43.0)	0.20 (0.065)	21.4 (13.7)	0.39 (<0.001)*	51.7* (53.8)
U-%iAs	-0.049 (0.49)	6.1 (3.9)	-0.021 (0.84)	6.0 (2.7)	-0.19 (0.051)	6.3 (4.8)
U-%MMAs	-0.13 (0.074)	6.4 (3.0)	-0.0034 (0.97)	6.6 (2.5)	-0.12 (0.21)	6.3 (3.3)
U-%DMAs	0.058 (0.43)	87.6 (6.1)	-0.069 (0.52)	87.7 (4.3)	0.14 (0.16)	87.5 (7.3)
Maternal OCM indicators						
B12	-0.12 (0.11)	127.4 (47.6)	-0.023 (0.83)	132.5 (48.0)	-0.039 (0.69)	123.0 (47.0)
Folate	-0.02014 (0.78)	40.6 (20.8)	-0.069 (0.52)	40.4 (20.3)	-0.043 (0.66)	40.9 (21.4)
Hcys	0.22 (0.0025)*	6.9 (2.2)	0.14 (0.19)	6.7 (2.2)	0.11 (0.26)	7.1 (2.3)

r_s= Spearman's rank correlation coefficient
*p<0.05

Table 6.4. Mediation analysis to model the effects of B12 deficiency in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), allowing for an interaction between the potential mediators of maternal iAs metabolism and B12 (%iAs, %MMAs, %DMAs).

Est[#]	%U-iAs <10ppb	%U-iAs >10ppb	%U-MMAs <10ppb	%U-MMAs >10ppb	%U-DMAs <10ppb	%U-DMAs >10ppb
NDE	-0.63 [-1.10, -0.15] (0.00099) [*]	-0.12 [-0.59, 0.35] (0.24)	-0.61 [-1.05, -0.17] (0.0067) [*]	-0.074 [-0.53, 0.38] (0.74)	-0.60 [-1.03, -0.17] (0.0068) [*]	-0.11 [-0.57, 0.36] (0.64)
NIE	-0.0089 [-0.052, 0.034] (0.68)	0.073 [-0.080, 0.23] (0.35)	0.00020 [-0.014, 0.015] (0.97)	0.023 [-0.053, 0.099] (0.54)	-0.0053 [-0.039, 0.028] (0.75)	0.061 [-0.071, 0.19] (0.36)
TE	-0.64 [-1.10 -0.17] (0.0073) [*]	-0.042 [-0.49, 0.41] (0.85)	-0.61 [-1.04, -0.17] (0.0067) [*]	-0.051 [-0.50, 0.40] (0.82)	-0.60 [-1.04, -0.17] (0.0067) [*]	-0.049 [-0.50, 0.40] (0.83)
PM	1.41%	-173.2%	1.4%	-151%	-51%	-125%
INT	-0.19 (0.067)	-0.063 (0.79)	0.081 (0.31)	-0.021 (0.75)	0.034 (0.21)	-0.00052 (0.99)
CDE[+]						
50[th++]	-0.54 [-0.98, -0.11] (0.014) [*]	0.0074 [-0.45, 0.47] (0.97)	-0.67 [-1.12, -0.22] (0.0034)	-0.059 [-0.51, 0.40] (0.80)	-0.60 [-1.04, -0.16] (0.0075) [*]	-0.028 [-0.48, 0.43] (0.90)
90th or 10[th +++]	-1.4 [-2.3, -0.45] (0.0032) [*]	-0.26 [-0.78, 0.27] (0.33)	-0.37 [-1.1, 0.26] (0.25)	-0.13 [-0.75, 0.48] (0.67)	-0.60 [-1.4, 0.21] (0.14)	-0.28 [-0.79, 0.28] (0.34)

Table 6.5. Mediation analysis to model the effects of B12 deficiency in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), without interaction between the potential mediators of maternal iAs metabolism and B12 (%iAs, %MMAs, %DMAs).

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
Est[#]	<10ppb	>10ppb	<10ppb	>10ppb	<10ppb	>10ppb
NDE	-0.61 [1.04, -0.17] (0.0063) [*]	-0.066 [-0.52, 0.39] (0.77)	-0.61 [-1.04, -0.176] (0.0058) [*]	-0.069 [-0.52, 0.38] (0.76)	-0.60 [-1.03, -0.17] (0.0068)[*]	-0.074 [-0.52, 0.37] (0.74)
NIE	0.00076 [-0.023, 0.025] (0.95)	0.017 [-0.044, 0.078] (0.58)	0.0047 [-0.034, 0.043] (0.81)	0.020 [-0.046, 0.086] (0.54)	-0.0053 [-0.037, 0.026] (0.73)	0.025 [-0.042, 0.092] (0.46)
TE	-0.60 [-1.04, -0.17] (0.0063) [*]	-0.049 [-0.50, 0.40] (0.83)	-0.604 [-1.04, -0.17] (0.0063) [*]	-0.049 [-0.50, 0.40] (0.83)	-0.60 [-1.04, -0.17] (0.0063)[*]	-0.049 [-0.50, 0.40] (0.83)
PM	-.13%	-35%	-.77%	-42%	-51%	0.88%

#NDE Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of B12 was changed from sufficient to deficient and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.

NIE Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure of B12 was changed from sufficient to deficient but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.

TE total effect, estimates how much birthweight z-score would change overall for a change in the exposure of B12 sufficient to deficient

PM proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.

INT the interaction coefficient and p-value from the interaction of B12 and arsenicals to birthweight.

CDE the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at fixed level (assigned).

++Mediator fixed at the 50th percentile (median value). Fixed at for 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .

+++ Mediator fixed at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7).

Table 6.6. Mediation analysis to model the effects of folate in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), allowing for an interaction between the potential mediators of maternal iAs metabolism and B12 (%iAs, %MMAs, %DMAs).

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
Est[#]	<10ppb	>10ppb	<10ppb	>10ppb	<10ppb	>10ppb
NDE	0.12 [-0.29, 0.53] (0.56)	-0.056 [-0.48, 0.36] (0.79)	0.097 [-0.31, 0.50] (0.63)	-0.054 [-0.47, 0.36] (0.79)	0.093 [-0.33, 0.52] (0.67)	-0.042 [-0.46, 0.38] (0.84)
NIE	0.021 [-0.072, 0.11] (0.65)	-0.0011 [-0.085, 0.083] (0.97)	0.056 [-0.13, 0.24] (0.55)	-0.0011 [-0.019, 0.017] (0.90)	0.049 [-0.11, 0.21] (0.54)	-0.0015 [-0.099, 0.070] (0.73)
TE	0.14 [-0.26, 0.54] (0.49)	-0.057 [-0.47, 0.35] (0.78)	0.14 [-0.26, 0.55] (0.49)	-0.056 [-0.47, 0.36] (0.79)	0.14 [-0.26, 0.54] (0.49)	-0.056 [-0.47, 0.36] (0.78)
PM	15%	1.9%	40%	2.0%	35%	2.7%
CDE[+]						
50[th++]	0.14 [-0.27, 0.55] (0.50)	-0.056 [-0.48, 0.36] (0.79)	0.096 [-0.31, 0.50] (0.64)	-0.071 [-0.49, 0.35] (0.73)	0.024 [-0.61, 0.66] (0.94)	0.0028 [-0.73, 0.74] (0.99)
90th or 10[th +++]	0.067 [-0.26, 0.55] (0.49)	-0.056 [-0.78, 0.67] (0.88)	0.055 [-0.65, 0.76] (0.87)	0.034 [-0.62, 0.69] (0.92)	0.12 [-0.29, 0.53] (0.56)	-0.042 [-0.46, 0.38] (0.84)

Table 6.7. Mediation analysis to model the effects of folate deficiency in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), without interaction between the potential mediators of maternal iAs metabolism and B12 (%U-iAs, %U-MMAs, %U-DMAs).

Est[#]	%U-iAs <10ppb	%U-iAs >10ppb	%U-MMAs <10ppb	%U-MMAs >10ppb	%U-DMAs <10ppb	%U-DMAs >10ppb
NDE	0.13 [-0.27, 0.53] (0.51)	-0.056 [-0.48, 0.36] (0.79)	0.097 [-0.31, 0.50] (0.63)	-0.055 [-0.46, 0.35] (0.79)	0.12 [-0.29, 0.53] (0.56)	-0.039 [-0.45, 0.38] (0.84)
NIE	0.090 [-0.030, 0.048] (0.65)	-0.0011 [-0.085, 0.083] (0.97)	0.045 [-0.044, 0.13] (0.48)	-0.0022 [-0.033, 0.028] (0.88)	0.024 [-0.041, 0.089] (0.46)	-0.0018 [-0.085, 0.049] (0.59)
TE	0.14 [-0.26, 0.54] (0.48)	-0.057 [-0.47, 0.35] (0.78)	0.14 [-0.26, 0.54] (0.48)	-0.057 [-0.47, 0.35] (0.78)	0.14 [-0.26, 0.54] (0.48)	-0.057 [-0.47, 0.35] (0.78)
PM	64%	1.9%	32%	3.9%	17%	3.2%

#NDE Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of Hcys was changed from below the median to above the median value (6.4) and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.

NIE Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure exposure of Hcys was changed from below the median to above the median value (6.4) but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.

TE total effect, estimates how much birthweight z-score would change overall for a change in the exposure of exposure of Hcys was changed from below the median to above the median value (6.4) .

PM proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.

Table 6.8. Mediation analysis to model the effects of Hcys deficiency in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), allowing for an interaction between the potential mediators of maternal iAs metabolism and B12 (%iAs, %MMAs, %DMAs).

Est[#]	%U-iAs <10ppb	%U-iAs >10ppb	%U-MMAs <10ppb	%U-MMAs >10ppb	%U-DMAs <10ppb	%U-DMAs >10ppb
NDE	-0.43 [-0.83, -0.018] (0.040)*	0.020 [-0.40, 0.44] (0.93)	-0.43 [-0.83, -0.018] (0.040)*	0.11 [-0.28, 0.50] (0.59)	-0.40 [-0.81, 0.0081] (0.054)	0.051 [-0.35, 0.45] (0.80)
NIE	-0.0052 [-0.040, 0.030] (0.76)	0.093 [-0.096, 0.28] (0.33)	-0.0052 [-0.040, 0.030] (0.76)	0.0087 [-0.078, 0.096] (0.84)	-0.028 [-0.11, 0.055] (0.50)	0.062 [-0.074, 0.20] (0.37)
TE	-0.43 [-0.84, -0.024] (0.038)*	0.11 [-0.28, 0.50] (0.57)	-0.43 [-0.83, -0.023] (0.038)*	0.11 [-0.27, 0.50] (0.56)	-0.43 [-0.84, -0.026] (0.037)*	0.11 [-0.28, 0.51] (0.56)
PM	1.2%	83%	8.7%	7.6%	6.6%	55%
INT	-0.063 (0.26)	-0.045 (0.54)	-0.066 (0.26)	-0.077 (0.33)	0.040 (0.17)	0.038 (0.42)
CDE[+]						
50[th++]	-0.41 [-0.82, -0.0049] (0.047)*	0.11 [-0.28, 0.51] (0.57)	-0.36 [-0.78, 0.059] (0.091)	0.13 [-0.25, 0.52] (0.49)	-0.65 [-1.3, 0.04] (0.067)	-0.14 [-0.66, 0.37] (0.58)
90th or 10[th +++]	-0.59 [-1.25, 0.075] (0.082)	-0.13 [-0.69, 0.43] (0.64)	-0.65 [-1.3, -0.024] (0.041)*	-0.11 [-0.65, 0.43] (0.68)	-0.39 [-0.81, 0.0183] (0.061)	0.13 [-0.26, 0.52] (0.52)

Table 6.9. Mediation analysis to model the effects of Hcys deficiency in strata of above or below the World Health Organization's recommended guideline for allowable levels of inorganic arsenic in drinking water (10ppb), without interaction between the potential mediators of maternal iAs metabolism and B12 (%U-iAs, %U-MMAs, %U-DMAs).

	%U-iAs	%U-iAs	%U-MMAs	%U-MMAs	%U-DMAs	%U-DMAs
Est[#]	<10ppb	>10ppb	<10ppb	>10ppb	<10ppb	>10ppb
NDE	-0.43 [-0.83, -0.023] (0.038)[*]	0.095 [-0.30, 0.49] (0.63)	-0.42 [-0.82, -0.0087] (0.045)[*]	0.11 [-0.28, 0.49] (0.58)	-0.42 [-0.82, -0.011] (0.044)[*]	0.092 [-0.30, 0.48] (0.64)
NIE	0.00013 [-0.019, 0.019] (0.98)	0.016 [-0.048, 0.079] (0.63)	-0.012 [-0.068, 0.045] (0.68)	0.0049 [-0.045, 0.054] (0.86)	-0.0096 [-0.066, 0.047] (0.74)	0.019 [-0.035, 0.074] (0.49)
TE	-0.43 [-0.83, -0.023] (0.038)[*]	0.11 [-0.28, 0.50] (0.57)	-0.43 [-0.83, -0.023] (0.038)[*]	0.11 [-0.28, 0.50] (0.57)	-0.43 [-0.83, -0.023] (0.038)[*]	0.11 [-0.28, 0.50] (0.57)
PM	-0.030%	14%	4.4%	-1.1%	0.88%	2.24%
INT	-0.063 (0.26)	-0.045 (0.54)	-0.066 (0.26)	-0.077 (0.33)	0.040 (0.17)	0.038 (0.42)

#NDE Natural direct effect, interpreted as how much birthweight z-score would change if the exposure of Hcys was changed from below the median to above the median value (6.4) and the mediator of arsenicals is kept at a level it would've taken in absence of B12 deficient exposure.

 NIE Natural indirect effect, interpreted as how much birthweight z-score would change on average if the exposure exposure of Hcys was changed from below the median to above the median value (6.4) but the mediator (arsenicals) were changed from the level it would've taken in absence of B12 deficiency.

TE total effect, estimates how much birthweight z-score would change overall for a change in the exposure of exposure of Hcys was changed from below the median to above the median value (6.4) .

PM proportion mediated (PM) that was calculated by the following formula: NIE/TE x 100.

INT the interaction coefficient and p-value from the interaction of B12 and arsenicals to birthweight.

CDE the controlled direct effect estimates how much the outcome of birthweight z-score would change on average if the mediator of arsenicals were controlled at fixed level (assigned).

++Mediator fixed at the 50th percentile (median value). Fixed at for 5.3 %U-iAs, 5.9 %U-MMAs, and 88.5 % U-DMAs .

+++ Mediator fixed at 90th percentile for %U-iAs (9.5) and %U-MMAs (9.6), and 10th percentile for %U-DMAs (81.7)

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CHAPTER VII: CONCLUSIONS

The research presented in this thesis focuses on a population exposed to arsenic (As) prenatally. The emphasis on this developmental window of exposure is of public health importance as the prenatal period represents a critical window of susceptibility to As exposure for the developing fetus. Moreover, exposure to As is a significant global public health problem as millions are exposed to elevated levels of As from inorganic arsenic (iAs) in drinking water. Of local importance, this exposure is also present in North Carolina from contaminated wells[1].

Individuals metabolize iAs differently, and importantly inter-individual differences in pregnant women's metabolism of iAs influences risk for adverse birth outcomes[2-4]. Once ingested, iAs undergoes hepatic methylation generating mono- and di-methyl arsenicals (MMAs and DMAs, respectively) – a process that facilitates urinary iAs elimination. Recent public health interventions have been investigating the use of nutritional supplementation of nutrients involved in one carbon metabolism (OCM) in an effort to facilitate metabolism of iAs, and thus reduce iAs-associated disease burden, [5-7].

OCM is the nutritionally regulated pathway essential for supplying methyl groups and may play an important role in both iAs methylation and *in utero* development. Because of these potentially important interactions between these nutritional factors and exposure to iAs during the prenatal period, the **central hypothesis of this research was that nutrients involved in one-carbon metabolism (B12, folate, and homocysteine) influence arsenic metabolism and birth outcomes.**

To investigate this hypothesis, I used existing data of biomarkers of iAs exposure and metabolism of iAs and collected new data of OCM biomarkers from banked maternal serum samples within the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort in Gómez Palacio, Mexico. This work was established based on previous studies in our lab where we demonstrated that pregnant women are exposed to potentially harmful levels of iAs from drinking water, with levels up to 236 ppb[3]. This finding is alarming as these drinking water samples greatly exceed the World Health Organization's (WHO) recommended guideline of 10 ppb. Furthermore, maternal urinary concentrations and proportions of MMAs (potentially representing maternal metabolism of iAs) were negatively associated with newborn birthweight[3]. The studies presented in this thesis, further investigated these relationships and included a key factor of maternal nutritional biomarkers of OCM within three study aims. The conclusions, strengths, limitations, and future directions are detailed below for each of the three aims investigated in this work.

Major findings of Chapter IV

The primary goal of the first aim of the thesis in this cross-sectional analysis was to examine the associations among the levels of maternal serum indicators of OCM and iAs. Specifically for the iAs measurements, maternal urinary markers of total iAs exposure, metabolism of iAs and the levels of neonatal cord serum iAs metabolites (both collected at delivery) were assessed. **The hypothesis to be tested was that maternal nutritional biomarkers of OCM indicators would be associated with both maternal and neonatal biomarkers of exposure to iAs and its metabolites.** To determine if maternal serum OCM indicators were associated with maternal exposure to iAs and maternal metabolism of iAs, there was a total of 197 pregnant women included in the analysis. Maternal exposure to iAs was assessed using the total amount of iAs in urine (U-tAs). Pregnant women's estimated

efficiency for metabolism of iAs was calculated using the percentages of the individual metabolites out of total iAs (i.e. %U-iAs, %U-MMAs, and %U-DMAs). For the assessment of neonatal exposure to iAs, there were 188 infants included, with exposure estimated based on neonatal cord serum total iAs (C-tAs) and cord serum metabolites, calculated from the percentages of the individual metabolites in neonatal cord serum out of the total iAs in neonatal cord serum (%C-iAs, %C-MMAs, and %C-DMAs). OCM indicators were defined by the levels of maternal serum B12, folate, and homocysteine (Hcys) from serum samples collected at the time of admission for delivery. Cutoffs of OCM indicators in maternal serum samples were used to determine pregnant women's nutritional status. Specifically, estimates for B12 deficiency/sufficiency were defined as <148 pmol, and ≥148 pmol (respectively), hyperhomocysteinemia/normal Hcys was defined as ≥10.4 and <10.4 nmol (respectively), and folate deficiency/ sufficiency was defined as <9 nmol and ≥ 9 nmol (respectively).

The first major finding from Chapter IV was that maternal urinary and fetal cord serum metabolites were significantly correlated, indicating that maternal-fetal transport of iAs and its metabolites across the placenta did indeed occur. Correlations between iAs in maternal blood and neonatal samples of cord blood and other tissues has been supported by others[7-9]. An additional finding of this work that may be of concern for iAs- related toxicity in that the average percentages of MMAs in neonatal cord serum were higher than the averages of %MMAs in maternal urine; this finding may require further investigation as higher proportions of MMAs are associated with an increased risk for iAs-associated diseases[10]. Additionally, the finding of overall higher proportions of the DMAs present in cord serum support that with increased methylation of iAs in the last trimester, majority of excreted As in urine is in the form of methylated DMAs, and therefore is the major

proportion of arsenicals transferred to the developing fetus *in utero*. This finding of higher

proportions of DMAs relative to other iAs metabolites has been demonstrated previously in

cord blood[7]. It is not yet known if the levels of arsenicals in cord serum represent a signature

of elevated exposure or how they relate to health outcomes, as at the time of this report these

results are the only ones of neonatal cord serum biomarkers of iAs and its metabolites. Taken

together, the levels of iAs and its metabolites found in cord serum indicate that this tissue

may serve as a biomarker of potential *in utero* exposure to iAs and its metabolites.

The second major finding from Chapter IV was that majority of the pregnant women

in the cohort displayed folate sufficiency, yet B12 deficiency. Specifically, there was a high

prevalence of folate sufficiency in the cohort, with 99% of the pregnant women having serum

folate levels ≥ 9 nmol/L. This finding is likely due to majority of the women reporting that

they took prenatal supplements during pregnancy (96%), and because of mandatory folic acid

supplementation of both corn and flour products in Mexico. Hyperhomocysteinemia was

relatively low, where 8% of women had Hcys levels ≥ 10.4 μmol/L. The finding that 74% of

the women displayed a deficiency in B12 (serum levels < 148 pmol/L) was surprising. The

potential causes of these deficiencies may be multifactorial. The most common reason for

B12 deficiencies during pregnancy occur due to dietary reasons, such as adhering to a

vegetarian and specifically a lactovegetarian diet[11-15]. Additional contributions of the B12

deficiencies found here may be due to intestinal parasite infections, as this is a growing

concern in many areas, and particularly for those living in developing countries.

Furthermore, standards of B12 deficiencies are the same across pregnancy, yet there are

many biological changes that occur over the course of pregnancy including, hemodilution,

active transport to the fetus, and changes in binding proteins, potentially contributing to

lower levels of B12[16]. However, the high rate of B12 deficiency is alarming and will need to be further addressed. This will be important for the health of the women, children in this study, and for any future intended pregnancies – as B12 deficiency is associated with numerous adverse pregnancy outcomes, including intrauterine growth restriction, preterm delivery, neural tube defects (NTD)[17, 18]. Additionally, exposure to B12 deficiencies *in utero* are also associated with later in life health outcomes in children, including immune function impairment, and neurological and cognitive functions, among others[19]. Furthermore, the results presented in this work are valuable in their overall contribution to the limited knowledge of B12 levels in pregnant women and the prevalence of B12 deficiencies in Mexico, as there are no global estimates for deficiencies for both B12 and folate.

The third major finding from Chapter IV is that contrary to what might be expected with such a high prevalence of B12 deficiency, such deficiencies were not associated with maternal metabolism of iAs. However, levels of B12 were associated with maternal total (U-tAs). Additionally, despite an overall high prevalence of folate sufficiency in pregnant women, children born to women in the lowest tertile of serum folate had significantly higher %C-MMA as compared to those born to folate replete women. This finding is interesting as it represents a potential dose response relationship between maternal serum folate levels and cord serum MMAs. Furthermore, maternal Hcys levels were positively associated with both total As and percentages of MMAs in neonatal cord serum. Higher levels of biomarkers of %MMAs in association with higher levels of Hcys biomarkers have been observed in other iAs exposed populations [7]. This finding could be related to the fact that with a moderate elevation in total homocysteine, there is a positively associated parallel increase in S-adenosylhomocysteine (SAH) concentrations, and it is known that SAH accumulation is a

strong inhibitor of methyltransferases[6], in particular S- Adenosylmethionine (SAM)[20]. Therefore, inhibition of SAM would lead to decreased methylation of iAs, resulting in higher proportions of MMAs. These findings are concerning as higher levels Hcys and higher levels of maternal percentages of MMAs are both associated with adverse birth outcomes. However, it is not yet known how potential alterations of cord serum arsenicals with increases in Hcys levels could influence neonatal health outcomes. The results from this study indicate that maternal OCM status may influence neonatal metabolites of iAs and warrant further investigation of these relationships as they relate to neonatal health.

Limitations of Chapter IV

There are several limitations from Chapter IV that may influence the interpretations and generalizability of these results. These include, two broad limitations: the first relates to the use of serum markers for estimates of OCM, and the second relates to potential residual confounding.

The first limitation from Chapter IV is that data regarding the nutritional biomarkers is limited by both partial nutritional information from the questionnaire data (as the original study was not designed to fully elucidate dietary data), and by a one-time measurement of the serum markers of the OCM indicators measured at delivery. These limitations could result in possible exposure misclassification, potentially underestimating the relationship between OCM indicators and maternal metabolism. Because levels of OCM indicators can change over the course of pregnancy[21], the findings here of deficiencies in B12, a general lack of folate deficiencies, and few women who had high Hcys levels should be interpreted with caution and warrant follow-up. However, despite potential variations in these serum levels, measurements of biomarkers for B12, folate, and Hcys are currently the standard for estimating deficiencies in clinical settings. Additionally, biomarkers in serum can be more

accurate and precise than other commonly used assessments of nutritional status such as

dietary recall data and/or food frequency data collected from surveys, questionnaires, and/or

interviews. Furthermore, in support of the biomarker data as they relate to potential dietary

intake of the nutrients, a recent study determined that among women who were of

reproductive age, daily B12 and folic acid intakes were positive predictors of levels of serum

B12 and folate[22]. Despite such correlations, without knowing the sources of foods that

contribute to the levels of OCM indicators measured in this study, we are limited in our

ability to extrapolate information regarding public health recommendations to potentially

reduce and mitigate nutritional imbalances for pregnant women in this area. In the likelihood

of having full dietary data, extrapolations of deficiencies would have their own limitations,

including inconsistencies in self-reporting of nutritional data, which could potentially

introduce recall bias. Furthermore, it would be difficult to fully elucidate the causes of the

deficiencies found in this study without further information as B12 and folate deficiencies are

multifactorial, and can be due to inadequate nutrient intake from foods, overall poor diet

quality, low bioavailability and/or gastrointestinal infections, differences in metabolism

based on genetic factors, and other factor[11, 23]. Therefore, future investigators will need to

consider all factors that may influence B12 deficiencies in this area and for other populations

during the prenatal period.

The second limitation from Chapter IV is that we did not capture other potential

biomarkers involved in OCM or other nutrients/nutritional statuses that may influence iAs

metabolism, as there are many interactions that can influence this process. This could

potentially underestimate the influence of OCM on iAs metabolism in this cohort, as OCM

depends on adequate intakes of multiple nutrients. For example, previous studies in iAs

exposed populations have found associations between the levels of urinary selenium and plasma α-tocopherol, folate, and cysteine with proportions of arsenic species in urine, and that dietary protein, iron, zinc, and niacin are associated with urinary MMAs and DMAs (as reviewed by [24]). In addition, there are other mechanisms of nutritional regulation of iAs metabolism, including influences of nutrients on oxidative stress and epigenetic regulation. For example, previous work has suggested that antioxidant levels in the body could protect against arsenic-induced toxicity, as toxic effects were reduced with the administration of ascorbic acid, a-tocopherol plant extracts, flavonoids, polyphenols, and/ or selenium (as reviewed by [24]). Such considerations will need to be factored into future study designs to fully elucidate the many nutritional associations with iAs metabolism.

The last limitation from Chapter IV is that we cannot rule out any potential residual and/or unmeasured confounding of the relationship between OCM indicators and shifts in iAs metabolism. An example of a potential unmeasured confounder is the influence of polymorphic variations in genes that may regulate metabolism of both nutrients involved in OCM and iAs. However, this is a limitation that we plan to address within this cohort (see future studies). Important to the findings of this work are that in attempts to reduce any residual confounding, adjustments were made for the available demographic data and included key factors that may influence both OCM and iAs metabolism, as were identified based on previous studies and a DAG. Furthermore, careful attention was made to assess changes in estimates based on the covariates added to the model, therefore the models are well specified. Another factor that could limit any residual confounding is that there was overall homogeneity within the population studied.

Given the potential for a large prevalence of B12 deficiency in this pregnant population and the possibility for adverse health impacts for both the mother and any future pregnancies from these deficiencies, these limitations will need to be considered for future studies.

Strengths of Chapter IV

There are many strengths of the work carried out in Chapter IV of this thesis that aide in informing potential biological mechanisms of how nutrients involved in one carbon metabolism influence the levels of iAs and may inform future studies and potential interventions of iAs-associated diseases.

The first strength of the work in Chapter IV is the use of two different matrices for biomarkers of prenatal iAs exposure, which supports that infants born to these women were exposed to iAs during a sensitive developmental period. Furthermore, the use of urinary markers for metabolism of iAs are well established biomarkers for exposure, and is non-invasive. Additionally, identifying metabolism of iAs represents key biological mechanisms of potential detoxification. While it is known that shifts in iAs metabolism occur in pregnancy with increases in metabolism efficiency towards the end of pregnancy, any differences between trimesters of pregnancy would not influence individual shifts, as all samples represented urinary collection immediately before delivery, therefore would likely not influence our findings. Furthermore, there was likely little misclassification of the implied source of exposure to iAs, as the women had high levels of iAs in their drinking water samples, and there was significant correlation between iAs in drinking water and iAs levels in urine (r_s=0.51, $p<0.001$). This is supported from previous research in the study area that also concluded that drinking water is the main source of iAs exposure in this area[25-28]. While other studies have demonstrated that dietary sources can influence iAs levels, a

previous assessment of dietary iAs exposures in this region estimated that most of iAs

exposure that did occur from foods, rather was dependent on the amount of water used and

cooking time, as most foods were boiled or cooked with water before consumption.

Furthermore, any contamination from seafood would be minimal as marine foods are not

usually consumed in this area[29]. This is also supported by the finding that few women (22%)

consumed seafood in this cohort. Therefore the exposure data collected here represent a vast

matrix of exposure classification via the levels of iAs from drinking water, maternal

metabolites of iAs in urine, and fetal cord serum iAs with little potential of differential

exposure misclassification.

The second strength of the work in Chapter IV is in the ability to generalize our

results to a large population of pregnant women – for most of the women in the study were

sufficient in folate. This is similar to many other pregnant populations, specifically in the US

and other populations living in the western world where foods are highly supplemented with

folic acid. This filled a significant research gap, as the only other previous studies to examine

pregnant women's OCM indicators status and iAs metabolism are from two populations in

Bangladesh where their exposure levels to iAs from drinking water is extremely high and the

women were severely malnourished.

The third major strength is that this study was conducted in a relative "low exposure"

to iAs population, therefore results may be more generalizable to several populations that are

exposed to iAs globally- particularly in the U.S, where low level exposures to iAs are

common.

The last major strength of the work in Chapter IV is in the statistical methods used to

investigate the differences in iAs metabolism as outcome. Previous studies have modeled iAs

metabolism without addressing the concern of applying traditional linear regression models to these types of data with boundaries of the interval (i.e., 0 or/and 1). We used beta regression modeling, which has been previously been used for percentage-scaled dependent variables due to its flexibility in capturing various skewed unimodal and bimodal distributions, especially when normalizing and other transformations do not work well[30].

Future directions related to Chapter IV

Future directions of research to investigate the relationship between OCM and iAs metabolism that goes beyond the scope of this work incudes utilizing existing data and/or banked samples to identity other nutritional impacts of iAs metabolism, addressing potential unmeasured confounding, integrating existing OMICs data into the data presented here, and identifying potential sources of deficiencies for future preventions of OCM deficiency associated outcomes. There is need to further investigate the relationships described in this present study, as despite our findings that many of the OCM indicators were not strongly associated in this cross-sectional analysis with maternal metabolism of iAs, we cannot rule out that OCM does not impact iAs metabolism in pregnant populations. Additionally, because we found associations with OCM indicators and fetal metabolites, further analysis within this dataset of these relationships is warranted. Given that there is potential to increase methylation capacity with OCM indicators (e.g. increases in %U-DMAs), thus reducing tissue exposures and concentrations of iAs and iAs-associated toxicity, investigations are important for future studies.

The first future direction for the research related to Chapter IV includes incorporating existing data that has been previously collected that may influence iAs metabolism in the BEAR cohort. Specifically, there is data on the levels of other metals that may act as either a co-toxicant or as other important nutritional markers, including selenium and manganese as

measured in maternal urinary samples. Selenium is another nutritional factor known to influence iAs metabolism. Furthermore, there is data on urinary creatinine concentrations, which has previously been used as a proxy of macronutrient status, as it is related to muscle mass and intake of meat – as protein intakes are known to influence iAs metabolism[6]. Previous studies observed that urinary creatinine was a strong predictor of As methylation,[31] and individuals exposed to iAs in drinking water with lower urinary creatinine levels are at increased risk for As-induced skin lesions[32]. Combining these data with the nutritional serum markers described in Chapter IV could be used to create a more complete nutritional profile of the women in the BEAR cohort and will be interesting to analyze, especially with the high prevalence of B12 deficiency.

The second future direction for research in relation to the findings of Chapter IV, would address genetic differences that may underlie the relationship between OCM and iAs metabolism, by genotyping the mothers for known polymorphisms that may influence both OCM and iAs metabolism. For example, it is well known that common polymorphisms in the gene that regulates enzymes for methylenetetrahydrofolate reductase *MTHFR* (C677T) play a critical role in OCM. Previous work has identified that individuals with the *MTHFR* 677TT genotype have 70% reduced activity of MTHFR in comparison to the *MTHFR* 677CC genotype, resulting in the reduction of the rate of 5-methylTHF production[33]. Additionally, previous studies have indicated that MTHFR influences iAs metabolism in adults[34]. Furthermore, in studies of mice, maternal genotype contributed to the sensitivity of iAs embryotoxicity – measured by implantation, resorption, congenital malformation and fetal birth weight in a *MTHFR* mouse model[35]. It has also been hypothesized that individuals with a genotype associated with less efficient methylation of arsenic, such as variants inorganic

arsenic methyltransferases (*AS3MT)* may be more sensitive to interactions of iAs metabolism with poor nutrition[24]. We have genotyped both the moms and infants in the BEAR cohort for variants of *AS3MT,* and therefore can investigate the relationship between genotype for *AS3MT* and OCM indicators, as well as OCM indicators associated genes such as *MTHFR* and *AS3MT* for gene-gene interactions. The support for the role of genotype of both OCM indicators and other iAs-associated genes in influencing iAs metabolism in human pregnancy cohorts has not been fully elucidated.

The third future direction for research in relation to Chapter IV would be to explore potential relationships between OCM indicators and OMICs data, as other studies within the BEAR cohort have focused on OMICs data. In a subsample of participants, there is data on the epigenome, transcriptome, proteome, and metabolome. We will estimate the influence OCM indicators on these OMICs biomarkers in relationship to iAs exposure and metabolism in this cohort. Interestingly, in preliminary data, I investigated the overlap of expressed microRNAs in neonatal cord blood in the BEAR cohort to B12 and folate and %U-MMAs, and there is evidence of shared microRNAs between %U-MMAs, folate, and B12. Specifically, the expression of microRNAS was negatively associated with B12 and positively associated with U-tAs (data not shown). Additionally, in preliminary investigations of a targeted analysis to examine the relationship between *KCNQ1* methylation and B12, we found that as the levels of B12 decrease, expression of KCNQ1 also decreases, and as levels of U-TAs increase, expression of *KCNQ1* increases (data not shown). These preliminary results are fascinating; methylation of *KCNQ1,* a maternally imprinted gene known to regulate birthweight – thus *KCNQ1* may be a candidate gene influencing iAs-

associated shifts in birthweight. Given these preliminary findings, further investigations of these relationships are warranted.

The last future direction for research in relation to Chapter IV would be to investigate other sources of B12 deficiency that would also influence iAs-associated diseases. Specifically, from pilot data in this cohort of measurements of maternal exposure to mycotoxins (aflatoxin), results suggest that some of the women in the BEAR cohort may have been exposed to aflatoxin – likely via contaminated corn and corn products. There is the potential to investigate this further as it relates to the levels of OCM indicators, as mycotoxins are also known to influence B vitamins and B12 deficiencies.

Such integration of these findings in the context of other data could represent an exciting and unique ability to investigate interactions between multiple mechanisms of OCM indicators, other nutritional data, genetic and epigenetic mechanisms, and other toxicants in relation to iAs exposure and metabolism. Results would significantly strengthen the investigations of biological mechanisms that underlie iAs metabolism, reduce any potential unmeasured confounding, and could be applied to better understand iAs-associated diseases.

Major findings of Chapter V

The second aim of the thesis builds off of both the findings presented here in Chapter IV and previously published work in our lab that maternal metabolism of iAs (%U-iAs and %U-MMAs) was associated with infants' birthweight in the Biomarkers of Exposure to Arsenic (BEAR) pregnancy cohort. Furthermore, it is known that nutrients involved in one carbon metabolism (OCM) can influence both iAs metabolism[5, 36, 37] and fetal growth[12, 21, 38-43]. Therefore, the work in this chapter sought out to clarify these two potential biological mechanisms on shifts in infant birthweights using a causal mediation approach. This work addressed two sub aims. The originally proposed aim of this work (**Aim 2a**) was to estimate

the total effect of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, homocysteine) as measured in maternal serum at delivery on infant birthweight, where the total effect is any effect that operates through the path of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, homocysteine), iAs metabolism, and birthweight. To address this aim using a causal mediation approach, we estimated the total effect (TE) as proposed, and the natural direct effects (NDE), of OCM indicators on infant birthweight, and controlled direct effects (CDE) of both OCM indicators and maternal metabolism of iAs on infant birthweight. **The hypothesis of this work was that levels of maternal serum OCM indicators would be associated with changes in overall infant birthweights, independent of iAs exposure**. Specifically, deficiencies in both folate and B12 and increases in Hcys were predicted to be negatively associated with infant birthweight. The original proposed **Aim 2b** was to estimate the indirect effect of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, cysteine, homocysteine), as measured in maternal serum at delivery, on infant birthweight that operated through iAs metabolism. To estimate this, the natural indirect effects (NIE) and proportions mediated (PM) by the arsenicals were estimated. **The hypothesis of this aim was that the effects of maternal nutritional biomarkers involved in one carbon metabolism (B12, folate, homocysteine) on birthweight operated via the pathway of iAs metabolism for changes in infant birthweights.** The investigation of these mechanisms in a causal framework had not previously been demonstrated.

The first major finding from Chapter V was that B12, folate, and Hcys when examined independently of one another, did not have a statistically significant effect on infant birthweight z-scores. However, maternal imbalances in B12 and folate together

influence birthweight adjusted for gestational age z-scores, where there were differences in mean birthweight z-scores across the four categories representing the interactions of lower and higher B12 and folate. Furthermore, this relationship was supported from tests for effect measure modification by median folate levels. Specifically, the average birthweight z-score was -0.74 [95% CI: -1.2, -0.31] lower for newborns born to women who were deficient in B12 relative to those born to women who were sufficient in B12-specifically for women who also had lower folate serum levels. These results suggest that the relationship between B12 deficiency and infant birthweight z-scores is modified by folate levels; therefore, future studies should consider this important biological factor in the relationship of B12 deficiency and infant birthweight. Specifically, _not_ including this important modifier (folate) of the relationship of B12 deficiency and infant birthweights could lead to incorrect estimates and effects of the relationship of maternal levels of B12 on infant birthweight.

The second major finding from Chapter V was that there were significantly stronger effects of B12 deficiency on infant birthweight z-scores when folate was lower, but not when folate was higher from the mediation analysis. Specifically, the natural direct effect estimates for B12 deficiency was a strong predictor of a negative association with birthweight z-scores when folate was lower (total effect of -0.74) compared to when folate was higher (total effect of 0.29). This is interesting as in the mediation analysis, without the consideration of folate, there was no evidence of statistically significant direct effects of B12 deficiency (total effect of -0.29). This finding further supports that levels of folate modify the effects of B12 deficiency on infant birthweights. Taken together these two major findings suggest that independent of iAs exposure, maternal folate serum levels modify the impacts of B12 deficiency on infant birthweight z-score in this population. Previous studies have also

indicated that imbalances in folate/vitamin B12 ratios identified in maternal red blood cells during and at the end of pregnancy have been associated with lower birth weight, spontaneous abortion, placental abruption and congenital malformations[14].

The third major finding from Chapter V was that results from the causal mediation analysis demonstrated that while there were no statistically significant mediated effects by maternal iAs metabolism in the relationship between OCM indicators and birthweight z-score, there is evidence of interaction between OCM indicators and iAs metabolism. These results suggest that within a B12 deficient population, maternal arsenic metabolism may interact with B12 levels, thus changing the effect of the B12 on birthweight z-score, with this effect further amplified when folate levels are lower. Specifically, when the levels of maternal arsenicals were set to levels of either the median and/or the 90th percentile (for %U-iAs, and %U-MMAs) and 10th percentile for %DMAs to simulate altered maternal metabolism of iAs (the controlled direct effect), we find that there is evidence of potential interaction of %U-iAs, %U-MMAs, and %U-DMAs and B12 deficiency on infant birthweight. These simulated findings are interesting as previous research from a clinical trial indicates that folate can increase arsenic metabolism[5]. These findings could have vast public health impacts in iAs exposed populations as they demonstrate that there are interactions between pregnant women's nutritional status and maternal metabolism of arsenic on infant birthweights. These complex biological interactions have not been previously identified in iAs exposed pregnant populations.

These estimated shifts of infant birthweight are ever important as they may lead to early life and later in life health effects. These susceptibilities to later in life diseases from prenatal exposures (e.g. fetal origins of adult disease (FOAD)) has been theorized to have

major impacts from birth and throughout the life course, and has been supported by several studies [44]. Additionally, while low birthweight is not the phenotype measured in this work, the data do represent potential shifts in birthweight that could indicate that lower birthweight could occur on a population level. While not assessed here, shifts in infant birthweights could be followed by "catch-up growth", a term that refers to exponential childhood growth – with associated increased risks for metabolic syndrome, obesity, insulin resistance, dyslipidemia, and hypertension[44-48]. This is a particular concern for the study population that this cohort represents, as the prevalence of both low birthweight and small for gestational age (SGA) is relatively high (~10%) in Mexican populations[49]. Additionally, shifts in birthweights on the other end of the spectrum can also represent impacts from being born bigger and/or large for gestational age (LGA), and these are associated with increased risks for diseases, including obesity and insulin resistance. Given the potential for a multitude of lifetime health effects from shifts in birthweights, establishing the underlying causes are critical in reducing these large public health burdens. Therefore, the results of this work may add critical information of some of the potential underlying mechanisms of birthweight shifts in this population in Mexico. With replication and further support, these results could influence public health interventions.

Limitations of Chapter V

Limitations of the work presented in Chapter V also encompass the limitations of the work for Chapter IV of this thesis (discussed above). However, specific to the results presented in this chapter, there are additional limitations in the exposure, mediator, and outcome assessment. These include limitations surrounding using OCM indicators as a proxy for nutritional data, the measurement of maternal metabolism of iAs as a potential mediator,

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and birthweight as an outcome. The second area of these limitations surrounds the causal interpretations of the findings, especially given the design of the study.

The first limitation of Chapter V is in the use of OCM indicators as a proxy of overall one carbon metabolism. This limits public health recommendations and the application of our findings for future interventions. From the literature, there is little data on the correlation of the levels of serum markers of these OCM indicators to extrapolate to the potential levels of nutritional supplements or diets that may have contributed to the serum levels of OCM indicators examined, particularly during the prenatal period. However, a recent study indicates that among women who were of reproductive age, daily intake of B12 and folic acid were positive predictors of serum B12 and serum folate[22]. Therefore, these data presented in this work likely represent what would have been current intakes of B12, folate, and their combined effects on the levels of Hcys. Furthermore, we can broadly make recommendations for women in this cohort to further examine their diets and nutritional status. As previously mentioned in the discussion of Chapter V, we cannot conclude that these levels are representative of the levels of serum OCM indicator levels over the course of pregnancy, as it is known that there are shifts in these markers – leading to potential misclassification. These shifts could not only influence our findings (as previously mentioned), but also our ability to make recommendation to remediate these potential nutritional deficiencies. However, currently, serum markers are the standard for OCM indicators and in determining deficiencies, regardless of trimester for pregnant women. Future studies, particularly for mediation analyses, would benefit from repeated measures of the OCM indicators over the course of pregnancy and to gather nutritional intake data to capture the amounts of nutrients foods and/or vitamin intake that contribute to serum OCM

indicators. Such correlations could be applied to associations with infant birthweights to make public health recommendations for pregnant women.

The second limitation of Chapter V that may influence the interpretations of causality are related to the study design. The cohort was developed to be a prospective cohort, and there is potential for follow up in this study; however, in the context of this work, the samples that contributed to the measurements of maternal serum estimates of exposure to OCM indicators, the mediator of maternal metabolism of iAs, and the outcome of infant birthweight were measured at the time of admission to the hospital for delivery and after delivery – therefore, the data are cross-sectional by design. This reverse causality between the mediator and the exposure cannot be fully dismissed, and therefore results should be interpreted with caution, as the causal criterion of temporality may be a factor that is not met by these data. However, the exposure of OCM indicators was measured in maternal serum, an indicator of recent OCM nutritional intake, and the potential mediator was iAs metabolism in maternal urine – also an indicator of recent iAs exposure, therefore it would be highly unlikely that the levels of iAs in urine would influence the OCM indicators in serum. Additionally, while the biomarkers of exposure and mediators were measured at the same time of the outcome of infant birthweight, indicators of exposure would precede the outcome. Furthermore, given that we did not find mediation of the relationship between B12 deficiency and infant birthweight by iAs metabolism, and instead potential evidence of interaction of iAs and B12, the simultaneous measurements may relax strict assumptions of temporality regarding mediation. Additionally, because recall bias (i.e. for typical questionnaire assessments for nutritional data) and potential measurement error (i.e. of the nutritional serum markers) is a major concern in using cross-sectional data (and in particular for

mediation analysis), such concerns would not be a factor in the interpretation of our findings

by using biomarker data of the nutritional OCM indicators. However, if there are changes in

OCM indicators and metabolism of iAs, the mediation of such could be underestimated,

especially if this occurs more readily in the first trimester of pregnancy. Given these

limitations, we acknowledge that the biological interpretations of the exposure-mediator

relationship are limited and assumptions of temporality in terms of measurements for

exposures and potential mediators are unlikely to accurately reflect longitudinal mediation

effects. Therefore, future studies will need to consider these limitations in study design and

attempt to obtain repeat measurements. While the design of a prospective cohort study to

gather repeat measures would be beneficial, would in itself introduce further complexity (to

intervene), as it would be unethical to not inform women of their exposures levels for both

OCM indicators and iAs.

The third limitation of Chapter V is in the assumption made that there is no

unmeasured confounding in regards to application of causal inference methods used here –

this could have potentially biased the results, and influence the interpretation of the findings

of Chapter V. Specifically, the estimation of direct and indirect effects, and subsequent

causal interpretations require that there be no unmeasured exposure-outcome, mediator-

outcome, or exposure-mediator confounders[50]. Some of the potential exposure-outcome

mediators that could be missing are mentioned above (e.g. other nutritional measures not

captured in this study); however, there may be others as well. There may also be unmeasured

potential confounders of the mediator-outcome relationship, and specific to these findings,

this can lead to biased estimates for the controlled direct effect[51].

The fourth limitation of Chapter V is in the small sample size of the study to detect statistically significant mediation and/or interaction. Furthermore, because we found modification of the main exposures of B12 and folate, we targeted the mediation analysis to describe effects of iAs metabolism within the different strata of B12/folate; this further reduced our sample size, limited our ability to detect statistical mediation and led to imprecise estimates, with very wide confidence intervals. Therefore the results should be interpreted with caution and replication in larger cohorts is warranted. This will be particularly important in the estimation of the total effect, as the association with the exposure in a given population depends on the population prevalence of the mediator, and despite the effort to simulate poorer metabolism, we were limited as in this population, most women were efficient at metabolizing iAs (a good thing). Additionally, because of the high prevalence of the exposure of B12 deficiency, this limited the ability to effectively model the data as a potential positive exposure change, specifically modeling shifts from B12 deficiency to sufficiency on the impacts for increased methylation capacity of iAs. Given the potential importance of interaction between OCM indicators and iAs metabolism on infant birthweight, future studies would benefit from modeling potential mediation of improved nutritional status on iAs metabolism as it relates to birth outcomes.

The last limitation of Chapter V surrounds the use of birthweight adjusted for z-scores as an outcome. Specifically, there is the potential for measurement error, and there are limitations in the interpretability of estimates that measure shifts of birthweight that are adjusted for z-scores. The benefits of using z-score birthweights is that it can counter issues surrounding adjusting for unstandardized birthweight. However, a caution for the use of z-scores in birthweight adjustments is that there could still be confounding the of coefficients

and there is a loss of interpretability[52]. There is also the potential of the misclassification of gestational age, which would influence the outcome of gestational age adjusted for z-scores. In this study, gestational age was estimated using the women's last menstrual period (LMP) verses other methods that may be more precise (such as ultrasound measurements). Therefore there may be some measurement error, and misclassification in the birthweight for z-score gestational ages used for our outcome. However, any estimates in this would likely be non-differential, thus potentially driving the estimates towards the null. Strengths for the use of birthweight for-gestational age z-score are that they do allow for comparison with other cohorts, domestic and international.

Given these limitations, the results and conclusions from the work in Chapter V should be interpreted with caution. In particular, from this work we may not be able to draw conclusions of causality, given the above limitations; therefore, the interpretations of the results presented in this chapter are based more on estimation and description of the potential effects of maternal shifts in vitamins and nutrients that influence OCM, maternal shifts in metabolism of iAs, and shifts in population infant birthweights.

Strengths of Chapter V

Despite the limitations of Chapter V, there are several strengths of this work; many of these are outlined in the conclusions of Chapter IV; however, there are further strengths unique to this chapter.

The first strength of Chapter V surrounds the choice of mediation methods applied to estimate both the direct and indirect effects of OCM indicators and maternal metabolism of iAs, on population shifts in infant birthweight z-scores. Specifically, by applying methods of causal mediation vs standard approaches (e.g. Baron and Kenny) to investigate mediation, we were able to model the relationships between the exposure, mediator, and outcome, allowing

for exploration of non-linear models and potential important exposure-mediator interactions[53]. Furthermore, by examining the controlled direct effects, we were able to model how much birthweight would change on average when the mediator of maternal metabolism of iAs (%U-iAs, %U-MMAs, %U-DMAs) was set to a level of poorer metabolism. The findings from these models supported that there is potential interaction between the exposure of OCM and maternal metabolism of iAs. The finding of interaction serves to answer questions for whom these causes and effects occur. The results from this chapter of the interactive models indicate that the effects of B12 deficiency on infant birthweight occur when populations are folate deficient, but are further affected when populations also have poorer iAs metabolism. These biological interactions are imperative to understanding mechanisms associated with prenatal iAs exposure and population shifts in birthweights, and may be applied to investigate other iAs-associated health outcomes.

The second strength of Chapter V is in the estimation of mediated effects of OCM and infant birthweight in the context of a modified relationship of B12 and infant birthweight by folate levels. The investigation of B12 and folate interaction is a huge strength of this study, as previous work has suggested that in populations supplemented with folic acid, there are interactions between imbalances of folate and B12. Such interactions are extremely relevant in multiple populations where the supplementation of folic acid is widespread and there are increases in B12 deficiencies.

The last strength of Chapter V is the use of biomarkers in the context of mediation analysis. While it is a limitation that we cannot necessarily extrapolate our results to target a public health intervention (i.e. because of not knowing the levels of the nutritional intakes for the OCM indicators), the strengths of using biomarkers are that they may better inform

mechanistic studies; therefore, future studies should incorporate both nutritional data and biomarker data to inform interventions. Furthermore, by using metabolism of iAs, instead of exposure alone, we are able to see the effects on and of a biological mechanism that has been demonstrated to be more important than the levels of exposure to iAs alone, and in particular for shifts in birthweight.

To my knowledge, only one other study has investigated the role of mediation in the context of environmental exposures during pregnancy[54], thus this is a highly unexplored use of these methods in the vast fields of both environmental and reproductive/perinatal epidemiology. This method is incredibly useful for the target population of pregnant women, as it is as period of high susceptibility for the impacts of exposures of both nutritional and toxic substances. Furthermore, many study designs make establishing causality in these target populations extremely difficult because of ethical and practical limitations of studying pregnant women and *in utero* exposures, and due to the many complex biological changes unique to this period. Therefore, the application of causal mediation and interaction methods are imperative to future studies of environmentally associated diseases from prenatal exposures.

Future directions related to Chapter V

Future directions of this work include studies to address some of the potential limitations of the presented here in Chapter V, and of those from Chapter IV. These include, the application of a sensitivity analysis to investigate any residual or unmeasured confounding, investigating other potential mediation/modification analyses, and replication of these findings in another cohort(s).

The first direction of future work related to Chapter V is to address the potential limitations of unmeasured confounding. One way to address potential unmeasured and/or

residual confounding is to employ sensitivity analyses. There are several methods to estimate

such for the measured effects of mediation, as have been outlined by Vanderweele[55]. While a

sensitivity analyses could be applied to all estimated effects, it will be particularly important

to do such for the controlled direct effects, as these findings highlight potential interactions

between the exposure of OCM indicators and iAs metabolism on infant birthweight. An

example of a potential way to address this could be to use newly collected genetic data that

may influence OCM and iAs metabolism, as proposed in the future studies section from the

conclusions of Chapter IV. Specially, there is the potential that polymorphisms in genes that

may influence both OCM and iAs metabolism may be unmeasured confounders or even

mediators of the relationship between the exposure (OCM indicators) and the mediator (of

iAs metabolism). Therefore, once key variants of polymorphisms are identified, we could

conduct sensitivity analyses to measure a potentially biased estimate and compare it to the

estimates presented in Chapter V. Furthermore, it may be that genotype for certain genes that

may regulate OCM could actually be investigated as intermediates of the aim 1 from Chapter

IV. We could test in a causal mediation approach whether genetic polymorphism may

mediate or modify the relationship between OCM and iAs metabolism. These analyses may

address a limitation of employing a cross-sectional analysis as genotype is fixed at birth and

therefore would not be caused by the exposure of OCM.

A second direction of future work related to Chapter V is to investigate the potential

for mediation and/or interaction as investigated here in Chapter V, but with neonatal cord

metabolites. This approach furthers the aims of this thesis, where the mediation analyses

presented in this Chapter could be applied to the findings of Chapter IV of the relationship

between maternal OMC indicators and the cord serum metabolites of iAs on infant

birthweights. This would be interesting as the findings from Chapter IV imply that maternal OCM indicators influence neonatal iAs metabolites. Furthermore, it is not yet known how the levels of fetal metabolites influence birth outcomes.

A third potential area of future research related the work of Chapter V would build on one of the proposed future studies suggested in Chapter IV, where we could develop a model of a nutrient phenotype constructed from the data used in this study in combination with other existing data/and or future data. Furthermore, there is the potential to measure other factors in banked samples, to address issues in instability of the B12 serum markers- for example, Holotranscobalamin has been demonstrated to be a potential sensitive indicator of vitamin B12 status, and in pregnant populations where B12 levels decreased over the course of the pregnancy, holoTC levels remained constant in women with an adequate intake of vitamin B12[56].

A fourth direction of future work related to Chapter V would be to replicate these findings in other pregnancy cohorts. Such replication will be carried out in another iAs exposed birth cohort from an existing collaboration with our lab within the prospective longitudinal cohort study, the New Hampshire Birth Cohort (NHBC) study. Benefits of replicating the mediation methods presented in this thesis in the NHBC cohort could potentially address many of the limitations presented here. Specifically, by investigating mediation analysis, the NHBC cohort would address the limitation of sample size of this thesis work presented here, as there are data on 1200 women and children exposed to low levels of iAs from drinking water. Furthermore, the limitation of temporality could be addressed, as nutritional data was collected before the iAs measurements and in the form of food frequency questionnaire data (with some repeated measures). There is also the potential

to use banked samples to measure OCM indicators from various tissue types in the NHBC cohort. Replication in this cohort could also potentially address unmeasured confounding, as the questionnaire data is vast. Depending on the distribution of the nutritional OCM indicators in the NHBC, there may also be the opportunity to target the differences by folate and B12 across the different categories (and exploration of other categories/cut points of folate levels). This would address a limitation of the mediation analysis presented in this chapter that did not capture the effects of higher B12 and higher folate, and it may be that iAs metabolism is different across these varying levels, as is suggested by the finding from Chapter IV that the %C-MMAs differed by tertiles of folate. Additionally, results from this chapter indicated that women in the higher B12 and higher folate levels had newborns with the lowest mean birthweight z-score. These results warrant further investigation. Using a US based cohort may also help with generalizability to the US population, as the findings relate to both the levels of iAs exposure in similar populations and country-wide nutritional supplementation. It will be interesting to compare results from two pregnancy cohorts and such findings could influence policy interventions.

The methods applied in Chapter V are a unique feature of this study, and given the opportunity to replicate the findings in other cohorts presents exciting opportunities to further investigate the interactions between factors contributing to one carbon metabolism and metabolism of iAs during pregnancy and their impacts on adverse birth and other pregnancy related outcomes. This also presents great prospects for potential public health interventions to reduce the health impacts of iAs associated diseases.

Major findings of Chapter VI

The work in Chapter VI builds on the work carried out in this thesis from chapters IV and V, where it was demonstrated that the levels of metabolites of iAs may be influenced by biomarkers of one carbon metabolism (see results Chapter IV) and that OCM indicators influence infant birthweight z-scores (see results Chapter V). The aims from Chapter VI focused on understanding how the overall hypothesis of this thesis was influenced by the levels of exposure to iAs, as measured in drinking water. This stemmed from the consideration that levels of exposure to iAs from drinking water may modify prenatal metabolism of iAs, and potential impacts between maternal metabolism of iAs and metabolism-associated changes in birthweight. The aim addressed in Chapter VI was **Aim 3: Investigate the total and indirect effects of maternal levels of nutritional biomarkers involved in one carbon metabolism (B12, folate, homocysteine) on infant birthweight by different levels of drinking water iAs.** This aim tested the hypothesis (***Hyp 3***): *The levels of iAs in drinking water influence the effect of maternal nutritional biomarkers and the health of her newborn.* Stratified mediation analysis were carried out to estimate the effects of all OCM indicators on infant birthweight z-scores and potential mediation by arsenic metabolism based on exposure to drinking water iAs below or at/above the EPA/ WHO guideline level of 10ppb. Knowing how OCM indicators influence birthweight across different levels of exposure could identify susceptible populations at risk. This information could be applied to risk assessments to better inform regulations for levels of iAs in drinking. This is important as regulations for levels of iAs allowed in drinking water vary by region, and are continually being reexamined. Additionally, in Mexico, iAs is currently regulated at 25 ppb. Furthermore, there is evidence that health effects of iAs may occur even at the level below the WHO guideline level.

The first major finding of Chapter VI was that Dw-iAs was significantly correlated with U-tAs in the participants of the entire cohort 0.60 (<0.001) (as previously reported), suggesting that drinking water is the primary source of exposure to iAs in this population. This positive correlation remained strong for those whose iAs levels of drinking water were above the WHO level; however, this was not observed for those with exposure to Dw-iAs below the WHO guideline level. We did not find that the urinary proportions of metabolites were significantly associated with drinking water across the cohort. Although not statistically significant, (p=0.07) %U-MMAs were negatively correlated with drinking water levels in all women, and %U-iAs was marginally significantly (i.e. slightly greater than *p* of 0.05) negatively correlated (*p*=0.051) with drinking water levels in those exposed to levels above the WHO guideline level. These results suggest that there are correlations between the levels of iAs in drinking water, but not across all indicators of maternal metabolism, nor in categories below the WHO guideline level.

The second major finding of Chapter VI is that homocysteine (Hcys) was significantly correlated with levels of iAs in drinking water. In the mediation analysis higher Hcys levels had a negative association with infant birthweight z-scores for those women with drinking water levels of iAs below the WHO guideline level of 10ppb, but not for those that who had drinking water levels of iAs higher then the WHO guideline level. Additionally, the effect estimate of higher Hcys levels (at or above the median of 6.4 μmol/L) compared to lower Hcy levels (below the median of 6.4 μmol/L) were negatively associated with infant birthweight. Additionally, the controlled direct effect of Hcys on infant birthweight when allowed to interact with %U-MMAs indicated that there was a negative relationship with infant birthweight. These data suggest the interaction of Hcys and maternal metabolism of

iAs may be particularly important at lower levels of exposure to iAs from drinking water. These complex biological interactions in the context of a dose response assessment have not been previously identified in iAs exposed pregnant populations.

Limitations of Chapter VI

There are limitations of the work carried out in Chapter VI that may influence the interpretations of the findings. Such limitations include those described more fully in Chapter V. However, there are additional limitations specific to Chapter VI.

The first limitation of Chapter VI is in the context of the statistical estimation of the relationship between drinking water levels of iAs and the proportions of metabolites in maternal urine. Specifically, we did not find that all the proportions of arsenicals were associated with drinking water levels from correlation tests. This lack of correlation should be interpreted with caution, as there are many other factors that may confound this relationship. Mainly, these were modeled in a bivariate assessment without the inclusion of other key predictors. Future studies will need to model these relationships in a manner that would account for potential confounders of drinking water exposure and metabolism of iAs and examine the potential for linear and/or non-linear trends.

The second limitation of the work from Chapter VI may lie in the choice for cutoffs of iAs drinking water levels that could influence the some of the null findings and imprecise estimates. Specifically, the differences in the relationships between OCM indicators deficiencies in the mediation analysis of the strata of those women with levels of drinking water iAs above the WHO guideline level should be interpreted with extreme caution, as the estimates are extremely imprecise.

The last limitation of Chapter VI is that we are underpowered to detect multiple modifiers/mediators in this study. Specific to the findings of this thesis, because we found

that the association between B12 deficiency and infant birthweight z-scores are modified by folate levels, future work will need to consider such interactions as they relate to the levels of exposure.

Strengths of Chapter VI

Despite these limitations, there are many strengths of the work carried out in Chapter VI, which include the previously mentioned strengths from Chapter V. Specific to this work, there was strong correlation between exposure to iAs in drinking water and the maternal biomarker of urinary total arsenic, suggesting that there is little exposure misclassification.

Future directions related to Chapter VI

Future work of Chapter VI would build on the future work described for previous chapters of this thesis. Specific to Chapter VI, the focus on further research would aim to address some of the limitations discussed above, and to better explain the impact of the underlying exposure levels of iAs from drinking water on metabolism and iAs-associated disease.

The direction for future work related to Chapter VI includes a more targeted analysis using multivariable regression models to include confounders not modeled in the bivariate assessment of this work to determine other factors that may explain the relationship between drinking water levels and iAs metabolism (gestational age, etc).

The second direction for future work related to Chapter VI would address the limitation of sample size and the potential of multiple modifiers/mediators of the effects of OCM on infant birthweight. This work would examine the potential interaction of folate and B12 in the different drinking water strata in a larger population. Although we are limited in sample size to carry this out in the BEAR cohort, such considerations could be applied to

other data – such as the potential to replicate these findings in other cohorts (as mentioned in the future direction of Chapter V).

The last direction for future work related to Chapter VI is that within this data set, we would explore other cutoffs of drinking water levels of iAs that could be used to inform policies specific to Mexico, as their MCL is much higher (at 25 ppb) than the WHO.

Overall conclusions Chapters IV, V, VI

The overall public health significance of the work of this thesis is that we have identified key factors that may explain susceptibility for shifts of infant birthweight in an arsenic exposed population. This included interactions between both nutritional and environmental exposures. The importance of understanding the underlying mechanisms of iAs-associated disease was a recent focus of the national institute of environmental health workshop aimed at identifying emerging issues and research needs to address the multi-faceted challenges related to arsenic and environmental health. The conclusions of this workshop were that there are still many research needs to address the underlying causes, and subsequent prevention, of iAs-associated diseases. This included the need to identify impacts of exposure in susceptible populations and of nutrition regulation in iAs metabolism for the potential of interventions.

The methods applied here are a unique feature to address research gaps, and given the opportunity to replicate the findings in other cohorts presents exciting opportunities to further investigate the impacts and interactions between factors contributing to one carbon metabolism and metabolism of iAs during pregnancy and their impacts on adverse birth and other health outcomes. This is a highly unexplored use of these methods in the vast fields of environmental and reproductive/perinatal epidemiology. The conclusions of this thesis work provide a great framework for potential public health interventions to reduce the health

impacts of iAs associated diseases. Importantly, because some variation of metabolism of iAs may be explained by exposure to iAs from drinking water, and/or levels of OCM metabolism attempts to elucidate dose–response still remain imperative in iAs-associated health outcomes.

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