ARSENIC, TARGETED METABOLOMICS AND DIABETES-RELATED OUTCOMES: CONNECTING THE DOTS IN THE STRONG HEART STUDY

by

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ABSTRACT

Diabetes, and related outcomes, is a global epidemic with an enormous cost both to the economy and in terms of lives lost. Exposure to inorganic arsenic, a ubiquitous naturally occurring environmental carcinogen, as well as the efficiency of its metabolism in the body, has been identified as a risk factor for diabetes development. One carbon metabolism (OCM), a biochemical pathway essential to numerous methylation reactions, including arsenic metabolism, appears to play an important role both in arsenic metabolism and diabetes-related outcomes. This dissertation aimed to better understand the relationships between each of these variables and determine whether arsenic metabolism is truly a risk factor for diabetes, or if the association is an epidemiological artifact confounded by OCM status. We used data from both the Strong Heart Study (SHS), a population-based cohort, as well as the Strong Heart Family Study (SHFS), a family-based extension of the SHS. Both populations are comprised of American Indian tribal members from Oklahoma, Arizona and North and South Dakota, exposed to low-moderate arsenic in drinking water and food, with high rates of diabetes and diabetes-related outcomes.

First, we conducted a cross-sectional analysis evaluating the association of dietary intake of OCM nutrients (folate and vitamins B_2, B_6 and B_{12}) with urinary arsenic methylation patterns (iAs%, MMA% and DMA%) in a subset (n=405) of participants from the SHS. Higher vitamin B_6 and B_2 were associated with higher DMA% and lower MMA% (i.e., a more efficient arsenic metabolism profile). We also observed an antagonistic interaction between folate and vitamin B_6 with higher folate being associated with higher DMA% and lower iAs% only in the presence of high vitamin B_6.

Second, we conducted a prospective analysis in 1,047 SHFS participants free of prevalent metabolic syndrome, evaluating the association of arsenic exposure and arsenic metabolism with incident metabolic syndrome and each of its individual components (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated fasting plasma glucose (FPG)). Arsenic exposure was associated with increased risk for elevated FPG but not with metabolic syndrome or other individual components. Arsenic metabolism patterns, independent of arsenic exposure, were associated with both incident metabolic syndrome and elevated waist circumference, but not with other components of the syndrome.

Third, we conducted a pilot (n=59) cross-sectional targeted metabolomic analysis in the SHFS. Eight metabolites were identified as having significant correlations with both a diabetes-related outcome (HOMA2-IR, FPG, waist circumference) and at least one arsenic metabolism biomarker (iAs%, MMA% or DMA%). Consistent with previous studies, higher MMA% was associated with lower HOMA2-IR and waist circumference, and higher DMA% was associated with higher HOMA2-IR and waist circumference After adjustment for the eight OCM-related metabolites, associations between arsenic metabolism and diabetes-related outcomes were substantially attenuated and no longer significant.

Fourth, we conducted a set of analyses using data from the SHFS to better understand the role OCM status plays in arsenic metabolism, diabetes-related outcomes and the relationship between the two. We first conducted cross-sectional analyses evaluating the association between OCM variables (both genetic and nutrient intake) and arsenic metabolism (iAs%, MMA% and DMA%). Next, we evaluated the associations between

both OCM nutrients and OCM-related genetic variants with diabetes-related outcomes (diabetes, metabolic syndrome, waist circumference and HOMA2-IR). OCM nutrients were not associated with arsenic metabolism in the SHFS, however, higher vitamin B_6 was consistently associated with three of the four diabetes-related outcomes studied (higher HOMA2-IR and increased risk for diabetes and metabolic syndrome). One OCM-related genetic variant (methionine synthase) was associated with both higher MMA% and lower HOMA2-IR per 5 years of follow-up. After adjustment for MMA% the association between the MTR variant and all diabetes-related outcomes were attenuated or reversed direction.

In conclusion, arsenic exposure and arsenic metabolism may be risk factors for diabetes-related outcomes, even at low-moderate arsenic exposure. OCM status may also be a risk factor for diabetes-related outcomes as well as for arsenic metabolism, although these associations may differ based on the underlying nutritional state of the population. OCM status, diabetes-related outcomes and arsenic metabolism appear to be linked; more research is needed to understand the direction of the associations, in order leverage these findings into diabetes preventative efforts.

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PREFACE

This dissertation is the final result of the research work conducted with my co-advisors, co-authors, and collaborators during my doctoral studies in the Department of Environmental Health and Engineering at the Johns Hopkins Bloomberg School of Public Health. This dissertation is organized in a manuscript format. First, we present our specific aims and then provide an overview of, and the motivations behind, this dissertation. We then review each of the analyses conducted, organized into four chapters. The first chapter evaluates the cross-sectional association between dietary intake of one carbon metabolism-related nutrients and arsenic metabolism in the Strong Heart Study, a cohort of American Indians. The second chapter evaluates the prospective association between arsenic exposure and arsenic metabolism with metabolic syndrome and the individual components that characterize the syndrome in the Strong Heart Family Study, a family-based extension of the Strong Heart Study. The third chapter uses targeted metabolomics to evaluate the association between metabolites and both arsenic metabolism and diabetes-related outcomes in the same cohort. In the fourth chapter, we investigate the cross-sectional relationship between one carbon metabolism-related nutrient intake and genetic variants with arsenic metabolism, as well as the prospective association with diabetes-related outcomes in the Strong Heart Family Study. Finally, the discussion provides an overview of the research findings, strengths and limitations to the analyses, implications of the research, proposed next steps and final conclusions.

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Finally, I would like to thank those not involved in my research, but who have supported me throughout the past four years: my fellow EHS cohort who went through each step of this experience alongside me; environmental epidemiology students ahead of me who generously provided guidance based on their experiences; my family, who have always been my biggest cheerleaders; and most importantly, my husband, who is endlessly supportive of me achieving my dreams, whatever it takes.

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ABBREVIATIONS

5-methyltetrahydrofolate (5-MTHF)
Arsenic Metabolism Principal Component 1 (As PC 1)
Arsenic Metabolism Principal Component 2 (As PC 2)
Arsenite methyltransferase (AS3MT)
Body Mass Index (BMI)
Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)
Confidence Interval (CI)
Cystathionine β-synthase (*CBS*)
Dietary Folate Equivalent (DFE)
Dimethylarsinic acid (DMA)
Directed acyclic graphs (DAGs)
Estimate Glomerular Filtration Rate (eGFR)
Fasting Plasma Glucose (FPG)
Food Frequency Questionnaire (FFQ)
Geometric mean ratio (GMR)
High Performance Liquid Chromatography/Inductively Coupled Plasma-Mass Spectrometry (HPLC/ICPMS)
Homeostasis model assessment (HOMA)
Homocysteine (Hcys)
Hypertension (HT)
Inorganic Arsenic (iAs)
Interquartile Range (IQR)
liquid chromatography tandem mass spectrometery (LC-MS/MS)
lysophosphatidylcholine (LPC)
Metabolic Syndrome (MetS)
Methionine synthase (*MTR*)
Methionine synthase reductase (*MTRR*)
Methylenetetrahydrofolate dehydrogenase (*MTHFD1*)
Methylenetetrahydrofolate reductase (*MTHFR*)
Monomethylarsonic acid (MMA)
One-Carbon Metabolism (OCM)
One-Carbon Metabolism Principal Component 1 (OCM PC 1)
One-Carbon Metabolism Principal Component 2 (OCM PC 2)
One-Carbon Metabolism Principal Component 3 (OCM PC 3)
One-Carbon Metabolism Principal Component 4 (OCM PC4)
Phosphatidylcholine (PC)
Phosphatidylethanolamine N-methyltransferase (*PEMT*)
Principal Component Analysis (PCA)
Randomized Controlled Trial (RCT)
S-adenosylhomocysteine (SAH)
S-adenosylmethionine (SAM)
Serine hydroxymethyltransferase 1 (*SHMT1*)
Single nucleotide polymorphisms (SNP)
Sphingomyelin (SM)

Strong Heart Family Study (SHFS)
Strong Heart Study (SHS)
Relative Risk (RR)
Total arsenic (∑As)
Triglycerides (TG)
Variance inflation factors (VIF)
Waist Circumference (WC)

SPECIFIC AIMS

Type 2 diabetes is a global epidemic with an estimated worldwide prevalence of 8.5%, a number which has doubled since 1980.[1] The disease affects countries at all income levels and results in an enormous global burden both in regard to the economy (an estimated US $1.7 trillion in direct and indirect costs projected over 20 years) and mortality (1.5 million deaths directly caused by diabetes in 2012).[1] Research has shown, however, that intensive interventions can prevent development of the disease. Given its magnitude and exponential growth, identifying effective preventions, particularly in high risk populations is a global priority. Individuals with metabolic syndrome have been identified as an important high-risk group, with those afflicted having five-fold greater risk of developing diabetes than those without the syndrome.[2] Homeostatic model assessment of β-cell function and insulin resistance (HOMA2-IR), a method to measure insulin resistance from glucose and insulin concentrations, has also been identified as an important predictor of diabetes.[3] Waist circumference and fasting plasma glucose (FPG), a continuous measure of diabetes, are additional useful metrics in the identification of high-risk groups.[1]

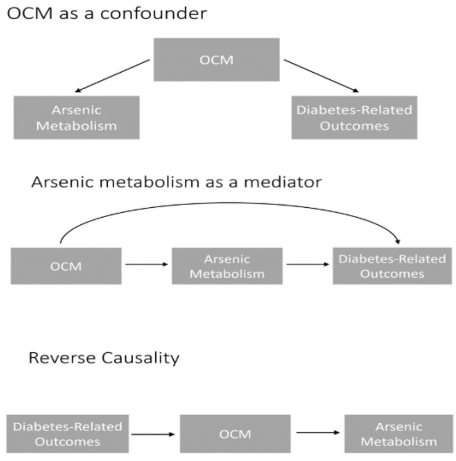
Inorganic arsenic exposure has been associated with numerous health outcomes, including cancer, adverse birth outcomes, cardiovascular disease and diabetes-related outcomes.[4-18]. After ingestion, arsenic is metabolized into mono- and di-methylated (MMA and DMA) arsenicals.[19-21] Evidence suggests that methylation of inorganic arsenic to DMA facilitates its excretion and detoxification; this is supported by findings that higher percentages of DMA (DMA%) and lower MMA% in the urine, reflecting a more efficient arsenic metabolism profile, has been associated with lower arsenic-related

health effects, particularly cardiovascular disease, skin lesions and cancer.[22-29] Randomized clinical trials have been implemented in highly arsenic-exposed regions intended to enhance arsenic metabolism (i.e., shift arsenic metabolism profiles towards higher DMA%).[30, 31] However, arsenic metabolism profiles present differently for diabetes-related outcomes, with higher DMA% and lower MMA% being associated with greater risk for diabetes, metabolic syndrome and BMI, including prospective studies.[9, 10, 14, 32-34] The mechanism behind these contrasting associations is not clear.

One carbon metabolism (OCM), an essential biochemical pathway critical to the generation of methyl groups, which are necessary for numerous biological processes, appears to play a role in both arsenic metabolism[21, 30, 35-39] and diabetes-related outcomes.[40-49] Little is known, however, on the interplay between OCM, arsenic metabolism and diabetes-related outcomes.

The main objectives of this dissertation were to understand how OCM affects (1) arsenic metabolism and (2) diabetes-related outcomes in populations exposed to low-moderate arsenic levels; and finally (3) the relationship between arsenic metabolism and diabetes-related outcomes, potentially serving as a mechanism to explain the contrasting associations between arsenic metabolism and diabetes-related outcomes versus other arsenic-related health outcomes (see Figure 1).

Figure 1. Potential relationship pathways between arsenic metabolism, OCM and diabetes-related outcomes



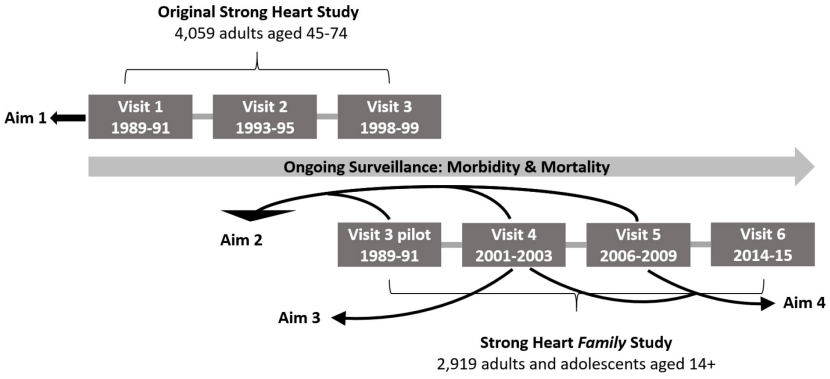
The **hypotheses** of this study were:

1) OCM status can affect an individual's arsenic metabolism efficiency, even at low-moderate levels of arsenic exposure
2) Arsenic exposure and arsenic metabolism are associated with diabetes-related outcomes, including metabolic syndrome and the individual components that make up the syndrome, in a US population chronically exposed to low-moderate arsenic levels through drinking water and food
3) OCM status, measured through OCM-related metabolites, polymorphisms or nutrient intake, can influence the association between arsenic metabolism and diabetes-related outcomes

To test these hypotheses, we used data from the Strong Heart Study (SHS), a population-based prospective cohort study designed to investigate cardiovascular disease, diabetes and their risk factors in American Indians residing in Arizona, North and South Dakota, and Oklahoma; as well as the Strong Heart Family Study (SHFS), an extension of the SHS designed to include family members of the original cohort. The SHS and SHFS serve as ideal populations to understand the relationship between OCM, arsenic metabolism, and diabetes-related outcomes given widespread exposure to arsenic through drinking water, a relatively high prevalence of diabetes and metabolic syndrome and the availability of multiple measures of OCM status.

The **specific aims** were the following (See **Figure 2**):

Figure 2. Flow Diagram of Studies



Aim 1: To evaluate the association between OCM (measured through intake of OCM-related vitamins) and arsenic metabolism. To complete this aim, we conducted a cross-sectional analysis in the SHS using data from baseline (visit 1) in 405 participants who participated in a food frequency questionnaire. This association was further evaluated in 935 participants in the SHFS, which is characterized by higher levels of OCM nutrient intake compared to the original SHS cohort.

Aim 2: To evaluate the prospective association between arsenic exposure and arsenic metabolism measured at baseline (visits 3 and 4) with incident metabolic syndrome (visits 4 and 5) as well as the five different components that characterize metabolic syndrome (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated FPG) using 1,047 SHFS participants free of prevalent metabolic syndrome at their baseline visit.

Aim 3: To evaluate potential mechanistic pathways between arsenic metabolism with diabetes-related outcomes using a targeted metabolomic approach with pilot data (n=59)

from visit 4 of the SHFS. The first part of this aim was to identify metabolites with significant correlations with both arsenic metabolism and diabetes related outcomes. The second part of this aim was to evaluate whether adjustment for those metabolites influenced the association between arsenic metabolism and diabetes-related outcomes

Aim 4: To evaluate the influence of OCM status (measured through intake OCM-related vitamins and OCM-related genetic variants from visit 4) on arsenic metabolism and diabetes-related outcomes (from visit 5). The first part of this aim was to evaluate the association between OCM-related variables and arsenic metabolism. The second part of this aim was to evaluate the association between OCM-related variables and diabetes-related outcomes. The final part of this aim was to evaluate whether the association between arsenic metabolism and diabetes-related outcomes changed after adjustment for OCM-related variables.

Given the growing epidemic of diabetes and diabetes-related conditions, efforts to understand its risk factors are critical. Arsenic exposure through contaminated drinking water and food is a significant global public health issue; both exposure and its subsequent metabolism have been identified as potentially important, but incompletely understood, modifiable risk factors for diabetes development. This dissertation provides a thorough investigation into the role OCM plays in the contrasting relationship between arsenic metabolism and diabetes-related outcomes compared with other arsenic-related health outcomes, presenting evidence that should be considered both in future arsenic

health effects prevention trials and in the context of identification of high risk groups for the development of diabetes.

INTRODUCTION

Overview

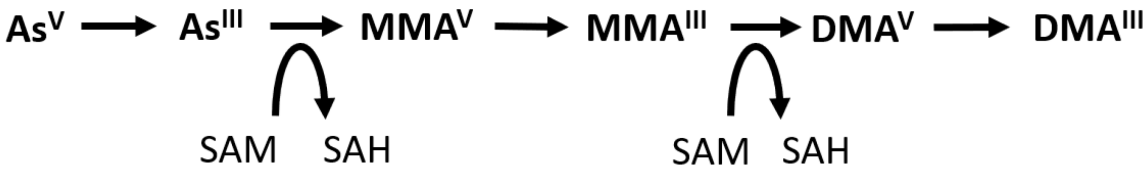
In this chapter we review the existing literature on the following topics: (1) the role of arsenic metabolism in the arsenic detoxification; (2) the association between arsenic exposure and arsenic metabolism and diabetes-related outcomes; (3) the association between one carbon metabolism (OCM) and arsenic metabolism; and (4) the association between OCM and diabetes-related outcomes

The Role of Arsenic Metabolism in Arsenic Detoxification

Arsenic metabolism is thought to play a major role in arsenic toxicity. After absorption, the inorganic forms of arsenic (arsenate and arsenite) are metabolized through reduction and methylation reactions into monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), which are excreted in the urine together with inorganic arsenic (**Figure 1**).[1-6] In humans, the distribution of arsenic metabolites in urine ranges 10-30% inorganic arsenic (iAs), 10-20% MMA and 60-80% DMA across populations.[7-11] DMA has a shorter circulating half-life and is more rapidly excreted in the urine than iAs.[1-6] Further, higher MMA% and lower DMA% in urine has been associated with increased risk of health outcomes, such as cancer, skin lesions and cardiovascular disease.[9, 12-18] Together, these findings have led to the characterization of an arsenic metabolism profile reflecting higher DMA% and lower MMA% as more efficient and protective against arsenic toxicity. Arsenic metabolism, however, is complicated by the generation of highly toxic reactive intermediates, including trivalent forms of MMA (MMA^{III}) and DMA (DMA^{III}). These trivalent forms oxidize rapidly in urine to

pentavalent forms making them difficult to measure.[19] For this reason, most epidemiological studies report concentrations of pentavalent and trivalent arsenic metabolites together. Some studies have suggested the increased risk for arsenic-related health outcomes associated with higher MMA% could be related to the high toxicity of MMA^{III}.[20-22] However, a different arsenic metabolism profile has been associated with diabetes-related outcomes. Higher DMA% has been associated with higher BMI and increased risk for diabetes and metabolic syndrome, including prospective evidence.[23-25] The mechanisms underlying this association are not well understood, particularly the converse relationship seen between arsenic metabolites and cardiovascular disease versus similar conditions, such as diabetes-related outcomes.

Figure 1. Arsenic Metabolism Proposed Pathway



Association Between Arsenic Exposure and Arsenic Metabolism with Diabetes-Related Outcomes

Epidemiologic evidence. Epidemiological studies at high (≥100 µg/L)[26-30] and moderate (<100 µg/L)[31-38] exposure levels have reported associations between arsenic and diabetes, although findings at low levels are mixed.[25, 39-41] Just two epidemiologic studies, conducted in highly exposed Taiwanese populations, have evaluated the

association between arsenic exposure and metabolic syndrome, both of which reported a positive relationship.[23, 42] Studies evaluating the association between arsenic exposure and individual components of metabolic syndrome, also have generally reported increased risk for these outcomes including hypertension,[43-47] triglycerides[48 37] and HDL[49, 50], particularly in high-exposure regions.

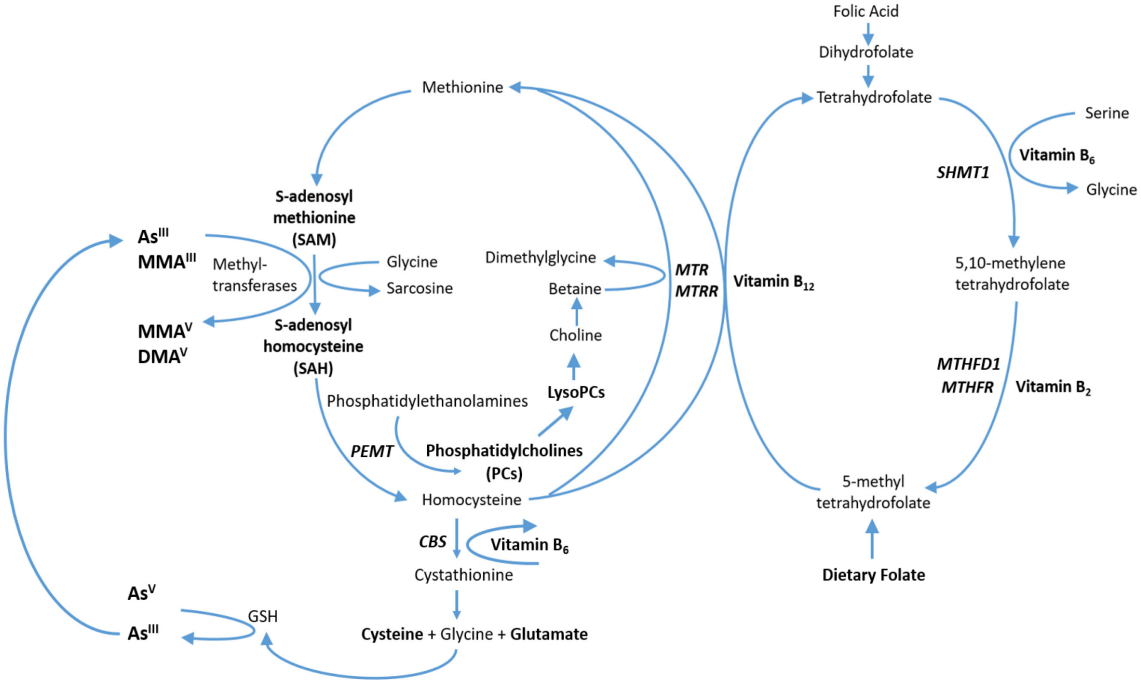
Less research has been conducted on the relationship between arsenic metabolism and diabetes-related outcomes, most of which have been cross-sectional and in highly exposed regions; however, they have consistently shown an association between an arsenic metabolism profile reflecting higher DMA% and lower MMA% with increased risk.[24, 51]

Experimental evidence. The role of arsenic in diabetes-related outcomes is supported by experimental and mechanistic evidence. Arsenic may induce diabetogenic effects through influencing the expression of gene transcription factors related to insulin signal transduction,[52-54] adipocyte differentiation and insulin sensitivity.[55, 56] Further, during arsenic metabolism, oxidative stress is induced through generation of ROS and free radicals, which can in turn activate insulin resistant pathways.[23, 42] More recent *in vivo* and *ex vivo* studies have shown that arsenic can interfere with glucose uptake at arsenic levels that are physiologically relevant to human exposures. [57-68] Together, these experiments suggest that arsenic induces insulin resistance and β cell dysfunction. *In vivo* evaluation of arsenic metabolism is difficult due to animals' faster metabolism and clearance of arsenic, resulting in lower internal doses of inorganic arsenic species and potentially making them less susceptible to arsenic toxicity than humans.[26]

Association Between One Carbon Metabolism and Arsenic Metabolism

OCM is critical in the biosynthesis of purines and thymidylate as well as the generation of methyl groups.[69] OCM facilitates the generation of S-adenosylmethionine (SAM) which serves as a methyl donor for numerous substrates which are essential to many biological processes including methylation reactions involved in the metabolism of inorganic arsenic into MMA and DMA.[69] Numerous gene-encoded enzymes are necessary for the proper functioning of the OCM pathway (**Figure 2**). Variants in OCM-related genes have been associated with variability in arsenic metabolism including methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*) and cystathionine-β-synthase (*CBS*).[70-74] OCM functioning, and adequate methyl group availability, is also dependent on essential nutrients including folate, vitamin B_{12}, vitamin B_6, and vitamin B_2, at various points in the pathway (**Figure 2**). For this reason, OCM-related vitamin intake has been hypothesized to affect arsenic metabolism efficiency. Randomized clinical trials (RCTs) conducted highly arsenic-exposed populations in Bangladesh have shown folic acid supplementation can increase DMA% in urine and reduce blood arsenic. [75, 76] Results from cross-sectional studies[77-79] have also shown associations between higher plasma folate and enhanced arsenic metabolism, consistent with findings from the RCTs. Collectively, these studies provide strong evidence for the temporality of these associations. Cross-sectional dietary intake studies of folate and arsenic metabolism from Bangladesh[80], Mexico[81] and the U.S.[82], however, have been inconsistent. Evaluation of OCM nutrients beyond folate, including vitamins B_2, B_6 and B_{12}, has not been as extensive, lacking evidence from RCTs, and findings have also been inconsistent.

Figure 2. One Carbon Metabolism Pathway with Study Measurements of One Carbon Metabolism and Arsenic Metabolism Bolded



Association Between One Carbon Metabolism and Diabetes-Related Outcomes

OCM also appears to be tightly linked to diabetes-related outcomes, although the direction of this relationship has not been established. Low OCM nutrient status has been associated with adverse health effects including diabetes incidence.[83-86] However, findings on the efficacy of OCM nutrient supplementation clinical trials to prevent diabetes have been null, [87, 88] with improvements seen only in smaller trials evaluating secondary diabetes outcomes.[89-93] Further, some observational studies have suggested high circulating folate levels may actually have deleterious effects on diabetes-related outcomes, including the offspring of mothers with high folate during pregnancy.[94-98] Studies evaluating the effect of intake of folate from foods or other OCM nutrients on diabetes-related outcomes are limited, mostly cross-sectional in design and findings are

even more conflicting,[83, 86, 99-106] highlighting the need for more research on this relationship. Genes encoding enzymes involved in OCM are also critical to the functioning of this pathway; variants in these genes have been associated with numerous diabetes-related outcomes[107-115], adding additional evidence to the link between OCM and diabetes as well as suggesting a direction of association.

Despite a clear indication that OCM-related genes play a role in risk for diabetes-related outcomes, experimental studies provide convincing evidence that the relationship between OCM status and diabetes may at least in part be due to reverse causality, and that an individual's metabolic state can also affect OCM functioning. Indeed, animal models have shown insulin resistance and diabetes can alter OCM-related metabolites and enzymes, including SAM, homocysteine and phosphatidylcholines, and that administration of insulin can prevent these perturbations.[116, 117] Together these findings highlight the complex nature of this relationship and the need for more research to better characterize the pathway between them.

CHAPTER 1

Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: evidence from the Strong Heart Study

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ABSTRACT

B-vitamins involved in one-carbon metabolism (OCM) can affect arsenic metabolism efficiency in highly arsenic exposed, undernourished populations. We evaluated whether dietary intake of OCM nutrients (including vitamins B_2, B_6, folate (B_9), and B_{12}) was associated with arsenic metabolism in a more nourished population exposed to lower arsenic than previously studied. Dietary intake of OCM nutrients and urine arsenic was evaluated in 405 participants from the Strong Heart Study. Arsenic exposure was measured as the sum of iAs, monomethylarsonic (MMA) and dimethylarsinic acid (DMA) in urine. Arsenic metabolism was measured as the individual percentages of each metabolite over their sum (iAs%, MMA%, DMA%). In adjusted models, increasing intake of vitamins B_2 and B_6 was associated with significantly lower iAs% and MMA%, and significantly higher DMA%. A significant interaction was found between high folate and B_6 with enhanced arsenic metabolism efficiency. Our findings suggest OCM nutrients may influence arsenic metabolism in populations with moderate arsenic exposure. Stronger and independent associations were observed with B_2 and B_6, vitamins previously understudied in relation to arsenic. Research is needed to evaluate whether targeting B-vitamin intake can serve as a strategy for the prevention of arsenic-related health effects at low-moderate arsenic exposure.

INTRODUCTION

Inorganic arsenic (iAs) in food and water is a major global health concern. An established carcinogen, chronic arsenic exposure also increases the risk of cardiovascular disease, respiratory disease, neurologic deficits, and diabetes.[1-7] After ingestion, iAs (arsenate and arsenite) is metabolized into mono- and di-methylated arsenicals (MMA and DMA); DMA has a shorter circulating half-life and is more rapidly excreted through the urine as compared to iAs.[8-13] The urinary distribution of arsenic metabolites across human populations ranges from 10-30% for iAs, 10-20% for MMA and 60-80% for DMA.[14-18] Higher percentages of iAs (iAs%) and MMA (MMA%) and lower percentages of DMA (DMA%) in urine are thought to reflect a less efficient arsenic metabolism profile and have been associated with higher risk of cancer, skin lesions and cardiovascular disease.[16, 19-25] Conversely, higher DMA% and lower MMA% have been associated with diabetes, metabolic syndrome and higher body mass index.[26-31] Understanding non-modifiable (genetics, sex, life-stage) and modifiable (smoking, alcohol intake, kidney function, body mass index, nutrition) determinants of arsenic metabolism is important given the role of arsenic metabolism in arsenic toxicity.[32-35]

Nutritional status is a major susceptibility factor for arsenic-related disease, at least in part through the impact of nutrition on one-carbon metabolism (OCM).[32] OCM, a network of interrelated biochemical reactions dependent on sufficient intake of vitamin B_2 (riboflavin), vitamin B_6, folate (vitamin B_9) and vitamin B_{12}, plays an essential role in methylation processes throughout the body, including the methylation reactions involved in arsenic metabolism (**Figure 1**).[36, 37] In studies from Bangladesh, both cross-sectional [38] and folic acid supplementation trials demonstrated [39, 40] that higher folate is associated

with increased arsenic methylation efficiency, resulting in higher DMA% and lower iAs% and MMA% in urine and in reduced blood arsenic concentrations. In cross-sectional studies, greater dietary intake of vitamins B_{12} and B_2 [41] and higher plasma B_{12} [42] have been associated with lower iAs% and higher MMA%, in Bangladeshi adults. Further, both epidemiologic [43-45] and experimental studies [46, 47] have reported OCM nutrients to be associated with lower risk for arsenic-related disease.

The generalizability of the OCM findings in Bangladesh to US populations with low-moderate arsenic exposure and different dietary patterns is unclear. We evaluated the association of OCM nutrients with arsenic metabolism biomarkers in the Strong Heart Study (SHS), a population-based cohort study initiated to assess cardiovascular risk factors in American Indian adults residing in Arizona, Oklahoma and North and South Dakota. We used dietary intake estimates of B_2, B_6, folate and B_{12} as measures of OCM nutrients and percentages of urinary inorganic arsenic (iAs%) and its methylated metabolites (MMA% and DMA%), as measures of arsenic metabolism. We also modeled the complexity of both arsenic metabolism profiles and nutrition intake through the use of principal component analysis (PCA).

METHODS

Study Population

The SHS recruited 4,549 American Indians from 13 tribes located in Arizona, Oklahoma and North and South Dakota. Eligible participants were men and women aged 45-74 years at the baseline visit in 1989-1991. The overall participation rate was 62%. All participants provided informed consent and study protocols were approved by

multiple institutional review boards, community members and The Indian Health Service. In 2016, one of the communities withdrew their consent for participating in future studies, reducing the overall sample size to 3,516. The final version of this manuscript, along with a lay summary, was sent to, and approved by, all remaining communities.

At the baseline visit (1989-1991), a random sample of 50 males and 50 females from each age decade and at each study site (n=722; 508 after excluding the community that withdrew consent) was selected to participate in a self-administered food frequency questionnaire (FFQ), which provided estimated long-term daily average intake of folate and vitamins B_2, B_6 and B_{12} in milligrams.[48] We excluded 94 participants with missing data on urine arsenic, and 9 participants missing data on education, alcohol intake, smoking status, body mass index (BMI), estimated glomerular filtration rate (eGFR), and urine creatinine, leaving 405 participants for this study. Participants included in this study were similar to the overall study population on most variables of interest, except that they were slightly older than the full cohort (**Supplemental Table S1**).

Data Collection

Baseline visits included bio-specimen collection, a physical exam, and an interview-administered questionnaire. Visits were performed by trained and certified examiners according to a standardized protocol. Details have been described previously.[49]

Urine Arsenic Metabolites

Morning spot urine samples were collected during baseline visit in polypropylene tubes, frozen within 1 to 2 hours of collection, shipped buried in dry ice and stored at <-70°C in the Penn Medical Laboratory, MedStar Research Institute, Washington, DC.[49] The freezers have been operating under a strict quality control system to guarantee secure sample storage. For arsenic analyses, urine samples were thawed in 2009-2010, and up to 1.0 mL from each urine sample was transferred to a small vial, transported on dry ice to the Trace Element Laboratory at Graz University, Austria and stored at <-70°C until analyses.[50]

Quality control and quality assurance methods for urine arsenic analysis have been described in detail.[50] Urine concentrations of arsenite, arsenate, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) were measured using high performance liquid chromatography/inductively coupled plasma-mass spectrometry (HPLC/ICPMS). The limits of detection were 0.1 µg/L for arsenite, arsenate, MMA and DMA. The inter-assay coefficients of variation for arsenite and arsenate, MMA, DMA and total arsenic were 5.6%, 6.3%, 3.5% and 4.4% respectively. Urine arsenobetaine concentrations (median (IQR): 0.73 (0.49, 1.47) µg/L), confirmed seafood consumption in this population was infrequent and were measured using cation-exchange HPLC/ICPMS together with other less common arsenic cations. The limit of detection for urine arsenobetaine was 0.1 µg/L and the inter-assay coefficient of variation was 6.9%. No participants in this analysis had arsenic species below the limit of detection.

Nutrition Variables

Dietary intake of OCM-related micronutrients was measured during the baseline visit through estimated daily averages of dietary intake of vitamins $B_{2,}$ $B_{6,}$ folate and B_{12} in the past-year. These variables, as well as total caloric intake, were measured through an interviewer-administered Block 119-item food frequency questionnaire (FFQ). The Block questionnaire is one of the most widely used questionnaires with demonstrated reliability and validity.[51] To enhance accuracy of the questionnaire in this cohort, additional questions relating to foods commonly consumed by American Indians were added.[51] Of note, folate was calculated based on dietary intake alone as doses of folic acid supplementation were not available (only whether supplements were taken or not) and mandatory folic acid fortification was not implemented by the date of FFQ administration.

Other Variables

Sociodemographic (age, sex, and education) and life-style (vitamin supplementation use and drinking and smoking status) study variables were ascertained through a standardized questionnaire (separate from the FFQ) by trained and certified interviewers.[49] Height and weight measurements for BMI calculation (weight in kilometers divided by height in meters squared) were conducted during the physical exam. Urine creatinine was measured from the spot urine samples collected for arsenic analysis using an automated alkaline picrate methodology.[49] eGFR was calculated from creatinine, age and sex using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, as previously described.[52]

Statistical Analyses

To evaluate arsenic metabolism, we computed the relative proportions of inorganic and methylated arsenic metabolites by dividing each metabolite concentration over the sum of those species x 100. In this way, a methylation profile was estimated for each participant, consisting of iAs%, MMA% and DMA%, totaling to 100%. We also evaluated arsenic metabolism using principal component analysis (PCA), as recently proposed.[33, 34] PCA was conducted using each arsenic species percentage, then scaling them. PCA is useful in the analysis of arsenic metabolism biomarkers because it removes the inter-dependence of the three biomarkers (iAs%, MMA% and DMA%) allowing for improved interpretation of each metabolite. The two resulting arsenic metabolism principal components (As metabolism PC1 and PC2) may be biologically meaningful, potentially reflecting the secondary and primary methylation steps, respectively.[33, 34] Inorganic arsenic exposure was estimated as the sum of urinary concentrations of iAs, MMA and DMA (∑As). ∑As concentrations were right skewed and log-transformed for the analyses.

The analysis of dietary estimates of OCM nutrients (vitamin B_2, vitamin B_6, folate and vitamin B_{12}) requires adjustment for total caloric intake in most analyses. Because nutrient and total caloric intake variables were measured from the same questionnaire, their errors are strongly correlated. To cancel out these errors and improve the validity of energy-adjusted nutrients, nutrient density and residual methods have been suggested over simply including caloric intake as a variable in regression models.[53] We used a residual analysis approach and regressed each log-transformed vitamin intake on log-transformed total caloric intake. We then added the mean log-transformed value of each

nutrient to the nutrient residuals to create calorie-corrected nutrient variables. Because some OCM nutrients share common dietary sources and they are metabolically inter-dependent, we also used PCA to identify major PCs across log-transformed, calorie-corrected OCM nutrients.

Arsenic methylation patterns were compared across sociodemographic, health, nutrition and behavioral characteristic variable categories using Kruskal-Wallis tests. Pearson correlation coefficients were conducted to estimate univariate correlations between iAs%, MMA%, DMA% and calorie-corrected vitamins (vitamin B_2, vitamin B_6, folate and vitamin B_{12}) (**Figure 2**).

The mean difference (95% CI) of each of arsenic species percentages (iAs%, MMA%, DMA%) and arsenic metabolism principal component (As metabolism PC1 and As metabolism PC2) comparing the two highest to the lowest tertile of each calorie-corrected nutrient variable (vitamin B_2, vitamin B_6, folate and vitamin B_{12}) was estimated using linear regression models. We used the following progressive adjustments for known arsenic metabolism and nutrition intake determinants in our models evaluating arsenic metabolism both as individual arsenic species' percentages as well as principal components: model 1 adjusted for ∑As (log-transformed) and urine creatinine (log-transformed); model 2 further adjusted for age (continuous), sex, study center (Arizona, Oklahoma, North/South Dakota), BMI (continuous), smoking status (never, former, current), alcohol use (never, former, current), and eGFR (continuous); model 3 further adjusted for the other OCM calorie-corrected nutrient variables. Because adjustment for all nutrients at once can be difficult due to possible collinearity, we also used OCM PCs

to evaluate the association of arsenic metabolism biomarkers with independent summary variables of calorie-corrected nutrients.

We conducted multiple sensitivity analyses. First, we evaluated the potential impact of outliers in our models using resistant regression with consistent results (data not shown). Second, we evaluated an alternative method of correcting nutrients for total caloric intake (log-transformed) using adjustment in the main regression analysis instead of the residual-based approach, with similar findings (data not shown). Third, we assessed the influence of urinary creatinine by running final models with and without adjustment for this variable with consistent results (data not shown). Main analyses include adjustment for urinary creatinine as it has been shown to affect arsenic metabolism, possibly through the competition for methyl groups in the synthesis of creatine, the precursor to creatinine.[40] Urinary creatinine reflects both dietary creatine intake (primarily from meat) and endogenous synthesis of creatine. Fourth, we used specific gravity as an alternative to urinary creatinine to account for urine dilution, with consistent results (**Supplemental Material Table S2**). In addition, we ran regressions with and without a participant with an outlying high (>100μg) value for B_{12}, again with consistent results (data not shown). We also ran regressions with and without participants reported to take folic acid or multivitamin supplements (n=9) with consistent results (data not shown). Further, to address potential concerns regarding DMA from food sources at low levels of arsenic exposure, we stratified by center due to geographical differences in exposure sources (water is a major source in Arizona and North and South Dakota, while in Oklahoma the predominant source is food). Results were similar in both areas of lower exposure (Oklahoma) and higher exposure (Arizona and Dakotas) (**Supplemental**

Material Table S2). Finally, exploratory analyses were conducted to investigate potential pairwise interactions between each OCM nutrient. A joint categorical variable reflecting high (equal to or greater than the median) and low (below the median) intake, as well as quantitative interaction terms, of each OCM nutrient pair, was created to assess these relationships (**Supplemental Material Table S3**).

Sample Size Justification

Our sample size was fixed as our analysis was based on previously collected data. Therefore, we conducted a minimally detectable effect estimate for each of our main outcomes (iAs%, MMA% and DMA%). We used a sample size of 405 and 80% power for each calculation. For iAs%, using a mean of 8.4 and standard deviation of 4.7, we computed the minimally detectable difference to be 0.66. For MMA%, using a mean of 14.7 and standard deviation of 5.3, we computed the minimally detectable difference to be 0.70. For DMA%, using a mean of 76.9 and standard deviation of 8.8, we computed the minimally detectable difference to be 1.23.

RESULTS

Participant Characteristics

Median (IQR) percentages for arsenic metabolism biomarkers were 7.1 (5.2-10.7)% for iAs%, 13.7 (11.3-17.7)% for MMA%, and 78.2 (71.6-83.3)% for DMA% (**Table 1**). Median (IQR) daily intake for OCM nutrients were 1.4 (0.9-2.0) mg for vitamin B_2, 1.2 (0.8-1.9) mg for vitamin B_6, 207 (120.4-336.7) µg for folate, and 3.0 (1.7-5.7) µg for vitamin B_{12}. Several characteristics were associated with higher iAs%, higher MMA% and lower DMA%, including being male, lower BMI, current smoker and

current alcohol drinker. Characteristics associated with just higher iAs% included being younger, having eGFR ≤ 60 mL/min/1.73 m^2 and having lower intake of B_6 and folate. Compared to tertiles 1 and 3, participants in the 2nd tertile of urine creatinine had lower iAs% and higher DMA% (**Table 1**). Pearson correlation coefficients were moderately positive for iAs% and MMA% (0.54), and strongly negative between both iAs% and MMA% with DMA% (-0.86 and -0.89, respectively) (**Figure 2**). All OCM nutrition variables were positively correlated with each other both before and after correcting for caloric intake. Calorie-corrected nutrient correlations ranged from 0.23 between vitamin B_{12} and vitamin B_6 to 0.70 between vitamin B_2 and folate. Correlations between OCM nutrients were negative with both MMA% and iAs% (ranging from -0.01 for iAs% and vitamin B_{12} to -0.13 for iAs% and vitamin B_6); and positive with DMA% (ranging from 0.03 with vitamin B_{12} to 0.13 with vitamin B_6)(**Figure 2**).

Variance in arsenic methylation patterns were summarized in two principal components. Arsenic metabolism principal component 1 (As PC1) explained 84.6% of the variance in arsenic species biomarkers and reflected higher DMA% and lower iAs% and MMA% (**Table 2**). Arsenic metabolism principal component (As PC2) explained 15.4% of the variance and reflected higher MMA% and lower iAs%, independent of DMA%. Variance in OCM nutrients was summarized in four principal components (**Table 2**). OCM PC1 explained 60.1% of the variance in OCM nutrients and reflected higher intake of all four B-vitamins. OCM PC2 explained 22.7% of the variance and reflected higher vitamin B_{12} intake and lower intake of vitamin B_6 and folate. OCM PC3 explained 10.3% of the variance and reflected higher folate and B_2 intake and lower

intake of vitamins B_6 and vitamin B_{12}. OCM PC4 explained 6.9% of variance and reflected higher intake of vitamin B_{12} and folate and lower intake of the other nutrients.

Association of One Carbon Metabolism Nutrients and Arsenic Metabolism Biomarkers

OCM nutrients, specifically vitamins B_6 and B_2, were associated with more efficient arsenic methylation profiles. In fully adjusted models (**Table 3**, Model 2), higher intake of vitamins B_2 and B_6 were associated with lower iAs%, lower MMA% and higher DMA%. Compared to tertile 1, participants in tertile 3 of vitamin B_2 intake had 1.00 (95% CI: -2.00, 0.00)% lower iAs%, 1.36 (95% CI: -2.48, -0.23)% lower MMA% and 2.36 (95% CI: 0.54, 4.18)% higher DMA%. Correspondingly, participants in tertile 3 of vitamin B_6 intake had 1.36 (95% CI: -2.35, -0.37)% lower iAs%, 1.57 (95% CI: -2.69, -0.45)% lower MMA% and 2.93 (95% CI: 1.13, 4.74)% higher DMA%. These associations, with the exception of vitamin B_2 and iAs%, remained statistically significant and similar in magnitude after further adjustment for all OCM nutrients (**Table 3**, Model 3).

In fully adjusted models without adjustment for other OCM nutrients (**Table 3**, Model 2), folate showed a similar association with arsenic metabolism biomarkers as vitamins B_2 and B_6, although they were weaker, not statistically significant, and stronger for the second tertile than the third. With further adjustment for other OCM nutrients, the association with tertile 2 of folate was attenuated, whereas tertile 3 reversed direction for all arsenic metabolism biomarkers, although the associations remained not-statistically

significant. The corresponding associations with vitamin B_{12} were weaker and not statistically significant in any model.

When modeling arsenic metabolism using PCA, tertile 3 of vitamins B_2 and B_6 intake were associated with higher As metabolism PC1, reflecting higher DMA% and lower iAs% and MMA% (**Table 4**). No significant associations were observed with any of the OCM nutrients and As metabolism PC2. In models using OCM PCs instead of the original nutrient variables, OCM PC1, reflecting higher intake of all OCM nutrients, was negatively associated with iAs% and MMA% and positively associated with DMA% and As metabolism PC1 (**Table 4**). OCM PC2, representing mostly high vitamin B_{12}, possibly reflecting meat intake, was not associated with arsenic methylation profiles. OCM PC3, reflecting higher intake of vitamin B_2 and folate with lower vitamin B_6, was positively associated with higher iAs% and MMA% and negatively associated with DMA% and As metabolism PC1, although none of the associations were statistically significant. OCM PC4, reflecting high folate and vitamin B_{12} and low vitamin B_6 and B_2, was positively associated with iAs% and MMA%, and negatively with DMA% and As metabolism PC1, however, only the association with MMA% was statistically significant. In analyses evaluating the joint effect of OCM nutrients in pairs, we found an independent additive association between intake of vitamins B_6 and B_2 with arsenic metabolism biomarkers. Mean DMA% was 2.76 (95% C:I 0.88, 4.65)% higher for participants with both vitamins B_6 and B_2 above (high) versus below (low) the median, 2.02 (95% CI: -0.10, 4.14)% higher for participants with only high B_6 and 1.08 (95%CI -1.09, 3.24)% higher for participants with only high B_2 (**Supplemental Material Table S3**). The joint association was not different from additive (p-value for interaction 0.83).

For vitamin B_6 and folate, compared to participants with low intake of both vitamins, high folate intake with low B_6 was associated with 1.39 (95% CI: 0.19, 2.58)% higher iAs% and 2.58 (95% CI: -4.77, -0.93)% lower DMA% while high folate intake with high vitamin B_6 was associated with 1.03 (95% CI: -2.01, -0.05) lower iAs% and 1.65 (95% CI: -0.14, 3.44)% higher DMA%. The p-value for interaction, supporting an antagonistic interaction, between folate and B_6 was 0.01 for iAs% and 0.001 for DMA% (no interaction was found for MMA%).

DISCUSSION

Dietary intake of OCM nutrients was associated with urinary arsenic methylation patterns in a population of rural American Indian adult men and women exposed to low-moderate levels of inorganic arsenic from drinking water and food. In general, higher intake of B-vitamins, in particular B_2 and B_6, was associated with lower percentages of iAs and MMA and higher percentages of DMA, a profile suggested to reflect enhanced arsenic metabolism. These associations persisted for vitamins B_2 and B_6 after adjustment for sociodemographic factors, smoking, alcohol intake, BMI, and kidney function, as well as all OCM nutrients. The joint association for vitamins B_2 and B_6 was independent and not different from additive. For vitamin B_{12}, a vitamin with a relatively high intake in the study population, there was no association with arsenic metabolism biomarkers, a finding that is consistent with results from other populations with generally adequate vitamin B_{12} intake.[38, 54] The association between folate intake and arsenic metabolism in main analyses was not clear. In joint analyses, an antagonistic association was found between folate and vitamin B_6, with higher folate being associated with higher DMA% and lower

iAs% only in the presence of high vitamin B_6. However, these results should be interpreted with caution given the high correlation between B_6 and folate.

OCM is critical in the biosynthesis of purines and thymidylate as well as the generation of methyl groups.[55] OCM facilitates the generation of S-adenosylmethionine (SAM) which serves as a methyl donor for numerous substrates which are essential to many biological processes including cellular signaling, DNA methylation, the synthesis of proteins, lipids, hormones and carbohydrates, and arsenic metabolism.[55] A product of SAM-dependent methylation reactions, s-adenosylhomocysteine, is hydrolyzed to homocysteine (Hcys), which can be remethylated to form methionine and activated to regenerate SAM.[55] OCM functioning, and adequate methyl group availability, is dependent on essential nutrients including folate, vitamin B_{12}, vitamin B_6, and vitamin B_2 (**Figure 1**).

Numerous studies conducted in Bangladesh have characterized the relationship between OCM nutrients and arsenic metabolism in populations exposed to high levels of arsenic. Randomized controlled trials (RCTs) [39, 40] have focused on folic acid given its key role in the recruitment of methyl groups, which are required for methylation reactions in the OCM pathway.[40] Results from cross-sectional studies [38, 56, 57] have also shown associations between higher plasma folate and enhanced arsenic metabolism, consistent with increases in urinary DMA% and decreases in iAs% and MMA% seen in RCTs. Collectively, these studies provide strong evidence for the temporality of these associations. Cross-sectional dietary intake studies of folate and arsenic metabolism from Bangladesh[41], Mexico[58] and the U.S.[59], however, have been inconsistent.

Evaluation of OCM nutrients beyond folate, including vitamins B_2, B_6 and B_{12}, all of which play important roles in OCM (**Figure 1**), has not been as extensive, lacking evidence from RCTs. Higher vitamin B_{12} (plasma[42] and dietary intake [41, 58]) was associated with lower iAs% and higher MMA% in some studies but not in studies with a lower prevalence of B_{12}-deficiency.[38, 39, 54] These results have led to the hypothesis that increases in vitamin B_{12} may promote the first step of arsenic methylation, leading to higher MMA% and lower iAs%.[36, 42] The study reporting plasma B_{12} levels being associated with lower iAs% and higher MMA%, by design, over-selected participants having moderate- to severe-B_{12} deficiency. The predomination of the methylation of iAs to MMA over MMA to DMA may be specific to under-nourished populations exposed to high levels of arsenic, due to a competition for available methyl groups.[36] In our population, characterized by adequate or even high vitamin B_{12} intake, we found no association with arsenic metabolism.

Three dietary intake studies, one conducted in the U.S.[59], one conducted in women from Mexico [58] and one conducted in Bangladesh,[41] reported null results for vitamin B_6. The Bangladeshi dietary intake study, however, found similar associations between vitamin B_2 and arsenic metabolism as had been reported for vitamin B_{12}; higher vitamin B_2 intake was associated with lower iAs% but higher MMA%.[41] Associations for vitamin B_2 were null in the other two dietary intake studies.[59]

Our analysis on the association between OCM nutrients and arsenic metabolism fills in gaps in our understanding of this relationship in a cohort relevant to the general U.S. population. The SHS population is better nourished (38% of participants from the Bangladeshi dietary intake study were underweight compared to just one participant in

our study) and exposed to lower levels of arsenic than the populations studied in Bangladesh. Further, dietary patterns differ substantially between the two populations: the SHS diet generally includes fewer fresh fruits and vegetables (significant sources of vitamin B_6 and folate) (**Figure 3**) and more meat/protein (significant sources of B_2 and B_{12} and intake and has been associated with arsenic metabolism [41, 59]) than typical Bangladeshi diets.[51, 60] This is reflected in our participants' higher levels of vitamins B_{12} and B_2 and lower levels of folate and vitamin B_6 than in Bangladesh (**Supplemental Table S4)**. Participants in the dietary intake study conducted among Mexican women had OCM nutrient intake patterns more similar to those in the Bangladeshi dietary intake study.[41, 58]

To our knowledge, only one other study has evaluated OCM nutrients and arsenic metabolism in a U.S. population.[59] This study, conducted in individuals from Western Nevada and Kings County, California, exposed to arsenic levels in drinking water generally above 10 μg/L[61], found no association between dietary intake of OCM nutrients and arsenic metabolism; however, the study was small (N=87), some OCM nutrients were not available (no evaluation of vitamin B_{12}), and the majority of the population was male (75%) with a large percentage having a history of bladder cancer (26%).[59]

In our study, the strongest associations between vitamin intake and arsenic metabolism were for the least extensively studied OCM nutrients, vitamins B_2 and B_6. Greater intake of vitamins B_2 and B_6 were associated with higher DMA% and As metabolism PC1 and lower iAs% and MMA%. The magnitude of increases in DMA% and decreases in MMA% for participants in the third versus first tertile of intake for B_2 and B_6 were modest (ranging from 1.5 to 3), yet similar in the magnitude of change for

MMA% reported in participants after receiving folic acid supplementation for 12 weeks in a Bangladesh-based folic acid supplementation trial.[39] Translating this into clinical relevance, increasing intake of vitamin B_2 by 0.96 mg, for example, reflecting the increase in intake needed to move those at the median of the first tertile to the median of the third tertile of vitamin B_2 intake, could result in MMA% 1-2 percentages lower. Although on the individual level, this may appear minimal, on the population level it could translate to significant effects on health outcomes. For example, a case-control study from Chile reported a 1.11 (1.06, 1.17) and 1.05 (1.00, 1.10) greater odds of skin cancer and bladder cancer, respectively, for each 1% increase in MMA%.[62]

Further, results from our pairwise interactions suggest there is an additive independent association for high intake of vitamins B_2 and B_6 with enhanced arsenic metabolism. Our findings add evidence that vitamins B_2 and B_6, less studied B-vitamins involved in OCM, may also affect arsenic metabolism in the same direction as has been reported for folate; and perhaps, play a stronger role in arsenic metabolism than folate in well-nourished protein-sufficient populations. This finding is also supported by the OCM PC analysis. OCM PC1, reflecting higher intake of all four vitamins, was associated with lower iAs% and MMA% and higher DMA% and As metabolism PC1. However, OCM PC4, reflecting low B_2 and B_6 intake and high folate and B_{12} intake, was significantly associated with higher MMA% and non-significantly associated with higher iAs% and lower DMA%. Thus, despite high folate and B_{12} intake, low intake of B_2 and B_6 (e.g., a diet with low intake of dairy, poultry and fruit but high intake of green vegetables and certain fish) resulted in lower arsenic metabolism efficiency. This result for PC4 is consistent with the joint analysis for folate and vitamin B_6, where high folate was

associated with lower arsenic metabolism efficiency in the presence of low vitamin B_6 but with higher arsenic metabolism efficiency with high B_6 intake. These findings suggest that high folate alone may not enhance arsenic metabolism in this population. Although we cannot disregard that these findings could be related to measurement error of folate nutritional status in the absence of plasma folate data, additional research is needed to evaluate if this pattern is consistent in other populations. Overall, these results suggest that, as it has been reported for plasma folate in undernourished and low-protein populations, increasing levels of OCM nutrients, specifically B_2 and B_6, can enhance arsenic methylation capacity in a well-nourished protein-sufficient population exposed to low-moderate arsenic.

In independent linear regressions analyses for folate, the stronger association with MMA% and DMA% for participants in the second tertile of folate rather than the third tertile, and the reversing of direction in tertile 3 after adjustment for other nutrients, is difficult to interpret. It could be related to measurement error from the difficulty of dietary folate measurement. We did not calculate dietary folate equivalents (DFEs), which are used to incorporate all sources of folate intake, in addition to accounting for the higher bioavailability of folic acid from supplements and fortified foods compared with naturally occurring folate in foods.[63, 64] However, sensitivity analyses excluding participants reporting vitamin supplementation use yielded consistent results, and mandatory fortification of foods with folic acid was not implemented in the United States until 1998 (almost ten years after data collection). It is also possible the patterns seen for folate and arsenic metabolism in our analysis are not consistent with other studies due to interactions with intake of other vitamins, unmeasured confounding, too narrow a

distribution of intake and/or oxidation of food folates during cooking. Studies in well-nourished populations exposed to a broad range of folate through food fortification and supplementation use, with measurements of both folate intake and biomarkers, are necessary to assess the dose-response relationship of folate intake with arsenic metabolism.

Limitations of our study include relying on self-reported dietary data from the FFQ, a dietary assessment tool that is associated with underestimates of intake, particularly energy and protein.[65, 66] Confirmation of findings through evaluation of nutrition biomarkers would have enhanced analyses and study interpretation. Further, B-vitamin intake was based solely on diet, lacking information on supplements. In addition, data were lacking on other nutrients involved in OCM, such as methionine and choline, which would have allowed a more comprehensive understanding of the full impact of OCM and arsenic metabolism. Although we were able to confirm that seafood intake in this population was low, we were unable to adjust for other food sources of inorganic arsenic that may confound the findings, such as rice intake. Since there are gene variants, for example, the common single nucleotide polymorphism in the gene for MTHFR, the 667 C→ T mutation, that lead to lower blood folate levels, the extent to which B-vitamin intake reflects internal levels of those vitamins, at least in part, is genetically determined. Additional research to evaluate the role of OCM related genetic variants and its impact on these relationships in this population would be useful.

CONCLUSIONS

Our study provides novel evidence that even at low levels of arsenic exposure and in well-nourished populations, OCM nutrient intake may affect the efficiency of arsenic metabolism. Further, our findings suggest vitamins B_6 and B_2, two previously understudied OCM vitamins, may play a stronger role in arsenic metabolism than folate in well-nourished protein-sufficient populations.

TABLES

Table 1. Participant Characteristics by Arsenic Metabolism (N=405)

	N	iAs% median (IQR)	P-value[a]	MMA% median (IQR)	P-value[a]	DMA% median (IQR)	P-value[a]
Overall	405	7.1 (5.2, 10.7)		13.7 (11.3, 17.7)		78.2 (71.6, 83.3)	
Age (years)							
<55	159	8.0 (5.8, 11.7)		13.3 (11.3, 18.0)		77.8 (70.6, 82.1)	
55 to <65	128	6.9 (5.3, 10.6)		13.5 (11.2, 17.5)		79.5 (71.0, 83.5)	
≥65	118	6.6 (4.4, 9.5)	0.007	14.6 (11.3, 17.4)	0.92	77.9 (73.1, 83.7)	0.46
Sex							
Male	181	9.2 (6.3, 12.7)		16.2 (13.0, 19.5)		75.1 (68.6, 80.2)	
Female	224	6.4 (4.6, 9.0)	<0.001	12.6 (9.6, 15.1)	<0.001	80.9 (75.7, 84.9)	<0.001
∑As (µg/L)							
0.70 – <7.10	135	7.0 (5.0, 10.5)		13.8 (11.3, 17.2)		78.8 (73.1, 82.4)	
7.10 – 14.0	136	6.9 (4.6, 10.5)		14.2 (10.6, 17.9)		78.7 (71.7, 84.3)	
> 14.0 – 87.2	134	8.0 (5.6, 11.5)	0.07	13.3 (11.5, 18.2)	0.87	77.3 (70.4, 82.6)	0.23
BMI (kg/m^3)							
<25	72	10.3 (7.7,		18.0 (13.2, 21.8)		71.6 (65.6, 77.3)	
25 to <30	141	12.4)		14.2 (11.8, 17.9)		77.9 (72.4, 82.5)	
≥30	192	7.1 (5.1, 10.6)	<0.001	12.8 (10.1, 15.9)	<0.001	80.6 (75.0, 84.7)	<0.001
		6.5 (4.6, 9.7)					
Smoking Status							
Never	114	6.1 (4.3, 9.1)		12.8 (9.4, 15.6)		80.3 (75.0, 86.1)	
Former	138	6.8 (4.8, 9.9)		13.5 (11.7, 17.7)		79.1 (72.6, 83.7)	
Current	153	9.3 (6.4, 12.6)	<0.001	14.7 (12.3, 18.6)	<0.001	76.1 (68.8, 80.8)	<0.001
Alcohol							
Never	66	5.9 (4.0, 9.2)		12.8 (9.7, 15.9)		80.4 (73.6, 85.5)	
Former	188	6.9 (4.6, 10.3)		13.3 (10.5, 17.2)		79.4 (73.3, 84.0)	
Current	151	8.8 (6.4, 12.3)	<0.001	14.7 (12.6, 18.8)	<0.001	76.2 (68.8, 81.0)	<0.001
eGFR (mL/min)							
>60	367	5.5 (3.8, 8.3)		13.3 (9.6, 16.8)		80.1 (75.1, 85.2)	
≤60	38	7.5 (5.4, 10.9)	0.001	13.7 (11.3, 18.0)	0.55	78.1 (71.2, 82.7)	0.06
Urine Creatinine (mg/dL)							
<0.95	136	8.5 (5.9, 11.9)		13.4 (11.4, 16.7)		78.0 (71.2, 81.9)	

0.95 – 1.50	134	6.4 (4.4, 9.3)		13.6 (10.5, 16.9)		79.4 (73.6, 85.0)	
>1.50	135	7.1 (5.0, 11.4)	<0.001	14.7 (11.9, 19.0)	0.12	78.2 (69.4, 83.1)	0.02
Total Caloric Intake (kcal)							
<1,230	135	7.5 (5.1, 10.5)		13.4 (11.3, 16.5)		78.7 (72.1, 83.3)	
1,230-1,968	135	8.1 (5.4, 11.9)		14.2 (10.8, 19.8)		77.0 (69.5, 83.4)	
>1,968	135	6.7 (5.0, 10.5)	0.14	13.7 (11.5, 17.3)	0.36	79.3 (72.5, 83.5)	0.24
Vitamin B_2 (mg)[b]							
<1.07	139	8.3 (5.4, 11.5)		14.1 (11.5, 18.8)		77.2 (69.9, 81.9)	
1.07-1.76	132	7.0 (4.9, 10.8)		13.3 (10.9, 17.8)		78.4 (72.0, 83.8)	
>1.76	134	6.9 (5.4, 10.1)	0.12	13.7 (10.8, 17.1)	0.39	79.0 (73.6, 83.5)	0.11
Vitamin B_6 (mg)[b]							
<0.93	135	8.5 (5.6, 12.9)		13.6 (11.5, 18.4)		76.9 (70.5, 82.1)	
0.93-1.68	135	7.7 (5.1, 11.4)		14.3 (11.4, 18.5)		77.8 (69.7, 82.6)	
>1.68	135	6.7 (4.9, 9.3)	0.005	13.2 (10.2, 16.9)	0.05	80.2 (74.8, 84.2)	0.005
Folate (µg)[b]							
<144.1	135	8.5 (5.7, 12.8)		14.1 (12.3, 18.0)		76.9 (69.9, 81.4)	
144.1-285.2	135	6.9 (4.9, 10.5)		13.2 (10.5, 17.7)		79.2 (72.1, 84.4)	
>285.2	135	6.9 (5.0, 10.2)	0.03	13.7 (10.0, 17.9)	0.22	79.3 (71.8, 83.7)	0.04
Vitamin B_{12} (µg)[b]							
<2.20	136	8.0 (5.3, 11.5)		14.2 (11.2, 18.7)		77.3 (70.7, 82.2)	
2.20-4.41	134	6.8 (4.9, 10.3)		13.3 (11.2, 17.1)		79.5 (72.6, 83.6)	
>4.41	135	7.2 (5.3, 11.2)	0.15	13.9 (11.3, 17.7)	0.53	78..2 (71.3, 83.5)	0.21

∑As, total urinary inorganic arsenic; BMI, body mass index; eGFR, estimated glomerular filtration rate
[a]Kruskal-Wallis tests were used to compare methylation medians across variable categories
[b]Vitamins are displayed as crude values, not calorie-adjusted

Table 2. Summary of Principal Components for Arsenic Species and Calorie-Adjusted Nutrition Variables

	As Metabolism PC1	As Metabolism PC2	OCM PC1	OCM PC3	OCM PC3	OCM PC4
Variance explained (%)	84.6	15.4	60.1	22.7	10.3	6.9
Standard deviation	1.59	0.68	1.55	0.95	0.64	0.52
Weights for iAs%	-0.55	-0.73	--	--	--	--
Weight for MMA%	-0.56	0.69	--	--	--	--
Weight for DMA%	0.63	-0.03	--	--	--	--
Weight for Vitamin B_2	--	--	0.57	0.13	0.47	-0.66
Weight for Vitamin B_6	--	--	0.53	-0.26	-0.79	-0.16
Weight for Folate	--	--	0.52	-0.46	0.37	0.62
Weight for Vitamin B_{12}	--	--	0.36	0.84	-0.12	0.39

As, arsenic; PC, principal component; OCM, one-carbon metabolism

Table 3. Mean Difference (95% CI) in iAs%, MMA% and DMA% by Calorie-Adjusted Nutrition Tertiles (N=405)

Model	Model 1[a]	Model 2[b]	Model 3[c]
Vitamin B_2 (mg)			
		iAs%	
<1.073	0.00 (ref)	0.00 (ref)	0.00 (ref)
1.073 – 1.43	-0.09 (-1.19, 1.01)	-0.21 (-1.20, 0.79)	-0.29 (-1.35, 0.77)
>1.43	-0.90 (-2.00, 0.20)	-1.00 (-2.00, 0.00)	-0.68 (-1.89, 0.53)
		MMA%	
<1.073	0.00 (ref)	0.00 (ref)	0.00 (ref)
1.073 – 1.43	-0.47 (-1.75, 0.81)	-0.59 (-1.71, 0.53)	-0.69 (-1.88, 0.50)
>1.43	-0.96 (-2.24, 0.33)	-1.36 (-2.48, -0.23)	-1.58 (-2.94, -0.22)
		DMA%	
<1.073	0.00 (ref)	0.00 (ref)	0.00 (ref)
1.073 – 1.43	0.57 (-1.53, 2.67)	0.80 (-1.01, 2.61)	0.98 (-0.99, 2.90)
>1.43	1.86 (-0.25, 3.97)	2.36 (0.54, 4.18)	2.26 (0.06, 4.46)
Vitamin B_6 (mg)			
		iAs%	
<0.936	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.936 – 1.4241	-0.21 (-1.29, 0.88)	0.08 (-0.90, 1.06)	0.11 (-0.69, 1.39)
>1.4241	-1.92 (-3.01, -0.84)	-1.36 (-2.35, -0.37)	-1.18 (-2.32, -0.03)
		MMA%	
<0.936	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.936 – 1.4241	-1.28 (-2.55, -0.02)	-0.78 (-1.89, 0.34)	-0.85 (-2.00, 0.30)
>1.4241	-2.29 (-3.56, -1.03)	-1.57 (-2.69, -0.45)	-1.80 (-3.09, -0.51)
		DMA%	
<0.936	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.936 – 1.4241	1.49 (-0.58, 3.56)	0.69 (-1.10, 2.49)	0.74 (-1.12, 2.60)
>1.4241	4.21 (2.14, 6.28)	2.93 (1.13, 4.74)	2.98 (0.89, 5.07)
Folate (µg)			
		iAs%	
<149	0.00 (ref)	0.00 (ref)	0.00 (ref)
149 - 266	-0.67 (-1.77, 0.42)	-0.40 (-1.40, 0.60)	-0.06 (-1.10, 0.98)
>266	-1.31 (-2.41, -0.21)	-0.80 (-1.79, 0.20)	0.14 (-1.07, 1.34)
		MMA%	
<149	0.00 (ref)	0.00 (ref)	0.00 (ref)
149 - 266	-1.50 (-2.82, -0.27)	-0.93 (-2.06, 0.20)	-0.29 (-1.46, 0.88)
>266	-0.70 (-1.97, 0.58)	-0.18 (-1.30, 0.93)	1.27 (-0.08, 2.62)
		DMA%	
<149	0.00 (ref)	0.00 (ref)	0.00 (ref)
149 - 266	2.22 (0.13, 4.32)	1.33 (-0.50, 3.16)	0.34 (-1.55, 2.23)
>266	2.01 (-0.09, 4.10)	0.98 (-0.83, 2.79)	-1.41 (-3.59, 0.78)
Vitamin B_{12} (µg)			
		iAs%	
<2.20	0.00 (ref)	0.00 (ref)	0.00 (ref)
2.20 – 3.93	0.36 (-0.74, 1.46)	0.52 (-0.47, 1.51)	0.74 (-0.31, 1.78)
>3.93	-0.27 (-1.37, 0.83)	-0.55 (-1.54, 0.44)	-0.06 (-1.16, 1.04)
		MMA%	
<2.20	0.00 (ref)	0.00 (ref)	0.00 (ref)
2.20 – 3.93	-0.51 (-1.80, 0.77)	-0.44 (-1.57, 0.68)	0.15 (-1.03, 1.32)
>3.93	0.01 (-1.28, 1.29)	-0.37 (-1.49, 0.76)	0.54 (-0.69, 1.78)
		DMA%	
<2.20	0.00 (ref)	0.00 (ref)	0.00 (ref)
2.20 – 3.93	0.15 (-1.96, 2.26)	-0.08 (-1.90, 1.74)	-0.89 (-2.79, 1.01)
>3.93	0.26 (-1.85, 2.38)	0.92 (-0.90, 2.74)	-0.48 (-2.48, 1.51)

[a]Adjusted for total log arsenic and log urinary creatinine
[b]Adjusted for total log arsenic, log urinary creatinine age, sex, center, smoking, alcohol, eGFR, BMI
[c]Adjusted for total log arsenic, log urinary creatinine age, sex, center, smoking, alcohol, eGFR, BMI, all b-vitamins of interest

Table 4. Mean Difference[a] (95% CI) in iAs%, MMA%, DMA% and Arsenic Principal Components by Nutrition Tertiles and OCM Principal Components

	As Metabolism PC1	As Metabolism PC2	iAs%	MMA%	DMA%
Calorie-Corrected Nutrition Tertiles					
Vitamin B_2					
<1.073	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
1.073 – 1.43	0.14 (-0.18, 0.47)	-0.05 (-0.20, 0.11)	-0.21 (-1.20, 0.79)	-0.59 (-1.71, 0.53)	0.80 (-1.01, 2.61)
>1.43	0.43 (0.10, 0.75)	-0.03 (-0.18, 0.13)	-1.00 (-2.00, 0.00)	-1.36 (-2.48, -0.23)	2.36 (0.54, 4.18)
Vitamin B_6					
<0.936	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.936 – 1.4241	0.12 (-0.20, 0.45)	-0.11 (-0.27, 0.04)	0.08 (-0.90, 1.06)	-0.78 (-1.89, 0.34)	0.69 (-1.10, 2.49)
>1.4241	0.53 (0.20, 0.86)	0.00 (-0.15, 0.16)	-1.36 (-2.35, -0.37)	-1.57 (-2.69, -0.45)	2.93 (1.13, 4.74)
Folate					
<149	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
149 - 266	0.24 (-0.09, 0.57)	-0.06 (-0.22, 0.09)	-0.40 (-1.40, 0.60)	-0.93 (-2.06, 0.20)	1.33 (-0.50, 3.16)
>266	0.18 (-0.15, 0.51)	0.10 (-0.06, 0.25)	-0.80 (-1.79, 0.20)	-0.18 (-1.30, 0.93)	0.98 (-0.83, 2.79)
Vitamin B_{12}					
<2.20	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
2.20 – 3.93	-0.02 (-0.35, 0.31)	-0.14 (-0.29, 0.02)	0.52 (-0.47, 1.51)	-0.44 (-1.57, 0.68)	-0.08 (-1.90, 1.74)
>3.93	0.17 (-0.16, 0.50)	0.04 (-0.12, 0.19)	-0.55 (-1.54, 0.44)	-0.37 (-1.49, 0.76)	0.92 (-0.90, 2.74)
One-Carbon Metabolism Principal Components					
OCM PC1	0.13 (0.04, 0.21)	0.00 (-0.04, 0.04)	-0.33 (-0.59, -0.07)	-0.38 -(0.67, -0.08)	0.71 (0.23, 1.18)
OCM PC2	0.03 (-0.11, 0.17)	-0.02 (-0.09, 0.04)	0.00 (-0.43, 0.43)	-0.17 (-0.65, 0.32)	0.17 (-0.62, 0.95)
OCM PC3	-0.14 (-0.35, 0.07)	0.02 (-0.08, 0.12)	0.29 (-0.34, 0.92)	0.48 (-0.23, 1.19)	-0.77 (-1.91, 0.38)
OCM PC4	-0.21 (-0.46, 0.05)	0.07 (-0.04, 0.20)	0.27 (-0.51, 1.05)	0.89 (0.01, 1.77)	-1.16 (-2.58, 0.27)

As, arsenic; PC, principal component; OCM, one-carbon metabolism

[a]Results adjusted for total log arsenic, log urinary creatinine, age, sex, center, smoking, alcohol, eGFR, BMI

FIGURES

Figure 1. One Carbon Metabolism, B vitamins, and Arsenic Metabolism. Methionine is activated by methionine adenosyltransferase to form S-adenosylmethionine (SAM). SAM provides the methyl group for most methylation reactions in the body (including arsenic methylation reactions), after which adenosylhomocysteine (SAH) is formed as a byproduct. SAH is hydrolyzed to homocysteine, which is then used in the transsulfuration pathway, or is regenerated into methionine via betaine or B_{12} dependent pathways. Dietary folate is converted to 5-methyl tetrahydrofolate through B_6 and B_2 dependent reactions, which provides the methyl group for the regeneration of homocysteine to methionine via the B_{12} pathway.

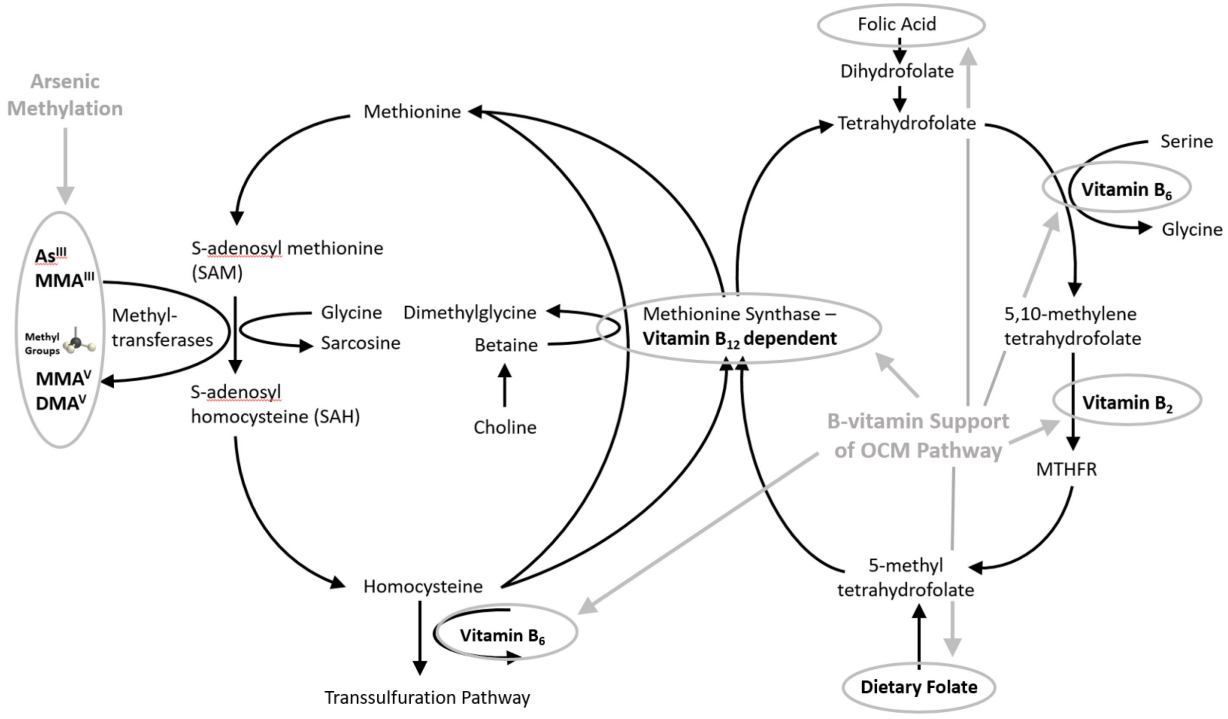


Figure 2. Pearson Correlation Matrix for Arsenic Species Percentages and Calorie-Corrected Nutrition Variables. Correlations and histograms of arsenic species percentages and calorie-corrected, using a residual analysis method of calorie-adjustment, nutrition variables (B_{12} outlier not included).

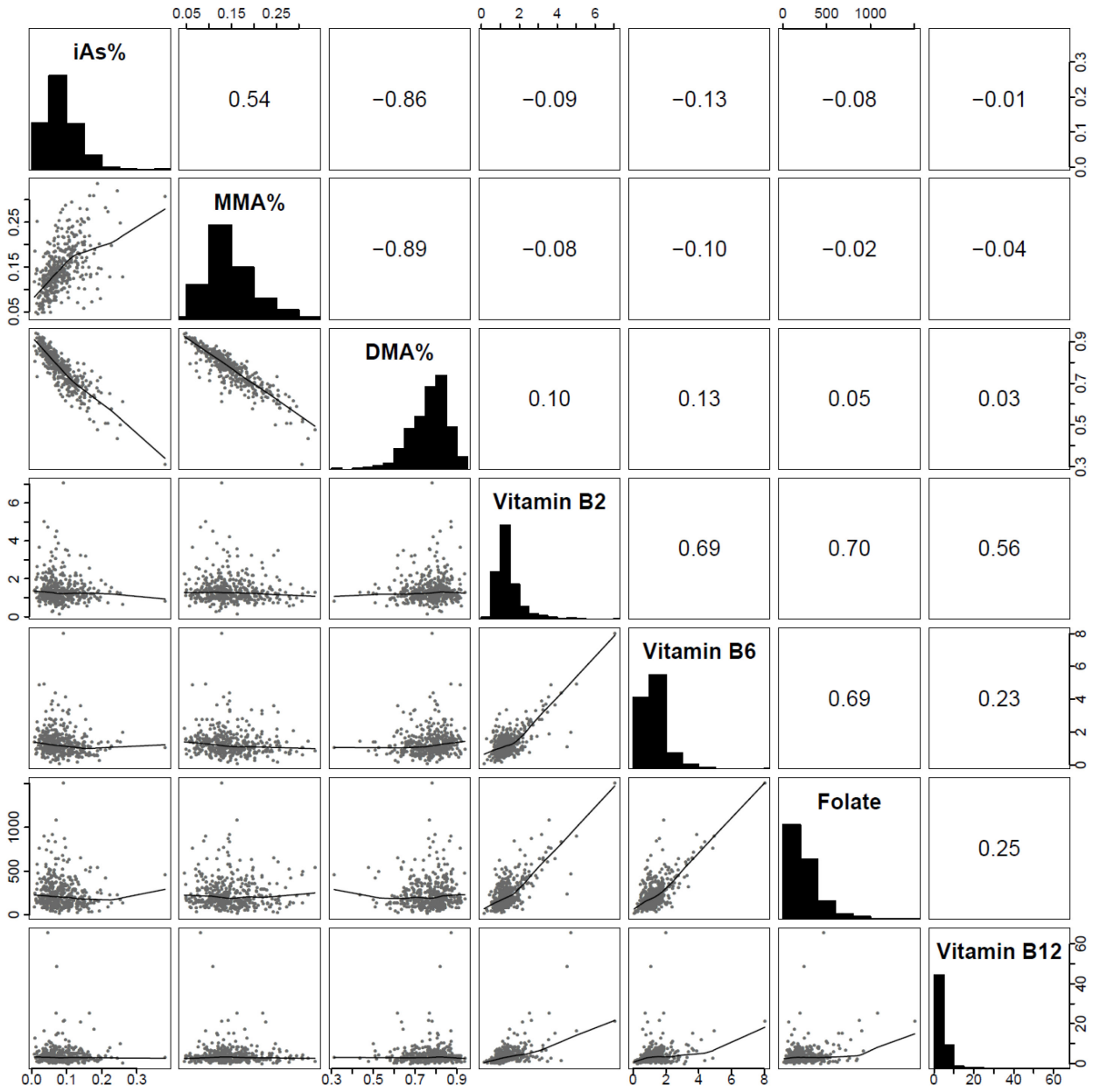
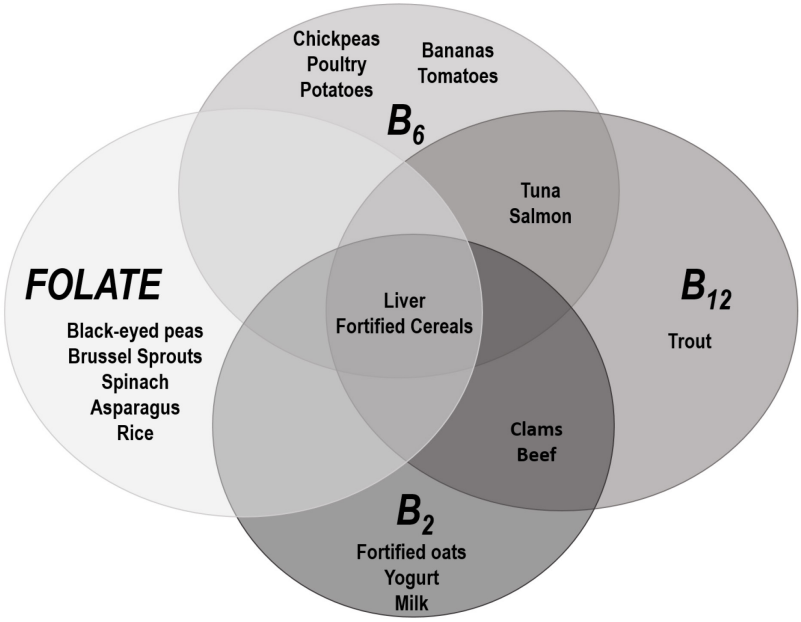


Figure 3. Major Dietary Sources of One Carbon Metabolism Nutrients. Foods listed under OCM nutrients are considered to be high dietary sources of that nutrient (provide 20% or more of the daily value)[67-70]



SUPPLEMENTAL MATERIAL

Supplemental Table S1. Baseline Participant Characteristics in Total Sample versus Nutrition Subset

	Total Sample (N= 3,047)	Subset (N=405)
Inorganic Arsenic Exposure (median, IQR)	9.5 (5.5, 17.3)	9.6 (6.1, 17.7)
Age (years) (n, %)		
<55	1,480 (48.5)	159 (39.3)
55- <65	1,009 (33.1)	128 (31.6)
≥65	560 (18.4)	118 (29.1)
Sex (n, %)		
Male	1,802 (42.0)	181 (44.7)
Female	1,769 (58.0)	224 (55.3)
BMI (kg/m^3) (median, IQR)	29.7 (26.4, 33.8)	29.7 (26.3, 33.5)
Smoking Status (n, %)		
Never	886 (29.1)	114 (28.2)
Former	1,022 (33.5)	138 (34.1)
Current	1,139 (37.4)	153 (37.8)
Alcohol (n, %)		
Never	453 (14.9)	66 (16.3)
Former	1,308 (43.0)	188 (46.4)
Current	1,279 (42.1)	151 (37.3)
eGFR (mL/min) (n, %)		
>60	2,678 (89.9)	367 (90.6)
≤60	300 (10.1)	38 (9.4)
Urine Creatinine (mg/dL) (median, IQR)	1.2 (0.8, 1.7)	1.3 (0.8, 1.7)

Abbreviations: BMI (body mass index); eGFR (estimated glomerular filtration rate)

Supplemental Table S2. Model Comparisons by Study Sites and Type of Urine Dilution Adjustment

Specific Gravity[a]	Creatinine[b]	Oklahoma (n=230)[b,c]	Dakotas/AZ (n=175)[b,d]
Vitamin B_2 (mg)			
		iAs%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.28 (-1.29, 0.73)	-0.21 (-1.20, 0.79)	-0.03 (-1.27, 1.21)	-0.47 (-2.17, 1.24)
-1.00 (-2.03, 0.02)	-1.00 (-2.00, 0.00)	-0.02 (-1.32, 1.28)	-2.01 (-3.67, -0.35)
		MMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.53 (-1.66, 0.59)	-0.59 (-1.71, 0.53)	-0.55 (-1.98, 0.89)	-0.77 (-2.65, 1.11)
-1.33 (-2.46, -0.19)	-1.36 (-2.48, -0.23)	-1.32 (-2.82, 0.18)	-1.64 (-3.46, 0.19)
		DMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.81 (-0.99, 2.61)	0.80 (-1.01, 2.61)	0.57 (-1.73, 2.88)	1.24 (-1.80, 4.28)
2.33 (0.51, 4.15)	2.36 (0.54, 4.18)	1.34 (-1.07, 3.75)	3.64 (0.69, 6.59)
Vitamin B_6 (mg)			
		iAs%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.24 (-0.76, 1.25)	0.08 (-0.90, 1.06)	0.27 (-0.98, 1.52)	0.21 (-1.44, 1.86)
-1.20 (-2.21, -0.19)	-1.36 (-2.35, -0.37)	-0.91 (-2.20, 0.38)	-1.50 (-3.17, 0.17)
		MMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.89 (-2.01, 0.23)	-0.78 (-1.89, 0.34)	-1.32 (-2.77, 0.13)	-0.48 (-2.30, 1.34)
-1.67 (-2.79, -0.54)	-1.57 (-2.69, -0.45)	-1.89 (-3.38, -0.40)	-1.14 (-2.98, 0.70)
		DMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.65 (-1.15, 2.44)	0.69 (-1.10, 2.49)	1.05 (-1.27, 3.37)	0.27 (-2.68, 3.22)
2.87 (1.06, 4.67)	2.93 (1.13, 4.74)	2.79 (0.41, 5.18)	2.64 (-0.34, 5.62)
Folate (µg)			
		iAs%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.37 (-1.39, 0.65)	-0.40 (-1.40, 0.60)	-0.42 (-1.72, 0.88)	-0.68 (-2.40,1.04)
-0.65 (-1.67, 0.36)	-0.80 (-1.79, 0.20)	-0.38 (-1.69, 0.93)	-1.07 (-2.68, 0.54)
		MMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.86 (-2.00, 0.28)	-0.93 (-2.06, 0.20)	-1.59 (-3.09, -0.08)	-0.43 (-2.31, 1.46)
-0.20 (-1.33, 0.93)	-0.18 (-1.30, 0.93)	-0.84 (-2.35, 0.67)	0.21 (-1.55, 1.97)
		DMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
1.23 (-0.60, 3.06)	1.33 (-0.50, 3.16)	2.01 (-0.40, 4.42)	1.10 (-1.98, 4.18)
0.85 (-0.96, 2.67)	0.98 (-0.83, 2.79)	1.22 (-1.21, 3.66)	0.86 (-2.01, 3.73)
Vitamin B_{12} (µg)			
		iAs%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
0.53 (-0.48, 1.54)	0.52 (-0.47, 1.51)	0.25 (-1.02, 1.53)	-0.12 (-1.89, 1.64)
-0.49 (-1.50, 0.52)	-0.55 (-1.54, 0.44)	-0.21 (-1.47, 1.06)	-0.93 (-2.67, 0.81)
		MMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.37 (-1.50, 0.77)	-0.44 (-1.57, 0.68)	-0.21 (-1.69, 1.27)	-1.14 (-3.06, 0.78)
-0.44 (-1.57, 0.69)	-0.37 (-1.49, 0.76)	0.40 (-1.07, 1.87)	-1.20 (-3.10, 0.70)
		DMA%	
0.00 (ref)	0.00 (ref)	0.00 (ref)	0.00 (ref)
-0.16 (-1.98, 1.66)	-0.08 (-1.90, 1.74)	-0.04 (-2.42, 2.33)	1.26 (-1.88, 4.40)

0.93 (-0.89, 2.74)	0.92 (-0.90, 2.74)	-0.19 (-2.55, 2.17)	2.13 (-0.97, 5.23)

[a]Model adjusted for total log arsenic, specific gravity, age, sex, center, smoking, alcohol, eGFR and BMI (vitamins are calorie-corrected)
[b]Model adjusted for total log arsenic, log urinary creatinine, age, sex, center, smoking, alcohol, eGFR and BMI (vitamins are calorie-corrected)
[c]Model run in participants from Oklahoma only
[d]Model run in participants from North/South Dakota and Arizona

Supplemental Table S3. Mean Difference[a] (95% CI) of iAs%, MMA% and DMA% by One-carbon Metabolism Nutrient Pairwise Interactions

Vitamins	iAs%	p-interaction	MMA%	p-interaction	DMA%	p-interaction
Folate and B_{12}						
Low Folate, Low B_{12}	0.00 (ref)		Ref		Ref	
Low Folate, High B_{12}	1.61 (-0.99, 1.31)		-0.60 (-1.90, 0.70)		0.44 (-1.67, 2.54)	
High Folate, Low B_{12}	0.08 (-1.06, 1.23)		-0.05 (-1.35, 1.24)		-0.03 (-2.13, 2.07)	
High Folate, High B_{12}	-0.66 (-1.79, 0.46)	0.27	0.03 (-1.24, 1.30)	0.47	0.63 (-1.42, 2.69)	0.88
Folate and B_6						
Low Folate, Low B_6	0.00 (ref)		0.00 (ref)		0.00 (ref)	
Low Folate, High B_6	0.52 (-0.68, 1.71)		-0.83 (-2.19, 0.53)		0.31 (-1.88, 2.49)	
High Folate, Low B_6	1.39 (0.19, 2.58)		1.20 (-0.17, 2.56)		-2.58 (-4.77, -0.39)	
High Folate, High B_6	-1.03 (-2.01, -0.05)	0.001	-0.62 (-1.74, 0.49)	0.32	1.65 (-0.14, 3.44)	0.01
Folate and B_2						
Low Folate, Low B_2	0.00 (ref)		0.00 (ref)		0.00 (ref)	
Low Folate, High B_2	-0.46 (-1.68, 0.75)		-1.76 (-3.12, -0.40)		2.23 (0.02, 4.43)	
High Folate, Low B_2	-0.20 (-1.40, 1.01)		-0.27 (-1.63, 1.08)		0.47 (-1.73, 2.67)	
High Folate, High B_2	-0.72 (-1.73, 0.28)	0.94	-0.37 (-1.49, 0.76)	0.09	1.09 (-0.74, 2.92)	0.31
B_{12} and B_6						
Low B_{12}, Low B_6	0.00 (ref)		0.00 (ref)		0.00 (ref)	
Low B_{12}, High B_6	-1.05 (-2.19, 0.10)		-1.72 (-3.01, -0.43)		2.76 (0.68, 4.84)	
High B_{12}, Low B_6	-0.30 (-1.45, 0.86)		-0.75 (-2.05, 0.55)		1.05 (-1.05, 3.15)	
High B_{12}, High B_6	-1.13 (-2.21, -0.05)	0.80	-1.18 (-2.39, 0.04)	0.17	2.31 (0.35, 4.27)	0.32
B_{12} and B_2						
Low B_{12}, Low B_2	0.00 (ref)		0.00 (ref)		0.00 (ref)	
Low B_{12}, High B_2	-0.43 (-1.61, 0.76)		-1.47 (-2.80, -0.13)		1.89 (-1.31, 3.02)	
High B_{12}, Low B_2	-0.08 (-1.26, 1.11)		-0.78 (-2.11, 0.56)		0.86 (-1.31, 3.02)	
High B_{12}, High B_2	-0.70 (-1.72, 0.32)	0.82	-0.78 (-1.93, 0.37)	0.13	1.48 (-0.39, 3.35)	0.41
B_2 and B_6						
Low B_2, Low B_6	0.00 (ref)		0.00 (ref)		0.00 (ref)	
Low B_2, High B_6	-0.57 (-1.73, 0.59)		-1.45 (-2.76, -0.14)		2.02 (-0.10, 4.14)	
High B_2, Low B_6	-0.05 (-1.24, 1.14)		-1.03 (-2.37, 0.31)		1.08 (-1.09, 3.24)	
High B_2, High B_6	-1.25 (-2.29, -0.21)	0.46	-1.51 (-2.68, -0.34)	0.31	2.76 (0.88, 4.65)	0.83

[a]Results adjusted for total log arsenic, log urinary creatinine, age, sex, center, smoking, alcohol, eGFR, BMI (vitamins are calorie-corrected)

Supplemental Table S4. Median Intake of OCM Nutrients compared to Recommended Dietary Allowance

Vitamin	Recommended Dietary Allowance (RDA)[67-70]	SHS Median[a] (IQR)	Bangladesh[41] Median[a] (IQR)
B_2	1.3 mg (Men) 1.1 mg (Women)	1.27 (1.01, 1.59) mg	0.90 (0.78, 1.07) mg
B_6	1.3 mg (Men and Women aged 19-50 years) 1.7 mg (Men aged 51+ years) 1.5 mg (Women aged 51+ years)	1.14 (0.83, 1.60) mg	3.38 (3.24, 3.57) mg
Folate	400 DFE	193 (130-309) µg[b]	263 (218, 323) µg
B_{12}	2.4 µg	2.91 (1.88, 4.91) µg	1.58 (1.04, 2.38) µg

[a]Intakes for both SHS and Bangladesh medians are calorie-adjusted, which were lower than non-adjusted values in our study

[b]We were unable to calculate Dietary Folate Equivalents (DFE) so comparison to RDA is difficult

CHAPTER 2

The Association of Arsenic Exposure and Arsenic Metabolism with the Metabolic Syndrome and its Individual Components: Prospective Evidence from the Strong Heart Family Study

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ABSTRACT

Inorganic arsenic exposure is ubiquitous and both exposure and inter-individual differences in its metabolism have been associated with cardiometabolic risk. The association between arsenic exposure and arsenic metabolism with metabolic syndrome and its individual components, however, is relatively unknown. We used poisson regression with robust variance to evaluate the association between baseline arsenic exposure (urine arsenic levels) and metabolism (relative percentage of arsenic species over their sum) with incident metabolic syndrome and its individual components (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension, elevated fasting plasma glucose (FPG)) in 1,047 participants from the Strong Heart Family Study, a prospective family-based cohort in American Indian communities. 32% of participants developed metabolic syndrome over follow-up. An IQR increase in arsenic exposure was associated with 1.19 (95% CI: 1.01, 1.41) greater risk for elevated FPG but not with other individual components or overall metabolic syndrome. Arsenic metabolism, specifically lower MMA% and higher DMA% was associated with higher risk of overall metabolic syndrome and elevated waist circumference, but not with any other component. These findings support there is a contrasting and independent association between arsenic exposure and arsenic metabolism with metabolic outcomes which may contribute to overall diabetes risk.

INTRODUCTION

Metabolic syndrome (MetS) affects a quarter of the global adult population and a third of US adults.[1, 2] Characterized by a clustering of abnormalities in waist circumference, triglycerides, cholesterol, blood pressure and glucose levels,[3] individuals with MetS have up to five-fold greater risk of suffering from coronary heart disease, stroke or diabetes.[2, 4] Efforts are needed to identify and intervene on preventable risk factors for MetS development.

Inorganic arsenic (iAs) is a carcinogen associated with increased risk of numerous health effects, including cardiometabolic outcomes.[5-22] After ingestion, iAs is converted to mono and dimethylated arsenic compounds (MMA and DMA) that are excreted in the urine.[23-25] Arsenic metabolism is measured by computing relative percentages of urine iAs, MMA, and DMA over their sum (iAs%, MMA%, DMA%). Inter-individual differences in arsenic metabolism are related to adverse health outcomes after controlling for arsenic exposure. Due to its relatively shorter half-life and rapid excretion through urine compared to iAs, higher DMA% is considered a more efficient arsenic metabolism profile and protective against arsenic toxicity. However, increasing evidence indicates that the association between arsenic metabolism and disease is more complex. Indeed, while higher urine MMA% and lower DMA% have been associated with greater risk for arsenic-related cancer[26-31] and cardiovascular disease,[32-34] lower MMA% and higher DMA% have been cross-sectionally associated with higher body mass index (BMI)[35] and prospectively with greater risk for insulin resistance,[36] diabetes[16, 37-39] and MetS.[11] The mechanism behind these contrasting associations is not clear, yet trends have been consistent across exposure levels and ethnicities.[40]

MetS is understudied in the context of arsenic and provides a useful opportunity to understand the conflicting associations reported for arsenic metabolism with metabolic outcomes versus the associations reported with cardiovascular disease, because of the clustering of inter-related but distinct components. To date, no studies have reported the relationship of arsenic with both MetS and its individual components. In this study, we provide a comprehensive evaluation of the prospective association of arsenic exposure and metabolism with MetS and its components in the Strong Heart Family Study (SHFS), a family-based cohort study of American Indian tribal members from Arizona, Oklahoma, and North/South Dakota. The study communities are affected by a high burden of MetS and exposed to low-to-moderate arsenic from drinking water and food.

METHODS

Study population

The SHFS recruited 2,919 participants in 1998-1999 and 2001-2003. Participants recruited in 1998-1999 (n=428) had follow-up visits in 2001-2003 and 2006-2009. Participants recruited in 2001-2003 (n=2,491) had a single follow-up visit in 2006-2009. For this study, we included participants free of diabetes at baseline with sufficient urine for arsenic analyses (n=1,720). We used urinary arsenic measured from baseline visits, and MetS data from 1-2 follow-up visits. Participants missing information on education, smoking, alcohol intake, BMI, estimated glomerular filtration rate (eGFR), and urine creatinine (n=18) were excluded. We further excluded participants missing MetS data (n=44) and prevalent MetS cases (n=611), resulting in 1,047 participants available for

incident MetS analyses. For incident individual components, we further excluded participants with prevalent cases of each component under analysis (**Figure 1).**

All participants provided informed consent and study protocols were approved by multiple institutional review boards, participating communities and The Indian Health Service.

Data Collection

Baseline and follow-up visits included bio-specimen collection, physical exams, food frequency questionnaire and an interview-administered questionnaire (age, sex, education, smoking history, alcohol use, medical history).[41] Waist circumference, blood pressure, height and weight, as well as urine and fasting blood sample collection, were performed during exams by centrally trained nurses following a standardized protocol.[42]

Urine arsenic

Morning spot urine samples were frozen within 1-2 hours of collection and stored at -70°C. For arsenic analyses, up to 1.0 mL from each urine sample was transported on dry ice to the Trace Element Laboratory at Graz University. Methods have been described.[43] Briefly, iAs, MMA, DMA and arsenobetaine were measured using high performance liquid chromatography/inductively coupled plasma mass spectrometry. Limits of detection were 0.1 μg/L for all four species. Arsenic species concentrations below the limit of detection (<5% for all species) were imputed as the limit of detection divided by √2. Arsenobetaine levels were low (median=0.51, IQR=0.34-1.00 μg/L),

confirming infrequent seafood consumption. Urine creatinine was analyzed using automated alkaline picrate methodology.

MetS

Fasting triglycerides, cholesterol, and glucose were measured using enzymatic methods. High-density lipoprotein cholesterol was measured by precipitation with heparin and manganese chloride.[42] The average of the last two of three systolic and diastolic blood pressure measurements taken on the right arm after 5 minutes of rest using a Baum mercury sphygmomanometer was calculated. Waist circumference was measured at the umbilicus while participants were in supine position.

MetS was characterized according to the National Cholesterol Education Program ATP III guidelines[3, 44], as recommended by the Indian Health Service,[45] and defined as ≥3 of the following: elevated waist circumference (≥102 centimeters for men, ≥88 centimeters for women); triglycerides ≥150mg/dL (or on medication); fasting plasma glucose ≥100mg/dL (or on medication); HDL cholesterol (<40mg/dL for men, <50mg/dL for women) (or on medication); systolic blood pressure ≥130mmHg or diastolic blood pressure ≥85mmHg (or on medication).

Other variables

Estimated daily average dietary intake of one carbon metabolism (OCM) micronutrients, including vitamins B_2, B_6 and folate, as well as total caloric intake and %kcal from fat and protein were measured at baseline through an interviewer-administered Block 119-item food frequency questionnaire.[46]

eGFR was calculated from plasma creatinine, age and sex using the Chronic Kidney Disease Epidemiology Collaboration formula.[47] BMI was calculated using weight in kilometers divided by height in meters squared.

We selected arsenite methyltransferase (*AS3MT)* single nucleotide polymorphism (SNP) rs12768205, available from the Illumina Cardio-Metabo DNA Analysis BeadChip (MetaboChip), because it showed the strongest association with arsenic metabolism biomarkers in the SHFS (index SNP).[48]

Statistical Analyses

Arsenic exposure, calculated as the sum of inorganic and methylated arsenic species (∑As) in urine, was right-skewed and log-transformed. Arsenic metabolism was computed by dividing each arsenic metabolite concentration over the sum of those species x 100 (iAs%, MMA% and DMA%). Differences in characteristics between participants with and without MetS were determined using Kruskal-Wallis and chi-square tests for continuous and categorical variables, respectively (**Table 1**). We assessed the prospective association of baseline urine arsenic measures (∑As, iAs%, MMA%, DMA%) with incident MetS and its individual components. We used modified poisson regression with robust variance[49] using generalized estimating equations with an independence working correlation structure to account for family clustering. Main analyses reported relative risk (RR) and 95% confidence intervals (95% CI) of MetS and each MetS component.

The associations of arsenic exposure with incident MetS and MetS components were reported per interquartile range (IQR) increase and with restricted cubic splines to

allow for flexibility in the dose-response. For arsenic metabolism, we conducted 3 types of analyses: 1) conventional model, 2) leave-one-out, and 3) principal component analysis. In conventional models, we evaluated each metabolite separately, reporting the relative risk for each outcome per 5 percentage point increase in each arsenic species. In leave-one-out models, for each arsenic species, we also included one of the species not being evaluated. For example, when evaluating iAs%, if we include MMA%, the regression coefficient estimates the relative risk associated with substituting DMA% for the equivalent iAs% while holding constant MMA% and ∑As. This leave-one-out approach has been used previously for arsenic metabolism.[37] Finally, we evaluated arsenic metabolism using principal component analyses, as recently proposed.[48, 50] Principal component analyses were conducted using each arsenic species percentage, then scaling them. This method is useful for arsenic metabolism because it removes the inter-dependence of the three biomarkers with the two resulting principal components allowing for potentially more biologically meaningful interpretation, by summarizing primary and secondary methylation steps, respectively.[48, 50] Although we cannot be certain the principal components truly reflect these methylation steps, as arsenic metabolism in the body is still debated, together with leave-one-out models, this modeling strategy contributes to a more comprehensive picture of how the three metabolites interact with respect to their association with health outcomes.

We used progressive adjustments for known arsenic and MetS determinants. For ∑As models, Model 1 adjusted for urine creatinine. Model 2 further adjusted for age (continuous), sex, region (Arizona, Oklahoma, North/South Dakota) and education (<12 years, 12+ years (or above/below appropriate level of schooling if aged <18 years)).

Model 3 further adjusted for BMI (<25, 25-<30, 30-<35, 35-<40, ≥40 kg/m^2), smoking status (never, former, current), alcohol use (never, former, current), and eGFR (continuous). For arsenic metabolism models, Model 1 additionally adjusted for log ∑As. In models evaluating waist circumference, BMI was not included as an adjustment for consistency with previous literature.[51]

Effect modification between arsenic metabolism and region, education, smoking status, BMI, ∑As, age, B vitamin intake and *AS3MT* genotype were conducted by adding interaction terms. Interaction p-values were obtained using Wald tests for multiple coefficients. Participants missing information on *AS3MT* (n=12) or vitamin intake (n=75) were excluded from interaction analyses.

We conducted several sensitivity analyses for arsenic metabolism and MetS models. First, we adjusted for number of MetS events present at baseline (0, 1 or 2), as the greater the number of baseline abnormalities, the fewer "new" abnormalities to be gained to develop MetS. Second, we adjusted for dietary OCM vitamin intake and total calories (both log-transformed). Third, we adjusted for %kcal from fat and protein. Finally, we adjusted for baseline waist circumference in models evaluating arsenic metabolism and elevated waist circumference.

RESULTS

Participant Characteristics

Of 1,047 participants without MetS at baseline, 338 (32%) developed MetS over a median follow-up period of 5.3 (IQR 4.7-6.5) years. Median age was 30.7 years with 56.4% female and 43.6% male. Median urine ∑As was 6.5 µg/L and 10.6%, 15.4% and

73.3%, respectively, for iAs%, MMA% and DMA% (**Table 1**). Compared to participants that remained free of MetS over follow-up, those who developed MetS were older, more educated, had higher BMI and DMA% and lower eGFR, iAs% and MMA% at baseline (P <0.05). The first arsenic metabolism principal component (PC1) explained 80.1% of variance in arsenic species and reflected higher DMA% and lower iAs% and MMA%, possibly representing overall methylation to DMA, or the secondary arsenic methylation step (**Table 2**). The second arsenic metabolism principal component (PC2) explained 19.9% of variance and reflected higher MMA% and lower iAs%, independent of DMA%, potentially reflecting the primary methylation step.

Association of Arsenic Exposure and Metabolism with Incident MetS

In adjusted models, ∑As was not associated with incident MetS (per ∑As IQR increase, RR=1.03 [95%CI: 0.90, 1.18]) (**Table 3**). For arsenic metabolism, in adjusted conventional models, the relative risk (95% CI) for incident MetS per 5% increase in iAs%, MMA% and DMA% was 0.94 (0.88, 1.01), 0.87 (0.79, 0.95) and 1.07 (1.02, 1.12), respectively. In iAs-fixed leave-one-out models for MMA% and DMA%, the relative risk (95% CI) for MMA% remained the same (0.87 (0.79, 0.97)) but strengthened to 1.14 (1.03, 1.27) for DMA% (**Table 3**). Consistently, in flexible dose-response analyses, DMA% showed a linear association with MetS when iAs% was included in the model (**Supplemental Figure S1**). The association for DMA%, however, was attenuated in the MMA%-fixed leave-one-out model. In both leave-one-out models for iAs% (fixing either MMA% or DMA%), there remained no significant association with MetS. When modeling arsenic metabolism using principal component analysis, an IQR increase in

PC1 (greater DMA%, lower MMA% and iAs%) was associated with a 1.19 (95% CI: 1.06, 1.34) increased risk for MetS, while PC2 (greater MMA%, lower iAs%, independent of DMA%) was associated with a 0.93 (95% CI: 0.84, 1.03) decreased risk (**Table 3**).

In interaction analyses of arsenic metabolism by subgroup characteristics on incident MetS (**Table 4**), there were interactions between BMI and DMA% (*P*-interaction=0.03) and between rs12768205 and MMA% (*P*-interaction=0.05). The association between DMA% and MetS was weaker with increasing BMI category and the inverse association between MMA% and MetS was markedly stronger for participants with rs12768205 AA genotype.

In sensitivity analyses including additional adjustments for baseline number of MetS criteria and B vitamins (**Supplemental Table S1**), as well as %kcal from fat and protein (data not shown), we observed consistent results.

Association of Arsenic Exposure and Metabolism with Incident MetS Components

In adjusted models, ∑As was associated with elevated FPG (RR=1.19 [95%CI: 1.01, 1.41]) (**Table 5**), but not with any other MetS component. ∑As remained associated with elevated FPG in flexible dose-response models (**Figure 2**).

For arsenic metabolism, the strongest association with individual MetS components was for waist circumference (**Figure 3, Table 5**). In the conventional model for MMA%, the relative risk of elevated waist circumference for a 5% increase in MMA% was 0.83 (95% CI: 0.76, 0.91), with consistent associations in both leave-one-out models. For DMA%, the relative risk for elevated waist circumference increased from

1.07 (95% CI: 1.01, 1.13) for a 5% increase in DMA% in the conventional model to 1.22 (95% CI: 1.10, 1.34) in the iAs%-fixed leave-one-out model. For iAs%, the only association with waist circumference was in the DMA%-fixed leave-one-out model (RR=1.25 [95% CI: 1.08, 1.45]). In the waist circumference sensitivity analysis additionally adjusting for baseline waist circumference, results were consistent (data not shown). Clear associations were not observed between arsenic metabolism and other MetS components beyond waist circumference although in conventional models, generally, higher DMA% and lower MMA% were non-significantly associated with higher risk for elevated triglycerides, reduced HDL, and hypertension (**Figure 3, Table 5**).

DISCUSSION

In American Indian men and women aged ≥14 years from Arizona, Oklahoma and North/South Dakota, arsenic exposure was associated with increased risk for elevated FPG but not with MetS or other individual components. Arsenic metabolism patterns, independent of arsenic exposure, were associated with both incident MetS and elevated waist circumference, but not with other components of the syndrome. The relative percentages of MMA appeared to be the main driver behind these associations. The distinct and independent associations between arsenic exposure and arsenic metabolism with MetS and its individual components suggest these are unrelated phenomena that could be contributing to overall diabetes risk at low-levels of arsenic exposure. For arsenic exposure, the association appears to be predominately with hyperglycemia. For arsenic metabolism, the association appears to be mainly with central adiposity. The

possible effect of arsenic exposure on hyperglycemia and of arsenic metabolism on central adiposity, could be underlying mechanisms for the observed associations between arsenic and diabetes in multiple populations.

MetS has been identified as the driving force behind the global epidemics of diabetes and cardiovascular disease.[2] Metabolic conditions continue to rise, with projections for global diabetes incidence alone to double by 2025.[2] Similar trends are evident in the US.[1, 52] While NHANES does not report trends in American Indians, an analysis in an older American Indian cohort (45-74 years) reported a MetS prevalence of up to 63%.[45] American Indians/Native Alaskans are reported to have the highest age-adjusted prevalence of diabetes.[53] Our study is the first to evaluate MetS from adolescence onwards among American Indians. With a median age of 30 years, our baseline MetS prevalence of 36%, and incidence of 32% over a median of 5.3 years of follow-up is undoubtedly high. The elevated risk for diabetes and MetS among American Indians compared with other US populations may be in part influenced by genetic background, however, environmental factors, including disproportionate exposure to arsenic in drinking water, have also been implicated.[54-58] Our study supports that exposure to environmental contaminants, in particular arsenic, could play a role.

The association between higher ∑As and elevated FPG supports previous experimental and epidemiologic evidence suggesting that iAs may have a diabetogenic effect. Although the mechanisms are not fully understood, experimental studies have shown that arsenic may induce diabetes through inhibiting insulin signaling and insulin-dependent glucose uptake, with both microRNAs and mitochondria proposed as potential mechanistic links.[21, 22, 59, 60] Epidemiological studies at high (≥100 µg/L)[22, 61-64] and

moderate (<100 μg/L)[16-18, 38, 65-68] exposure levels have reported associations between arsenic and diabetes, although findings at low levels are mixed.[37, 69-71]

The association between arsenic exposure and MetS, as well as other components beyond FPG, has been less studied. In contrast to our null findings between ∑As and MetS, two studies,[11, 12] conducted in highly exposed Taiwanese populations, found a positive association. Similarly, studies conducted in high-exposure regions suggest arsenic exposure is associated with increased risk for hypertension,[14, 20, 32, 72, 73] with mostly null findings in low-exposure populations,[16, 19, 74] consistent with our results for hypertension. A few studies have suggested an association between arsenic exposure with triglycerides[75 16] and HDL[76, 77] in contrast to our null findings. However, epidemiological studies evaluating low-moderate arsenic and triglycerides, HDL or hypertension have been cross-sectional. Finally, the association between arsenic exposure and waist circumference has not been evaluated before. In our study, we found no association.

For arsenic metabolism, our finding that lower MMA% and higher DMA% is prospectively associated with both an increased risk for incident MetS and elevated waist circumference is consistent with studies that have evaluated these relationships,[11, 35] and related outcomes such as diabetes[16, 37, 39, 68] and BMI,[35, 78, 79] although most have been cross-sectional. To our knowledge, our evaluation of arsenic metabolism and MetS in a low-moderate arsenic-exposed population is novel, as well as our prospective assessment of arsenic metabolism with waist circumference. Further, our leave-one-out models add to prospective evidence showing that greater DMA% due to reductions in MMA%, are related to incident diabetes.[37]

Mechanistic understanding of the increased risk reported for metabolic outcomes with lower MMA% is limited. MMA and DMA in this study, as in most epidemiologic studies, includes pentavalent and trivalent forms. Trivalent forms oxidize rapidly in urine to pentavalent forms making them indistinguishable.[80] Trivalent methylated arsenicals are considered the most toxic metabolites and MMA^{III} has been proposed as a mechanism for the associations between higher MMA% and increased risk for cancer, cardiovascular disease and skin lesions.[21, 26, 34] In turn, some studies have suggested the association between higher DMA% and increased risk for metabolic-related outcomes may be due to DMA^{III}.[16, 37, 68] Other studies have suggested confounding by diet may explain these associations. For example, a nutritionally sufficient diet with high fat and protein intake may enhance arsenic metabolism efficiency while also having adverse metabolic effects.[39] In our study, however, adjusting for %kcal from protein and fat did not affect results. Diet has also been suggested to play a role through OCM.[37] OCM, regulated by B vitamins, is responsible for providing methyl groups necessary for methylation reactions in arsenic metabolism and could be driving the association between arsenic metabolism and metabolic outcomes.[25] However, we saw no evidence of confounding or effect modification by estimated B vitamin (folate, vitamin B_2 and vitamin B_6) intake. .

In pregnant women, DMA% increases as women progress through pregnancy.[81, 82] Because adiposity increases during pregnancy, it has been suggested that adiposity may be driving shifts in arsenic metabolism patterns.[35] Our finding that waist circumference had the strongest associations with arsenic metabolism supports adiposity playing a key role in the pathway between arsenic metabolism and metabolic outcomes. Our association was prospective and remained after adjustment for baseline waist circumference,

suggesting that adiposity may be a consequence of arsenic metabolism, not a cause, still we cannot discard the possibility that reverse causality explains the association. Of note, however, we saw a significant interaction for DMA% and MetS by BMI, with increasing strength of association with lower BMI. This could be interpreted as evidence against reverse causality, as we are seeing an association between higher DMA% and MetS among those with normal BMI at baseline. In a previous study in this cohort, participants with *AS3MT* rs12768205 AA genotype had lower MMA%.[48] We observed an interaction between MMA% and *AS3MT* genotype, with risk for MetS further reduced with increasing MMA% with each additional A allele, suggesting the inverse association between MMA% and MetS is stronger among those with genetically lower MMA%.

Our study strengths include broad age distribution, a well-established cohort, high-quality laboratory methods and consideration of genetic contributions. Like most epidemiological studies, we were unable to differentiate between trivalent and pentavalent forms of MMA and DMA. We confirmed seafood intake in our population was infrequent and adjusted for B vitamins, however, we cannot discount the potential for remaining confounding by food sources. Further, we did not have information on vitamin B_{12}, an important vitamin in the OCM pathway. Because we do not have repeated measures of arsenic exposure and metabolism we cannot evaluate how changes in adiposity and fasting glucose are related to changes in arsenic over time, and therefore cannot totally exclude the possibility of reverse causality despite its prospective design.

CONCLUSIONS

Our findings support previous evidence that arsenic has diabetogenic effects even at low-level exposures and may be contributing, in part, to the high burden of diabetes in arsenic-exposed populations. Further, our results suggest disruption of glucose regulation maybe a key pathway driving the association between low-level arsenic exposure and cardiometabolic outcomes. In addition, we found arsenic metabolism patterns, specifically lower MMA%, are prospectively associated with increased risk for development of MetS and elevated waist circumference. These findings support the importance of preventing low-level arsenic exposure and the need to better elucidate underlying mechanisms of the contrasting and independent associations for arsenic exposure and metabolism with MetS.

TABLES

Table 1. Baseline Participant Characteristics of American Indians in the Strong Heart Family Study by Incident Metabolic Syndrome Status at Follow-up, 1998-2009

	Overall	No MetS	MetS	P-value
Total, N (%)	1047	709 (67.7)	338 (32.3)	
Age (years), Median (IQR)	30.7 (20.6-41.8)	28.4 (19.5-40.9)	34.0 (24.4 – 44.4)	<0.001
Sex, N (%)				
Female	590 (56.4)	402 (56.7)	188 (55.6)	
Male	457 (43.6)	307 (43.3)	150 (44.4)	0.80
Education, N (%)				
<12 years	363 (34.7)	259 (36.5)	104 (30.8)	
12+ years	684 (65.3)	450 (63.5)	234 (69.2)	0.08
Smoking Status, N (%)				
Never	451 (43.1)	308 (43.4)	143 (42.3)	
Ever	185 (17.7)	123 (17.3)	62 (18.3)	
Current	411 (39.3)	278 (39.2)	133 (39.3)	0.91
Alcohol Intake, N (%)				
Never	119 (11.4)	88 (12.4)	31 (9.2)	
Ever	222 (21.2)	143 (20.2)	79 (23.4)	
Current	706 (67.4)	478 (67.4)	228 (67.5)	0.20
BMI (kg/m^2), Median (IQR)	27.4 (23.9-31.6)	26.1 (22.9-30.1)	30.1 (26.5-34.4)	<0.001
Urinary Creatinine (mg/dL), Median (IQR)	1.5 (1.0-2.1)	1.6 (1.0-2.2)	1.5 (1.1-2.1)	0.42
eGFR, Median (IQR)	122 (111-134)	123 (112-134)	120 (108-132)	0.02
∑As (μg/L), Median (IQR)	6.5 (4.2-10.8)	6.3 (4.2-10.4)	7.0 (4.2-11.7)	0.21
iAs%, Median (IQR)	10.6 (7.5-14.9)	10.9 (7.6-15.3)	9.9 (7.1-13.5)	0.01
MMA%, Median (IQR)	15.4 (12.2-19.0)	16.0 (12.7-19.8)	14.2 (11.2-17.6)	<0.001
DMA%, Median (IQR)	73.3 (66.3-78.9)	72.1 (65.6-78.2)	75.1 (69.1-80.5)	<0.001
MetS Components				
Waist Circumference (inches), Median (IQR)	36.6 (32.7-40.6)	35.4 (31.5-39.0)	39.0 (35.4-43.6)	<0.001
Elevated Waist Circumference[a], N (%)	473 (45.2)	270 (38.1)	203 (60.1)	<0.001
Triglycerides (mg/dL), Median (IQR)	104 (80.0-135)	96.0 (75.0-125)	119.5 (95.2-146.8)	<0.001
Elevated Triglycerides[b], N (%)	166 (15.9)	88 (12.4)	78 (23.1)	<0.001
HDL (mg/dL), Median (IQR)	53.0 (44.0-62.0)	54.0 (46.0-63.0)	50.0 (42.0-59.8)	<0.001
Reduced HDL[c], N (%)	255 (24.4)	148 (20.9)	107 (31.7)	<0.001
Systolic Blood Pressure (mmHg), Median (IQR)	115 (108-124)	114 (107-122)	119 (111-128)	<0.001
Diastolic Blood Pressure (mmgHg), Median (IQR)	74.0 (67.0-80.0)	73.0 (66.0-79.0)	76 (69.0-82.0)	<0.001
Hypertensive[d], N (%)	196 (18.7)	112 (15.8)	84 (24.9)	0.001
Fasting Glucose (mg/dL), Median (IQR)	90.0 (85.0-95.0)	90.0 (85.0-95.0)	92.0 (86.0-96.0)	0.001
Elevated FPG[e], N (%)	110 (10.5)	77 (10.9)	33 (9.8)	0.67

[a]Meets criteria for elevated waist circumference component of MetS ((≥102 cm in men and ≥88 cm in women)
[b]Meets criteria for elevated triglyceride component of MetS (≥150 mg/dL (or on medication))
[c]Meets criteria for reduced HDL of MetS (≥100 mg/dL (or on medication))
[d]Meets criteria for hypertensive component of MetS (<40 mg/dL for men and <50 mg/dL for women (or on medication))
[e]Meets criteria for elevated FPG component of MetS (systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg (or on medication))
SI conversion factors: To convert HDL cholesterol to mmol/L, multiply values by 0.0259
Abbreviations: MetS (metabolic syndrome), IQR (interquartile range), BMI (body mass index), eGFR (estimated glomerular filtration rate), FPG (fasting plasma glucose)

Table 2. Summary of Principal Components of Arsenic Species, The Strong Heart Family Study, 1998-2009

	PC1	PC2
Variance in arsenic species explained (%)	80.1	19.9
Standard deviation	1.55	0.77
Weight for iAs%	-0.55	-0.67
Weight for MMA%	-0.53	0.74
Weight for DMA%	0.64	0.04

Abbreviations: dimethylarsinic acid (DMA); inorganic arsenic (iAs); monomethylarsonic acid (MMA); PC1 (principal component 1); PC2 (principal component 2)

Table 3. Relative Risk of Incident Metabolic Syndrome per IQR increase in ∑As, 5% increase in Arsenic Metabolism Biomarkers (iAs%, MMA% and DMA%), and IQR increase in Arsenic Metabolism Principal Components, The Strong Heart Family Study, 1998-2009 (N=1047)

	Model 1[a]		**Model 2[b]**		**Model 3[c]**	
	RR	*CI*	*RR*	*CI*	*RR*	*CI*
∑As, IQR (10.8 vs 4.20)	1.13	0.99, 1.29	1.05	0.92, 1.19	1.03	0.90, 1.18
iAs% (5% increase)						
Conventional Model	0.90	0.84, 0.97	0.91	0.85, 0.97	0.94	0.88 1.01
Leave-one-out Models						
MMA% fixed	0.97	0.91, 1.04	0.97	0.90, 1.04	0.98	0.91 1.05
DMA% fixed	1.21	1.04, 1.40	1.19	1.02, 1.39	1.12	0.96 1.30
MMA% (5% increase)						
Conventional Model	0.79	0.72, 0.87	0.80	0.72, 0.88	0.87	0.79 0.95
Leave-one-out Models						
iAs% fixed	0.81	0.73, 0.89	0.81	0.73, 0.91	0.87	0.79 0.97
DMA% fixed	0.83	0.71, 0.96	0.84	0.72, 0.98	0.89	0.77 1.04
DMA% (5% increase)						
Conventional Model	1.11	1.06, 1.17	1.11	1.06, 1.17	1.07	1.02 1.12
Leave-One-Out-Models						
iAs% fixed	1.24	1.12, 1.38	1.23	1.10, 1.37	1.14	1.03 1.27
MMA% fixed	1.03	0.96, 1.10	1.04	0.96, 1.11	1.02	0.95 1.10
PC1, IQR (1.04 vs -0.92)	1.32	1.17, 1.48	1.32	1.16, 1.49	1.19	1.06 1.34
PC2, IQR (0.5 vs -0.38)	0.90	0.82, 0.98	0.91	0.82, 1.00	0.93	0.84 1.03

Abbreviations: ∑As (iAs+MMA+DMA); confidence interval (CI); dimethylarsinic acid (DMA); inorganic arsenic (iAs); IQR (interquartile range); monomethylarsonic acid (MMA); PC1 (arsenic metabolism principal component 1); PC2 (arsenic metabolism principal component 2); relative risk (RR)

[a]Model 1 adjusts for log total arsenic (except in models where ∑As is the predictor of interest), urinary creatinine

[b]Model 2 further adjusts for sex, center, education

[c]Model 3 further adjusts for alcohol intake, smoking status, kidney function and BMI

Table 4. Relative Risk of Incident Metabolic Syndrome per 5% increase in Arsenic Metabolism Biomarkers (iAs%, MMA% and DMA%) by Levels of other Risk Factors, The Strong Heart Family Study, 1998-2009 (N=972)

	Cases/ Controls	iAs%[a]	P-interaction	MMA%[a]	P-interaction	DMA%[a]	P-interaction
Age (years)							
≤ 30	121 / 353	0.99 (0.90, 1.11)		0.95 (0.82, 1.11)		1.02 (0.94, 1.09)	
> 30 – 50	141 / 242	0.88 (0.79, 0.99)		0.83 (0.73, 0.93)		1.12 (1.04, 1.20)	
> 50	49 / 71	0.97 (0.78, 1.21)	0.32	0.73 (0.59, 0.90)	0.10	1.13 (0.98, 1.30)	0.16
Gender							
Male	133 / 288	0.93 (0.84, 1.04)		0.80 (0.70, 0.92)		1.09 (1.02, 1.19)	
Female	178 / 378	0.95 (0.87, 1.05)	0.71	0.90 (0.79, 1.02)	0.24	1.05 (0.99, 1.13)	0.43
Education							
≤ 12 years	96 / 243	0.97 (0.86, 1.08)		0.79 (0.62, 1.01)		1.06 (0.98, 1.15)	
> 12 years	215 / 423	0.92 (0.84, 1.02)	0.55	0.80 (0.69, 0.93)	0.90	1.08 (1.01, 1.15)	0.74
Smoking Status							
Never	131 / 284	0.96 (0.84, 1.08)		0.82 (0.70, 0.95)		1.08 (0.99, 1.17)	
Ever	59 / 119	0.95 (0.80, 1.13)		0.87 (0.70, 1.07)		1.06 (0.95, 1.20)	
Current	121 / 263	0.92 (0.83, 1.04)	0.92	0.88 (0.77, 1.00)	0.76	1.07 (1.00, 1.15)	0.97
BMI kg/m^{2}[b]							
Normal	53 / 280	0.86 (0.70, 1.05)		0.75 (0.61, 0.92)		1.17 (1.03, 1.32)	
Overweight	104 / 208	0.90 (0.79, 1.01)		0.82 (0.70, 0.95)		1.11 (1.02, 1.21)	
Obese	154 / 178	1.02 (0.92, 1.12)	0.14	0.93 (0.82, 1.06)	0.13	1.01 (0.95, 1.08)	0.06
Folate (µg)							
< 352	155 / 333	0.97 (0.87, 1.08)		0.90 (0.79, 1.03)		1.04 (0.97, 1.13)	
≥ 352	156 / 333	0.92 (0.83, 1.02)	0.45	0.84 (0.74, 0.95)	0.43	1.09 (1.02, 1.16)	0.35
Vitamin B$_6$ (mg)							
< 1.7	151 / 334	1.00 (0.90, 1.12)		0.87 (0.76, 0.99)		1.04 (0.97, 1.13)	
≥ 1.7	160 / 332	0.90 (0.81, 0.99)	0.14	0.84 (0.75, 0.95)	0.74	1.09 (1.03, 1.17)	0.27
Vitamin B$_2$ (mg)							
< 1.7	143 / 318	0.97 (0.87, 1.09)		0.89 (0.76, 1.02)		1.05 (0.97, 1.13)	
≥ 1.7	168 / 348	0.92 (0.84, 1.01)	0.44	0.83 (0.73, 0.93)	0.47	1.09 (1.03, 1.16)	0.36
∑As (µg/L)							
≤ 4.20	77 / 168	0.93 (0.81, 1.08)		0.83 (0.68, 1.01)		1.08 (0.98, 1.20)	
> 4.20 – 6.58	68 / 188	1.05 (0.90, 1.23)		0.86 (0.71, 1.05)		1.02 (0.92, 1.14)	
> 6.58 – 10.84	82 / 154	0.90 (0.76, 1.05)		0.86 (0.72, 1.04)		1.09 (0.99, 1.21)	
> 10.84	84 / 156	0.90 (0.79, 1.03)	0.43	0.88 (0.76, 1.01)	0.98	1.08 (1.00, 1.17)	0.80

AS3MT							
GG	155 / 330	0.97 (0.90, 1.05)		0.90 (0.80, 1.00)		1.04 (0.98, 1.11)	
GA	134 / 264	0.87 (0.76, 1.00)		0.83 (0.71, 0.96)		1.12 (1.03, 1.22)	
AA	22 / 72	1.09 (0.70, 1.72)	0.33	0.54 (0.35, 0.83)	0.06	1.15 (0.90, 1.49)	0.34
Overall	311 / 666	0.94 (0.87, 1.02)		0.85 (0.78, 0.93)		1.07 (1.02, 1.13)	

Abbreviations: Arsenite methyltransferase (*AS3MT*); ∑As (iAs+MMA+DMA); BMI (body mass index); dimethylarsinic acid (DMA); inorganic arsenic (iAs); monomethylarsonic acid (MMA); BMI (body mass index)

P-values for interactions were obtained using Wald tests for multiple coefficients

[a]Models adjusted for log urinary total arsenic, log urinary creatinine, age, sex, center, smoking status, alcohol intake, B vitamin intake, caloric intake, education, BMI, and kidney function

[b]Normal = <25, overweight = 25- <30, obese = ≥30

Table 5. Relative Risk (95% Confidence Interval) of Incident Metabolic Syndrome Components per IQR increase in ∑As and 5% increase in Arsenic Metabolism Biomarkers (iAs%, MMA% and DMA%), The Strong Heart Family Study, 1998-2009

Arsenic Biomarker by Model and Outcome	No.	Model 1[a]		Model 2[b]		Model 3[c]	
Elevated Waist Circumference	574	*RR*	*CI*	*RR*	*CI*	*RR*	*CI*
∑As IQR (10.84 vs 4.2)		0.99	0.86, 1.14	0.99	0.86, 1.14	0.98	0.85, 1.13
iAs%							
Conventional Model		0.96	0.88, 1.04	0.97	0.89, 1.06	0.97	0.89, 1.06
Leave-one-out Model (MMA% fixed)		1.02	0.94, 1.12	1.02	0.94, 1.12	1.03	0.94, 1.12
Leave-one-out Model (DMA% fixed)		1.29	1.10, 1.51	1.25	1.07, 1.45	1.25	1.08, 1.45
MMA%							
Conventional Model		0.80	0.74, 0.88	0.83	0.76, 0.91	0.83	0.76, 0.91
Leave-one-out Model (iAs% fixed)		0.80	0.72, 0.88	0.82	0.75, 0.91	0.82	0.75, 0.91
Leave-one-out Model (DMA% fixed)		0.78	0.66, 0.91	0.80	0.69, 0.93	0.80	0.69, 0.93
DMA%							
Conventional Model		1.08	1.03, 1.14	1.07	1.01, 1.13	1.07	1.01, 1.13
Leave-one-out Model (iAs% fixed)		1.26	1.14, 1.39	1.22	1.10, 1.34	1.22	1.10, 1.34
Leave-one-out Model (MMA% fixed)		0.98	0.89, 1.06	0.98	0.90, 1.06	0.97	0.89, 1.06
Elevated Triglycerides	881						
∑As IQR (10.84 vs 4.2)		0.96	0.81, 1.14	1.00	0.84, 1.19	0.98	0.82, 1.19
iAs%							
Conventional Model		0.95	0.86, 1.04	0.93	0.84, 1.03	0.93	0.84, 1.03
Leave-one-out Model (MMA% fixed)		0.96	0.87, 1.06	0.95	0.86, 1.06	0.94	0.84, 1.05
Leave-one-out Model (DMA% fixed)		0.99	0.82, 1.18	1.01	0.84, 1.22	0.99	0.81, 1.19
MMA%							
Conventional Model		0.95	0.85, 1.06	0.92	0.82, 1.03	0.93	0.83, 1.04
Leave-one-out Model (iAs% fixed)		0.97	0.86, 1.09	0.94	0.83, 1.06	0.96	0.84, 1.08
Leave-one-out Model (DMA% fixed)		1.01	0.84, 1.21	0.99	0.82, 1.19	1.01	0.84, 1.23
DMA%							
Conventional Model		1.04	0.98, 1.10	1.06	0.99, 1.13	1.05	0.99, 1.13
Leave-one-out Model (iAs% fixed)		1.03	0.92, 1.16	1.06	0.94, 1.20	1.05	0.92, 1.18
Leave-one-out Model (iAs% fixed)		1.04	0.94,1.15	1.05	0.94, 1.17	1.06	0.95, 1.18
Reduced HDL	792						
∑As IQR (10.84 vs 4.2)		1.07	0.91, 1.25	0.96	0.81, 1.14	0.95	0.80, 1.14
iAs%							
Conventional Model		0.89	0.81, 0.98	0.90	0.82, 0.99	0.92	0.84, 1.02
Leave-one-out Model (MMA% fixed)		0.94	0.86, 1.04	0.93	0.85, 1.03	0.94	0.85, 1.04

Leave-one-out Model (DMA% fixed)		1.11	0.93, 1.34	1.04	0.86, 1.25	1.00	0.83, 1.22
MMA%							
Conventional Model		0.82	0.73, 0.93	0.87	0.77, 0.98	0.91	0.81, 1.03
Leave-one-out Model (iAs% fixed)		0.85	0.75, 0.96	0.90	0.79, 1.02	0.94	0.82, 1.07
Leave-one-out Model (DMA% fixed)		0.90	0.75, 1.08	0.96	0.80, 1.16	1.00	0.82, 1.21
DMA%							
Conventional Model		1.11	1.04, 1.18	1.09	1.02, 1.16	1.06	1.00, 1.13
Leave-one-out Model (iAs% fixed)		1.18	1.04, 1.34	1.12	0.98, 1.27	1.06	0.93, 1.22
Leave-one-out Model (iAs% fixed)		1.06	0.96, 1.16	1.07	0.97, 1.18	1.06	0.96, 1.17
Hypertension	851						
∑**As** IQR (10.84 vs 4.2)		1.11	0.91, 1.36	1.05	0.90, 1.23	1.03	0.88, 1.20
iAs%							
Conventional Model		1.00	0.93, 1.09	0.98	0.90, 1.06	1.00	0.92, 1.08
Leave-one-out Model (MMA% fixed)		1.00	0.91, 1.09	1.00	0.91, 1.10	1.01	0.91, 1.12
Leave-one-out Model (DMA% fixed)		0.97	0.80, 1.18	1.09	0.89, 1.33	1.05	0.85, 1.30
MMA%							
Conventional Model		1.02	0.90, 1.16	0.92	0.83, 1.03	0.96	0.86, 1.08
Leave-one-out Model (iAs% fixed)		1.02	0.89, 1.18	0.92	0.81, 1.05	0.96	0.84, 1.10
Leave-one-out Model (DMA% fixed)		1.03	0.85, 1.25	0.92	0.75, 1.12	0.95	0.77, 1.17
DMA%							
Conventional Model		0.99	0.93, 1.05	1.03	0.98, 1.09	1.01	0.96, 1.06
Leave-one-out Model (iAs% fixed)		0.98	0.85, 1.12	1.08	0.95, 1.23	1.04	0.91, 1.19
Leave-one-out Model (iAs% fixed)		1.00	0.92, 1.10	1.00	0.91, 1.10	0.99	0.89, 1.09
Elevated Fasting Plasma Glucose	937						
∑**As** IQR (10.84 vs 4.2)		1.48	1.28, 1.73	1.25	1.07, 1.46	1.19	1.01, 1.41
iAs%							
Conventional Model		1.04	0.95, 1.13	1.01	0.92, 1.10	1.03	0.95, 1.12
Leave-one-out Model (MMA% fixed)		1.07	0.98, 1.17	1.04	0.95, 1.13	1.03	0.94, 1.12
Leave-one-out Model (DMA% fixed)		1.21	1.01, 1.44	1.16	0.97, 1.38	1.03	0.86, 1.23
MMA%							
Conventional Model		0.92	0.82, 1.03	0.91	0.81, 1.03	1.01	0.90, 1.14
Leave-one-out Model (iAs% fixed)		0.89	0.78, 1.00	0.90	0.79, 1.02	1.00	0.88, 1.14
Leave-one-out Model (DMA% fixed)		0.83	0.69, 0.99	0.86	0.72, 1.03	0.97	0.82, 1.16
DMA%							
Conventional Model		1.01	0.95, 1.07	1.02	0.96, 1.09	0.98	0.93, 1.04
Leave-one-out Model (iAs% fixed)		1.13	1.00, 1.27	1.12	0.98, 1.27	1.00	0.88, 1.13
Leave-one-out Model (iAs% fixed)		0.93	0.86, 1.02	0.97	0.88, 1.05	0.97	0.89, 1.06

Abbreviations: ∑As (iAs+MMA+DMA); confidence interval (CI); dimethylarsinic acid (DMA); inorganic arsenic (iAs); IQR (interquartile range); monomethylarsonic acid (MMA); relative risk (RR)
[a]Model 1 adjusts for log total arsenic (except in models where ∑As is the predictor of interest), urinary creatinine
[b]Model 2 further adjusts for age, sex, center, education
[c]Model 3 further adjusts for alcohol intake, smoking status, kidney function and BMI (except in waist circumference models)

FIGURES

Figure 1. Study Flow Diagram. Flow diagram of study participants for Metabolic Syndrome analyses as well as individual Metabolic Syndrome component (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated fasting plasma glucose) analyses. Abbreviations: fasting plasma glucose (FPG); metabolic syndrome (MetS); triglycerides (TG); waist circumference (WC)

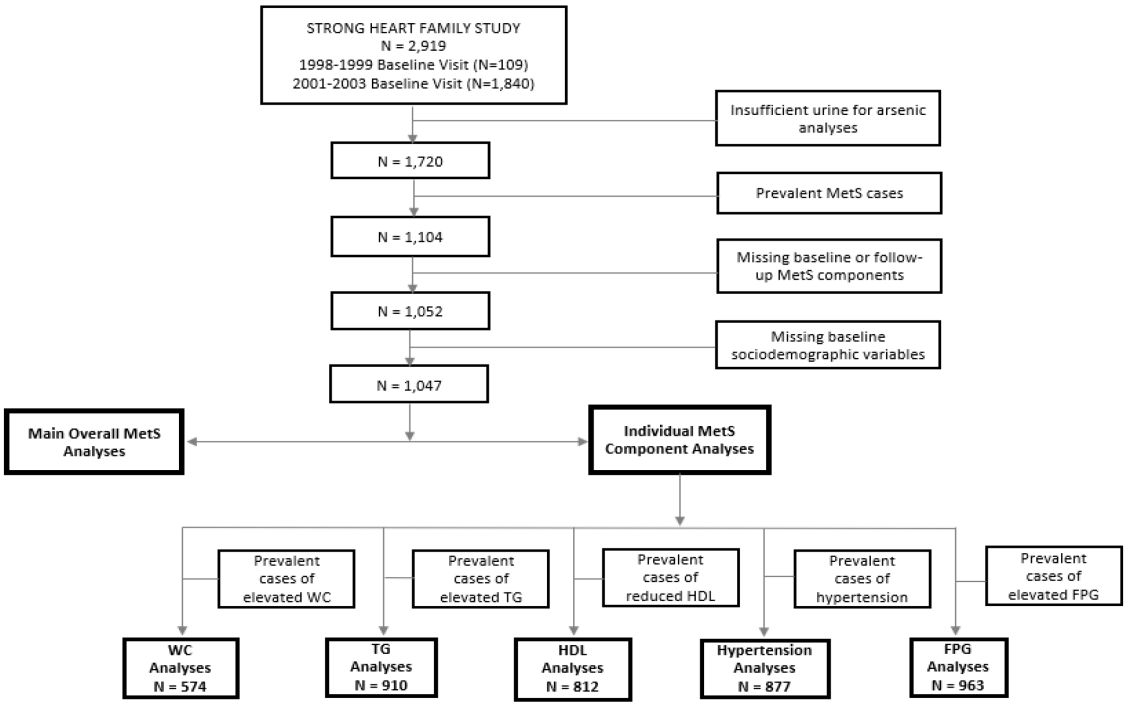


Figure 2. Relative risk (95% Confidence Interval) for Metabolic Syndrome and its individual components by ∑As using restricted cubic splines, The Strong Heart Family Study, 1998-2009. The lines represent adjusted relative risks of incident metabolic syndrome, elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated FPG based on restricted cubic splines for ∑As with knots at the 10th, 50th and 90th percentiles of the ∑As distribution. Shaded areas surrounding the lines represent the 95% confidence intervals. The reference was set at the 10th percentile of each arsenic percentage distribution. Relative risks were adjusted for log urinary creatinine, age, sex, body mass index (excluding waist circumference models), educational level, tribal center, smoking status, alcohol intake and kidney function. Abbreviations: ∑As (arsenic exposure: sum of urinary iAs, MMA and DMA concentrations); dimethylarsinic acid (DMA); fasting plasma glucose (FPG); inorganic arsenic (iAs); metabolic syndrome (MetS); monomethylarsonic acid (MMA).

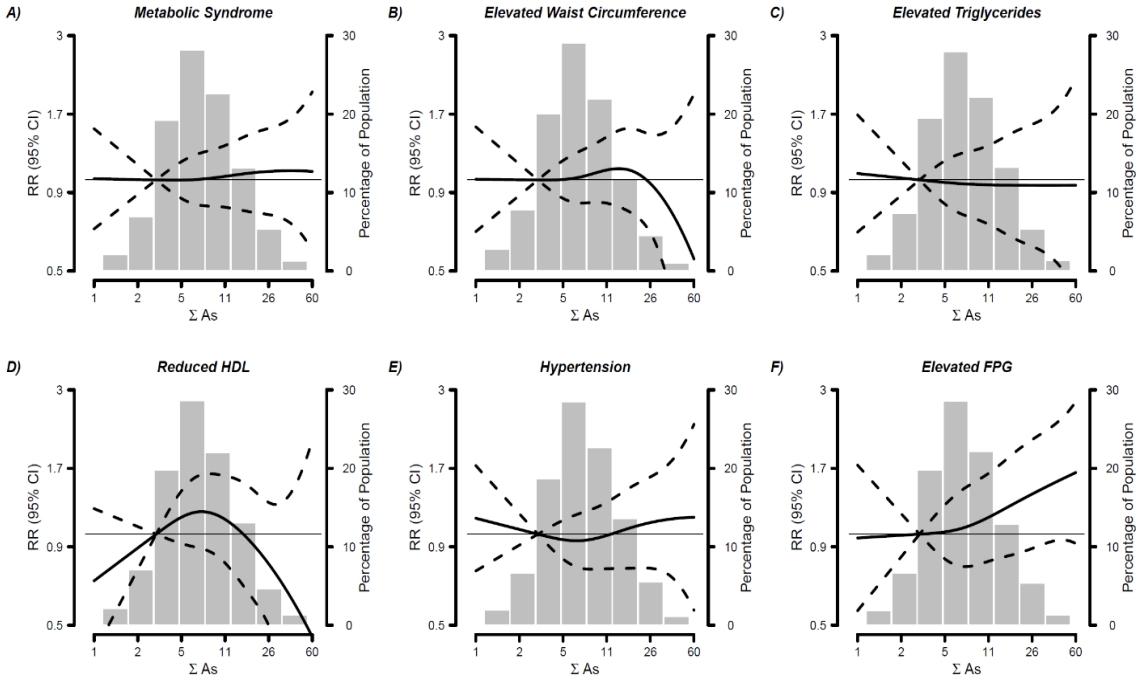
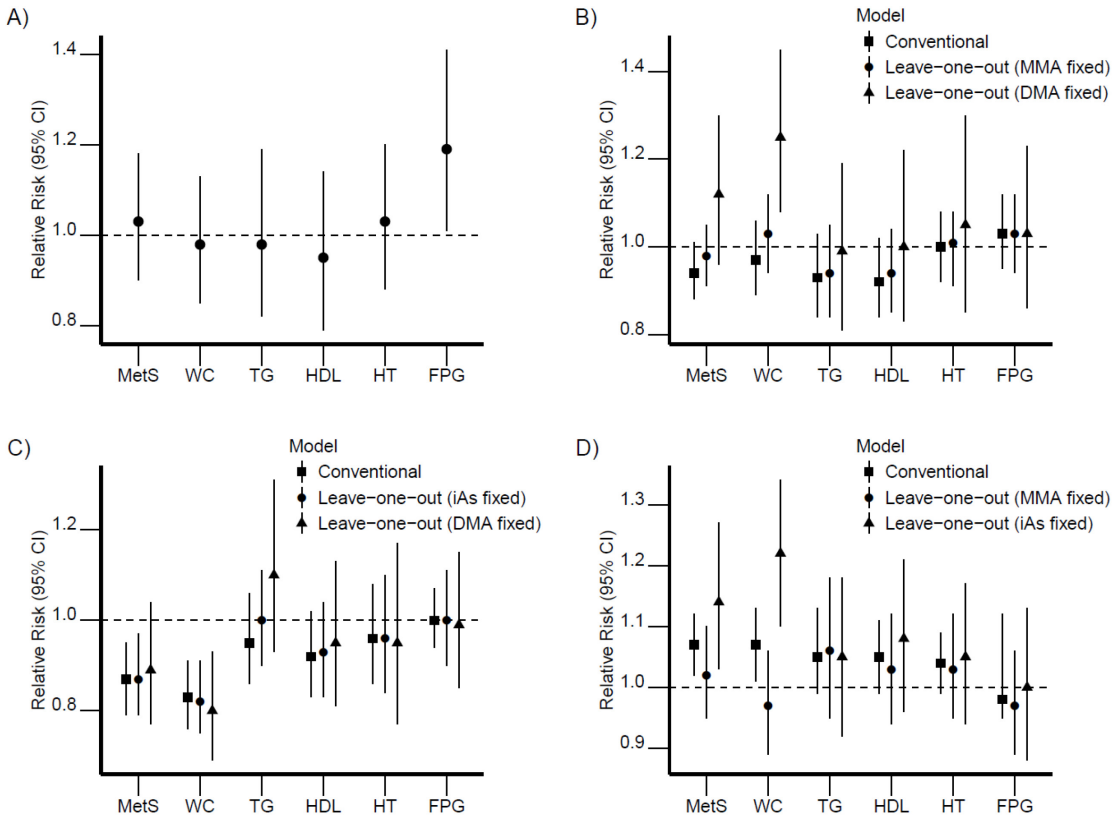
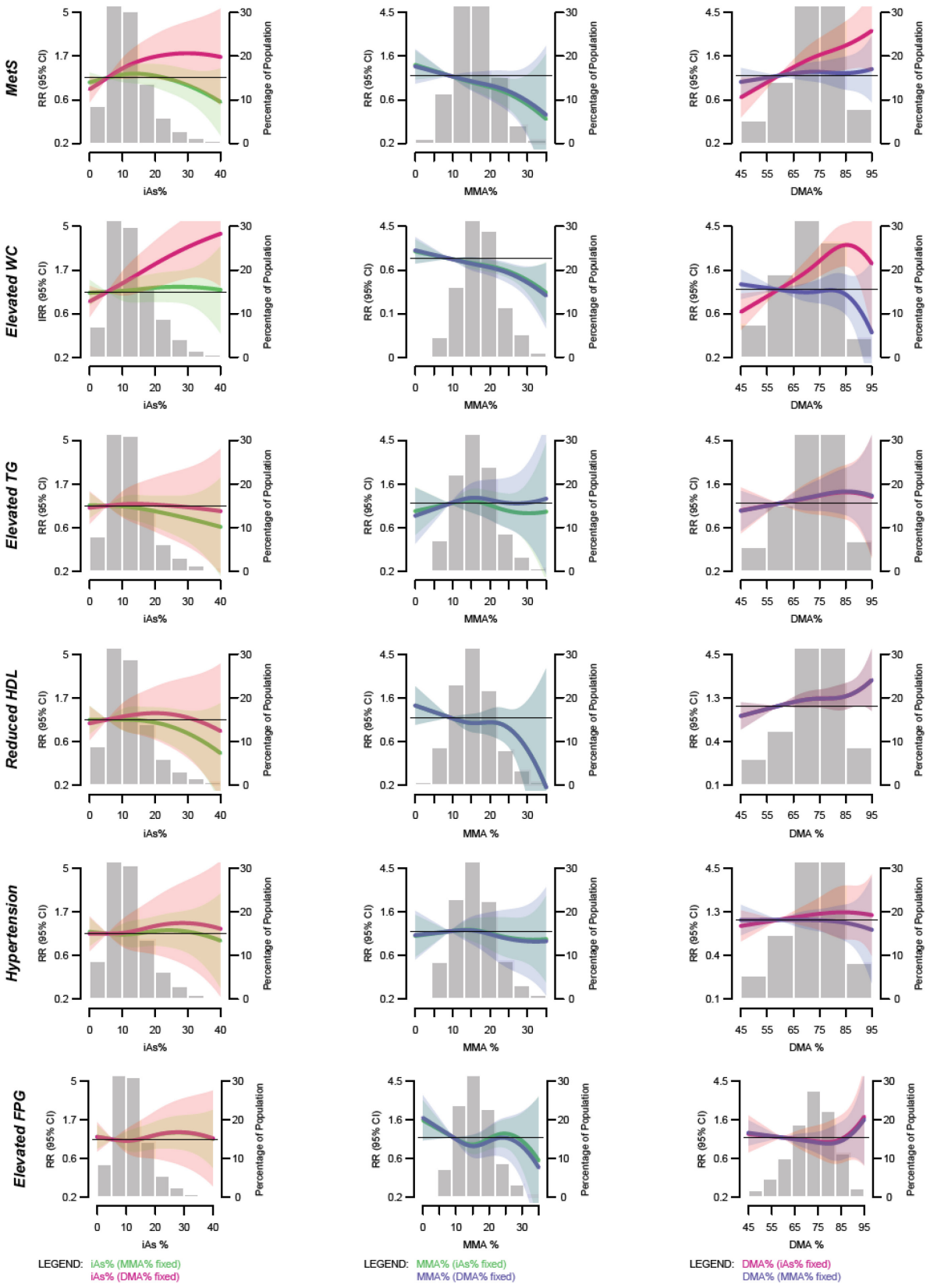


Figure 3. Relative Risk (95% Confidence Interval) for Metabolic Syndrome and its Individual Components per IQR Increase in ∑As and 5% Increase in Arsenic Metabolism Biomarkers (iAs%, MMA%, DMA%), The Strong Heart Family Study, 1998-2009. A) Relative risk for metabolic syndrome and its individual components (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated fasting plasma glucose) by IQR increase in ∑As. Points (circles) represent relative risk and lines represent 95% confidence intervals. Relative risk for metabolic syndrome and its individual per 5% increases in B) iAs%, C) MMA% and D) DMA%. The three different point types in figures B-D reflect three different models used to estimate the relative risk: the conventional model (square) and the two leave-one-out models (circle and triangle). Lines represent 95% confidence intervals. Relative risks were adjusted for log ∑iAs (excluding exposure models), log urinary creatinine, age, sex, body mass index (excluding waist circumference models), educational level, tribal center, smoking status, alcohol intake and kidney function. Abbreviations: ∑As (arsenic exposure: sum of urinary iAs, MMA and DMA concentrations); dimethylarsinic acid (DMA); fasting plasma glucose (FPG); hypertension (HT); inorganic arsenic (iAs); metabolic syndrome (MetS); monomethylarsonic acid (MMA); triglycerides (TG); waist circumference (WC).



Supplemental Material

Supplemental Figure S1. Leave-one out models reporting relative risk (95% Confidence Interval) for Metabolic Syndrome and its individual components by arsenic metabolism biomarkers (iAs%, MMA%, DMA%) using restricted cubic splines, The Strong Heart Family Study, 1998-2009. The lines represent adjusted relative risks of incident MetS, elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated FPG based on restricted cubic splines for each arsenic percentage with knots at the 10th, 50th and 90th percentiles of each arsenic percentage distribution. Shaded areas surrounding the lines represent the 95% confidence intervals. The reference was set at the 10th percentile of each arsenic percentage distribution. Relative risks were adjusted for log ∑As, log urinary creatinine, age, sex, body mass index (excluding waist circumference models), educational level, tribal center, smoking status, alcohol intake and kidney function. In models for iAs%, green and pink results are additionally adjusted for MMA% and DMA%, respectively. In models for MMA%, green and purple results are additionally adjusted for iAs% and DMA%, respectively. In models for DMA%, pink and purple results are additionally adjusted for iAs% and MMA%, respectively. Histogram bars represent the distribution of each arsenic percentage. For this figure, extreme tails in the histograms have been truncated for fit (iAs% > 40 = 3, MMA% > 35 = 1, DMA% < 45 = 10). Abbreviations: ∑As (arsenic exposure: sum of urinary iAs, MMA and DMA concentrations); dimethylarsinic acid (DMA); fasting plasma glucose (FPG); inorganic arsenic (iAs); monomethylarsonic acid (MMA); metabolic syndrome (MetS); triglycerides (TG); waist circumference (WC)


MetS
Elevated WC
Elevated TG
Reduced HDL
Hypertension
Elevated FPG
RR (95% CI)
IRR (95% CI)
Percentage of Population
iAs%
MMA%
DMA%
MMA %
DMA %
iAs %
LEGEND: iAs% (MMA% fixed)
iAs% (DMA% fixed)
LEGEND: MMA% (iAs% fixed)
MMA% (DMA% fixed)
LEGEND: DMA% (iAs% fixed)
DMA% (MMA% fixed)

Supplemental Table S1. Relative Risk (95% CI) of Incident Metabolic Syndrome per IQR increase in ∑As and 5% increase in Arsenic Metabolism Biomarkers (iAs%, MMA% and DMA%), The Strong Heart Family Study, 1998-2009 (Sensitivity Analyses)

	Model 1[a] (N=1047)		**Model 2[b] (N=1047)**		**Model 3[c] (N=972)**	
	RR	*CI*	*RR*	*CI*	*RR*	*CI*
∑As (IQR increase)						
10.84 vs 4.20	1.03	0.90, 1.18	1.02	0.89, 1.16	1.02	0.88 1.17
iAs% (5% increase)						
Simple Model	0.94	0.88 1.01	0.95	0.88, 1.02	0.95	0.88, 1.02
Leave-one-out Models						
MMA% fixed	0.98	0.91 1.05	0.98	0.91, 1.05	0.99	0.92, 1.07
DMA% fixed	1.12	0.96 1.30	1.10	0.94, 1.27	1.15	1.00, 1.33
MMA% (5% increase)						
Simple Model	0.87	0.79 0.95	0.88	0.80, 0.98	0.86	0.78, 0.94
Leave-one-out Models						
iAs% fixed	0.87	0.79 0.97	0.89	0.80, 0.99	0.86	0.78, 0.95
DMA% fixed	0.89	0.77 1.04	0.91	0.78, 1.06	0.87	0.75, 1.00
DMA% (5% increase)						
Simple Model	1.07	1.02 1.12	1.06	1.01, 1.11	1.07	1.02, 1.12
Leave-One-Out-Models						
As% fixed	1.14	1.03 1.27	1.12	1.01, 1.24	1.15	1.05, 1.28
MMA% fixed	1.02	0.95 1.10	1.02	0.95, 1.10	1.01	0.93, 1.09

Abbreviations: ∑As (iAs+MMA+DMA); dimethylarsinic acid (DMA); inorganic arsenic (iAs); IQR (interquartile range); monomethylarsonic acid (MMA)

[a]Model 1 adjusts for log urinary total arsenic (except in models where ∑As is the predictor of interest), log urinary creatinine, age, sex, center, smoking status, alcohol intake, education, BMI, and kidney function

[b]Model 2 includes Model 1 adjustments and further adjusts for number of components at baseline (0, 1, or 2)

[c]Model 3 includes Model 1 adjustments and further adjusts for dietary estimates (FFQ) of folate, vitamin B_2, vitamin B_6 and caloric intake at baseline

CHAPTER 3

Targeted Metabolomics to Understand the Association between Arsenic Metabolism and Diabetes-Related Outcomes: Preliminary Evidence from the Strong Heart Family Study

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ABSTRACT

Background: Inorganic arsenic exposure is ubiquitous and both exposure and inter-individual differences in its metabolism have been associated with cardiometabolic risk. A more efficient arsenic metabolism profile (lower MMA%, higher DMA%) has been associated with reduced risk for arsenic-related health outcomes. This profile, however, has also been associated with increased risk for diabetes-related outcomes.

Objectives: The mechanism behind these conflicting associations is unclear; we hypothesized the one-carbon metabolism (OCM) pathway may play a role.

Methods: We evaluated the influence of OCM on the relationship between arsenic metabolism and diabetes-related outcomes (HOMA2-IR, waist circumference, fasting plasma glucose) using metabolomic data from an OCM-specific and P180 metabolite panel measured in plasma, arsenic metabolism measured in urine, and HOMA2-IR and FPG measured in fasting plasma. Samples were drawn from baseline visits (2001-2003) in 59 participants from the Strong Heart Family Study, a family-based cohort study of American Indians aged ≥14 years from Arizona, Oklahoma, and North/South Dakota.

Results: In unadjusted analyses, a 5% increase in DMA% was associated with higher HOMA2-IR (geometric mean ratio (GMR)= 1.13 (95% CI: 1.03, 1.25)) and waist circumference (mean difference=3.66 (0.95, 6.38). MMA% was significantly associated with lower HOMA2-IR and waist circumference. After adjustment for OCM-related metabolites (SAM, SAH, cysteine, glutamate, lysophosphatidylcholine 18.2, and three phosphatidlycholines), associations were attenuated and no longer significant.

Conclusions: These preliminary results indicate that the association of lower MMA% and higher DMA% with diabetes-related outcomes may be influenced by OCM status, either through confounding, reverse causality, or mediation.

INTRODUCTION

Inorganic arsenic is a known human carcinogen and chronic exposure has been associated with increased risk for numerous health outcomes including metabolic effects, such as type 2 diabetes and the metabolic syndrome.[1-6] After ingestion, inorganic arsenic is metabolized through multiple oxidative methylation and reduction reactions ultimately converting inorganic arsenic (As^{III} and As^{V}) to the methylated metabolites monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), which are excreted in the urine together with inorganic arsenic.[7-9] Typically, arsenic metabolism is evaluated in epidemiological studies by computing relative percentages of inorganic arsenic, MMA, and DMA over their sum (iAs%, MMA%, DMA%).[10-13]

Inter-individual differences in methylation capacity have been associated with risk for subsequent health outcomes. Several studies have reported higher percentages of MMA (MMA%) and lower percentages of DMA (DMA%) in the urine to be associated with greater risk for many arsenic-induced health effects including skin lesions[14-16], cancers of the skin, bladder, lung[17-22] and cardiovascular disease,[23-25] even after controlling for arsenic exposure levels. However, for metabolic-related health outcomes, including diabetes and metabolic syndrome, higher MMA% and lower DMA% is associated with a lower risk.[3, 26-28] The reasons for the contrasting association between arsenic metabolism and metabolic outcomes versus other arsenic-associated health outcomes are not clear. Some studies have suggested that one-carbon metabolism (OCM) may play a role given its strong association with both metabolic outcomes[29-35] and arsenic metabolism.[8, 9, 36-42] OCM, a biochemical pathway that is dependent on folate, facilitates

the generation of S-adenosylmethionine (SAM), a central metabolite, which serves as the methyl donor for numerous methylation reactions including the methylation of arsenic.[43]

In this study, we explored potential mechanistic pathways that may explain the associations between arsenic metabolism and metabolic outcomes through the targeted evaluation of specific metabolites. The association between arsenic metabolism and metabolic profiles has been relatively unexplored. However, metabolic profiling has already identified metabolites, including those related to OCM, that can predict risk for diabetes beyond traditional diabetes risk factors.[44-48] Our analyses expanded on these findings by evaluating associations between OCM- and diabetes-associated metabolites, arsenic metabolism biomarkers, and diabetes-related outcomes including waist circumference, fasting plasma glucose and HOMA2-IR. Our selection of these outcomes was informed by previous work conducted in our study population that have reported associations between arsenic metabolism and diabetes[3], a homeostasis model assessment index (HOMA2-IR),[49] body mass index (BMI)[50], waist circumference[51] and metabolic syndrome[51]; all of these outcomes are linked to insulin resistance. Previous evaluation of the association between arsenic metabolism and other metabolic outcomes in this study population, including triglycerides and high-density lipoprotein cholesterol, yielded null results and were therefore not evaluated in this analysis.

Using data from the Strong Heart Family Study (SHFS), a family-based cohort study comprised of American Indian tribal members aged 14 years and older from Arizona, Oklahoma, and North and South Dakota, we conducted a pilot study including 59 participants. We analyzed nine OCM-specific metabolites in addition to the Biocrates P180 metabolite panel (188 endogenous metabolites including amino acids, lipids, and

carbohydrates, many of them related to OCM) in baseline plasma samples collected in 2001-2003. For our analyses, of the 188 metabolites, we *a priori* selected only metabolites that are involved in OCM or have been prospectively associated with incident diabetes in previous studies (n=31 metabolites).[44-48, 52-57] Given the small sample size, our analyses are considered exploratory and aim to describe the relationship of OCM-specific metabolites and previously identified incident diabetes-associated metabolites with arsenic metabolism and metabolic outcomes including fasting glucose levels, insulin resistance and waist circumference.

METHODS

Study population

The SHFS recruited a total of 3,838 participants for baseline visits in 1998-1999 and 2001-2003 from three centers: Arizona, Oklahoma and North/South Dakota. The age range of the participants in the SHFS was 14 to 93 (mean 42) years. At each visit, in-person interviews, physical examinations and biological specimens were obtained. Methods have been described in detail previously.[58] For this pilot study, we randomly selected 20 participants per study region among those with a baseline visit in 2001-2003 and with complete data on the following variables: urinary arsenic and creatinine, education, smoking status, BMI, eGFR, alcohol intake, dietary intake information and telomeres. One participant was excluded because of a missing sample, leaving 59 participants in the pilot evaluation of plasma metabolites. All participants provided informed consent and study protocols were approved by multiple institutional review boards, participating communities and The Indian Health Service.

Data Collection

Urinary Arsenic

Morning spot urine samples were collected in polypropylene tubes, frozen within 1 to 2 hours of collection, shipped buried in dry ice and stored at -70°C in the Penn Medical Laboratory, MedStar Research Institute, Washington, DC for up to 18 years. The freezers have been operating under a strict quality control system to guarantee secure sample storage. For arsenic analyses, urine samples were thawed and up to 1.0 mL from each urine sample was transferred to a small vial, transported on dry ice to the Trace Element Laboratory at Graz University, Austria and stored at -80°C until analyses. The urine concentrations of arsenite, arsenate, MMA and DMA were measured using high performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICPMS). The limits of detection were 0.1 μg/L for arsenite, arsenate, MMA and DMA. The inter-assay coefficients of variation for arsenite, arsenate, MMA, DMA and total arsenic were 14.7%, 6.9%, 6.4%, 6.0% and 4.7% respectively. For participants with concentrations below the limit of detection (less than 5% for all species) for total arsenic or for the arsenic species we imputed the corresponding limit of detection divided by the square root of two.

Blood Collection and Measurements

Participants were asked to fast for 12 hours before sample collection. Biological specimen aliquots were stored at -70°C at the Texas Biomedical Research Institute (Texas Biomed) in San Antonio, TX. Most baseline laboratory determinations have been performed at the MedStar Health Research Institute, Washington DC under strict quality control procedures including enzymatic methods for fasting plasma creatinine and

glucose and radioimmunoassay for fasting insulin. HOMA2-IR was used as a surrogate measure of insulin resistance and was calculated with the computed solved model for HOMA2-IR[59] using fasting glucose and insulin values.[60, 61] Plasma samples were used to measure OCM nutrients and nutrient intermediates. Some OCM intermediates can be affected by freeze-thaw cycles and were measured in samples that had never been thawed before.

OCM Metabolomics and P180 Panel

Targeted OCM metabolomic analysis of plasma was by liquid chromatography tandem mass spectrometery (LC-MS/MS) at the Center of Metabolomics, Baylor Research Institute under the direction of Dr. Teodoro Bottiglieri. Targeted metabolomic analysis of SAM, S-adenosylhomocysteine (SAH), methionine, choline, betaine, cysteine, cystathionine, homocysteine (Hcys), 5-methyltetrahydrofolate (5-MTHF), in plasma samples were performed using validated LC-MS/MS methods.[62-64] This metabolic panel provides a quantitative comprehensive assessment of OCM. Each plasma sample (<300 µL) was processed by addition of labeled-isotope internal standards followed by deproteinization. Processed extracts of samples were transferred to a 96 well microtiter plate for analysis. The compounds were detected by multiple reaction monitoring using positive-electrospray ionization as previously described.[62-64] Sample injection and separation was performed by a Shimadzu Nexera UPLC System interfaced with a 5500QTRAP® (Sciex). All data were collected and analyzed using Analyst software version 1.5.2. Stable isotopes were used for each metabolite to account for matrix effects related ion-suppression, and the quantitative values obtained were expressed as µmol/L.

Quality control samples (2 levels) were positioned at the start, in the middle and at the end of each batch of samples analyzed. The inter assay coefficients of variability for all metabolites were <20%, with most <15%.

The P180 Panel (Biocrates Life Sciences, Innsbruck, Austria) covers carefully selected metabolic pathways which includes 188 metabolites from 6 compound classes including hexoses, amino acids, biogenic amines, acylcarnitines, glycerophospholipids and sphingolipids. For a more targeted analysis, we selected metabolites that have been previously associated with incident diabetes.

Covariates

A standardized questionnaire was conducted during the in-person interviews and included sociodemographic data (age, sex), smoking history, alcohol use, and medical history.[65] Physical exam measures (waist circumference, height and weight) were performed by centrally trained nurses following a standardized protocol; this visit included collection of spot urine and fasting blood samples.[58] Estimated daily averages of dietary intake of OCM related micronutrients, including vitamins B_2, B_6 and folate, as well as supplement data, were measured during the baseline visit through an interviewer-administered Block 119-item food frequency questionnaire (FFQ). The Block questionnaire is one of the most widely used questionnaires with demonstrated reliability and validity.[66] To enhance accuracy of the questionnaire in this cohort, additional questions relating to foods commonly consumed by American Indians were included.[66]

Statistical Analyses

Arsenic exposure was estimated as the sum of inorganic (arsenite, arsenate) and methylated (MMA and DMA) arsenic species adjusted by urine creatinine to correct for urine dilution (∑As). ∑As was log-transformed to better approximate a normal distribution. Arsenic metabolism was estimated as percentages of inorganic arsenic, MMA and DMA, (iAs%, MMA%, DMA%) by dividing the corresponding concentration for each species by the sum of the inorganic and methylated species.[67, 68] Arsenic species percentages were modeled in their original scale.

Arsenic, metabolites and outcome related variables were coded into related groups in the analyses: 1) Arsenic exposure and metabolism biomarkers (∑As, iAs%, MMA% and DMA%) ; 2) OCM-specific metabolites (Hcys, cysteine, methionine, betaine, choline, SAM, SAH, SAM/SAH, cystathionine and 5-MTHF); 3) Amino-acids (glutamine, glutamate, glycine, histidine, isoleucine, leucine, phenylalanine, serine, tryptophan, tyrosine, valine); 4) Acylcarnitines (propionylcarnitine); 5) Glycerophospholipids (lysophosphatidylcholine (LPC) a C16:0, LPC a C18.2, phosphatidylcholine (PC) aa C32:1, PC aa C36:1, PC aa 38:3, PC aa C40:5, PC ae C34:3, PC ae C40:6, PC ae C44:4 and PC ae C44:5); 6) Sphingolipids (sphingomyelin (SM) C16:1); 7) Diabetes related outcomes (fasting plasma glucose (FPG), HOMA2-IR and waist circumference).

First, we computed Spearman correlations for metabolites, biomarkers and outcomes within and between the seven group types listed in the previous paragraph and displayed those correlations graphically in correlation globes (**Figure 1**). Correlations greater than |0.15| were represented by colored lines between the two metabolites and were displayed as solid colored lines for correlations with p-values < 0.05 and as

transparent colored lines for p-values ≥ 0.05. Metabolites with correlation p-values < 0.05 between both an arsenic metabolism biomarker (iAs%, MMA%, DMA%) and a metabolic outcome (HOMA2-IR, FPG, waist circumference) were considered metabolites of interest and evaluated further. P-values were not corrected for multiple comparisons due to the limited sample size. Median levels of metabolites of interest were compared across sociodemographic (age, gender, center), BMI, nutrition (OCM vitamin dietary and supplement intake), behavioral (smoking and alcohol intake status) and diabetes-related outcome characteristic variable categories using Kruskal-Wallis tests (**Figure 2**). Waist circumference and FPG categories were based on National Cholesterol Education Program ATP III Guidelines for metabolic syndrome components (≥40 inches for men, ≥35 inches for women for elevated waist circumference and ≥100mg/dL (or on medication) for elevated FPG).[69] Correlations between metabolites of interest and both arsenic metabolism biomarkers and diabetes-related outcomes were also graphically and numerically displayed through a correlation and scatterplot matrix (**Figure 3**).

Linear regression analyses were conducted to evaluate the associations of diabetes-related outcomes, including HOMA2-IR, FPG and waist circumference, separately, with each metabolite of interest as well as with the arsenic metabolism biomarkers iAs%, MMA% and DMA% (**Table 2**). HOMA2-IR was log-transformed in analyses to better approximate a normal distribution. FPG and waist circumference were normally distributed and therefore included in analyses in their original scale. Metabolites of interest were also normally distributed; however, they were standardized (N,0) to reduced variability. Results are presented as the geometric mean ratios (GMR) for HOMA2-IR, and mean differences for waist circumference and FPG, comparing the 75th

to 25th percentile (IQR increase) of each metabolite of interest or 5% increase in arsenic metabolism biomarker. Crude models report unadjusted associations and adjusted models report associations fully adjusted for all other metabolites. Variance inflation factors (VIF) were used to evaluate multicollinearity in adjusted models which included all OCM metabolites. Finally, we constructed directed acyclic graphs (DAGs) of possible pathways between arsenic metabolism, one carbon metabolism and diabetes-related outcomes based on our findings (**Figure 5**).

RESULTS

The median age of the study population was 35 years with slightly more females (53%) than males (**Table 1**). Most participants were overweight or obese (76%) and met or exceeded the recommended daily allowance (RDA) for intake of vitamins B_2[70] and B_6[71] despite most participants not taking supplements of either vitamin (78%). The majority of participants were below the RDA for of folate intake[72] (63%) and did not take folate supplements (75%), although RDAs used to categorize inadequate folate intake are based on dietary folate equivalents (DFEs), which incorporate different forms of folate (e.g., folic acid) and could not be measured in this dataset. Therefore, the true percentage of participants with inadequate folate intake is likely lower. Evaluation of plasma folate (5-MTHF) confirmed no participants were folate deficient (5-MTHF ≤9 nmol/L) (data not shown). Further, only seven participants had hyperhomocysteinemia (homocysteine >11.4 μmol/L and 10.4 μmol/L for men and women, respectively) suggesting vitamin B12 status was also likely sufficient in this population as well (data not shown). Median (IQR) plasma folate and homocysteine levels were 51.3 (40.2, 63.9) and 7.53 (5.85,

8.98), respectively. Forty-two percent of participants were never smokers, 22% were former smokers and 36% were current smokers. A total of 14% of participants reported never drinking alcohol, 25% reported former drinking and 61% reported current drinking. Median (IQR) for ∑As, iAs%, MMA% and DMA% were 3.66 (2.64, 8.27) µg/g creatinine, 10.7 (7.29, 16.4)%, 15.3 (11.3, 19.9)% and 72.9 (65.9, 78.8)%, respectively. Median (IQR) for diabetes-related outcomes were 1.44 (1.06, 2.62) for HOMA2-IR, 93.0 (89.0, 101.5) mg/dL for FPG and 105.0 (91.5, 117.5) cm for waist circumference. Participant characteristics were similar in the pilot sample compared to the full cohort.

Eight metabolites were identified as "metabolites of interest" due to correlations with both an arsenic metabolism variable (iAs%, MMA% or DMA%) and a metabolic outcome (HOMA2-IR, FPG, waist circumference) that reached statistical significance $p<0.05$. These metabolites included SAM, SAH, cysteine, glutamate, LPC a C18.2, PC ae C34:3, PC ae C40:6 and PC aa 38:3. Comparing median levels of these metabolites across sociodemographic and behavioral characteristics, some trends emerged (**Figure 2**). Plasma levels of SAM, SAH and glutamate were higher in male participants. Levels of SAM were also higher in those with sufficient dietary folate intake. Among participants who were overweight or obese, levels of SAM, SAH and glutamate were higher compared with participants with a normal BMI. Similarly, among participants with elevated waist circumference, levels of SAM, glutamate and PC aa 38:3 were higher compared with participants who had a normal waist circumference. Further, levels of LPC 18:2, PC ae 40:6 and PC ae 34:3 were lower in both participants who were overweight/obese and had an elevated waist circumference compared with participants who had a normal BMI or a normal waist circumference, respectively. Median metabolite

levels did not vary significantly by age, center, smoking status, drinking status, FPG status, intake sufficiency or supplement use of vitamins B_6 and B_2 or folate supplement use.

Spearman correlations between arsenic metabolism variables, diabetes-related outcomes and metabolites of interest are displayed in **Figure 2**. SAM, SAH, cysteine, glutamate and PC aa 38:3 were positively correlated with adverse diabetes-related outcomes and DMA%, and negatively correlated with MMA% and iAs%. LPC 18:2 and acyl-alkyl PCs (PC ae 34:3 and PC ae 40:6) were negatively correlated with adverse diabetes-related outcomes and DMA% and positively correlated with MMA% and iAs%. DMA% had a significant positive correlation with HOMA2-IR (0.34) and waist circumference (0.36). MMA% was significantly negatively correlated with HOMA2-IR, FPG and waist circumference.

In unadjusted regression analyses evaluating HOMA2-IR as the outcome, a 5% increase of MMA% was associated with a GMR of 0.75 (95% CI: 0.63, 0.89) (**Table 2**). After adjustment for all eight metabolites (SAM, SAH, cysteine, glutamate, LPC a C18.2, PC ae C34:3, PC ae C40:6 and PC aa 38:3), higher MMA% remained associated with reduced HOMA2-IR although the association was markedly attenuated and no longer significant (0.90 (0.74, 1.09)). For DMA%, the positive association with HOMA2-IR (GMR 1.13, 95% CI: 1.03, 1.25) became entirely null (GMR 0.99, 95%CI 0.89, 1.11) after adjustment for the other metabolites. Six of the eight metabolites were also significantly associated with HOMA2-IR in unadjusted models (increased risk for SAM, SAH, glutamate and PC aa 38:3; decreased risk for LPC 18:2 and PC ae 40:6). After adjustment for other metabolites, associations were attenuated and no longer significant

except for PC aa 38:3 which remained borderline significantly associated with increased HOMA2-IR. iAs% was not significantly associated with HOMA2-IR or any other diabetes-associated outcome (data not shown).

Similarly, in unadjusted analyses for waist circumference, an inverse association was observed with a 5% increase in MMA% (-7.83, 95% CI: -12.8, -2.90, cm) and a positive association was observed with a 5% increase in DMA% (3.66, 95% CI: 0.95, 6.38, cm) (**Table 2**). After adjustment for the other metabolites, the association with MMA% was attenuated to the null (0.93, 95% CI: -4.04, 5.90, cm) and the association with DMA% even reversed direction (-1.16, 95% CI: -3.99, 1.67, cm). Several other metabolites were also significantly associated with waist circumference in unadjusted models in the same direction as the associations seen between these metabolites and HOMA2-IR. After adjustment for the other metabolites, glutamate remained significantly associated with increased waist circumference and both LPC 18:2 and PC ae 40:6 remained significantly associated with reduced waist circumference. The direction of associations between MMA%, DMA% and OCM metabolites with FPG was the same as for the other diabetes-related outcomes, however none of the associations were significant. Variance inflation factor (VIF) values were below 4 for all variables in fully OCM-adjusted models, and therefore, multicollinearity was not considered a significant concern.

DISCUSSION

In this pilot study of 59 men and women from the SHFS, we observed significant correlations between eight metabolites (SAM, SAH, cysteine, glutamate, LPC 18:2, PC ae 34:3, PC ae 40:6 and PC aa 38:3) and both a metabolic outcome (HOMA2-IR, FPG,

waist circumference) and at least one arsenic metabolism biomarker (iAs%, MMA% or DMA%). Before adjustment, higher MMA% was associated with lower HOMA2-IR and waist circumference, and higher DMA% was associated with higher HOMA2-IR and waist circumference. Eight metabolites identified as "metabolites of interest" due to correlations with both arsenic metabolism and a diabetes-related outcome (HOMA2-IR, FPG, waist circumference) included SAM, SAH, cysteine, glutamate, LPC a C18.2, PC ae C34:3, PC ae C40:6 and PC aa 38:3. After adjustment for these eight metabolites, associations between arsenic metabolism and diabetes-related outcomes were substantially attenuated and no longer significant. For the metabolites of interest, glutamate, LPC 18:2 and PC ae 40:6 remained statistically significantly associated with waist circumference even after adjustment for other metabolites and for arsenic metabolism biomarkers. Together, our results suggest that these metabolites play a key role in the relationship between arsenic metabolism and diabetes-related outcomes and may explain the difference in association seen between arsenic metabolism and diabetes-related outcomes compared to the associations reported for arsenic metabolism and other health outcomes.

Inorganic arsenic exposure has been associated with numerous health outcomes including cancer, cardiovascular disease, skin lesions and birth outcomes.[1, 2, 15, 73-76] Further, an arsenic metabolism profile reflecting higher percentages of MMA% and lower percentages of DMA%, have been associated with greater risk for these outcomes, even after controlling for exposure levels.[14, 77-79] These findings, as well as the shorter half-life and rapid excretion in the urine of DMA compared to iAs, has led to the characterization of higher DMA% and lower iAs% and MMA% in urine as a more

efficient arsenic metabolism profile.[80] Inorganic arsenic exposure has also been associated with diabetes at high (≥100 μg/L)[81-85] and moderate (<100 μg/L)[27, 28, 67, 86-90] levels of exposure, with mixed findings at low levels.[3, 91-93] However, in contrast to other arsenic-associated health outcomes, a more efficient arsenic metabolism profile (i.e., higher DMA% but lower iAs% and MMA%) has been associated with increased risk for diabetes[3, 79] and other diabetes-related outcomes including higher BMI,[50] insulin resistance[94], elevated waist circumference[51] and metabolic syndrome.[95] The mechanism behind the reported contrasting associations is unclear. Some studies have suggested the association between higher DMA% and increased risk for metabolic-related outcomes may be due to DMA^{III}, as trivalent methylated arsenicals are considered the most toxic metabolites; this intermediate is difficult to measure analytically as it rapidly oxidizes to DMA^{V}.[3, 27, 28] Other studies have considered confounding by diet, particularly greater intake of OCM nutrients.[3, 26] All eight of the metabolites of interest in our study that were related both to arsenic metabolism and to a metabolic outcome are connected to the OCM pathway, as outlined below (study metabolites underlined) and in **Figure 4**, and provide a potentially relevant mechanism behind the association between arsenic metabolism and diabetes-related outcomes.

OCM facilitates the generation of <u>SAM</u>, the methyl donor for numerous substrates, which are essential to many biological processes including cellular signaling, DNA methylation, the synthesis of proteins, lipids, hormones and carbohydrates, and arsenic metabolism.[43] <u>SAH</u> is a product of SAM-dependent methylation reactions, and is hydrolyzed to Hcys, which can be remethylated to form methionine and activated to regenerate SAM.[43] SAH is a potent inhibitor of most transmethylation reactions,

including arsenic, through its binding with methyltransferases.[41] SAH is removed from methyltransferases when Hcys is pulled forward for the remethylation of Hcys to form methionine or diverted to the transsulfuration pathway for glutathione synthesis. Glutathione is a tripeptide consisting of cysteine, glutamate, and glycine and is critical to the body's antioxidant response (in its reduced form as GSH), including detoxification of heavy metals.[96] OCM is dependent on essential nutrients - including folate, vitamin B12, vitamin B6, vitamin B2 and choline – for the recruitment and transfer of methyl groups.[9] Indeed, in our study we observed greater SAM in participants with sufficient folate intake versus those with low intake. Choline can provide methyl groups for the remethylation of Hcys through conversion to betaine. Choline is also used in the synthesis of phosphatidylcholine (PC), an abundant phospholipid involved in maintenance of hepatic lipid metabolism.[97] Four of the eight OCM-related metabolites included in this analysis were PCs, which may also be synthesized through the methylation of phosphatidylethanolamine using SAM as the methyl donor.[98] aaPCs consist of glycerol linked to phosphocholine and two fatty acid residues, and removal of one fatty acid produces LPCs. aePC's comprise an ether linkage to one alkyl chain and one polyunsaturated fatty acid.[48]

In our study, SAM, SAH, glutamate and cysteine were all significantly associated with increases in HOMA2-IR and waist circumference, two of the diabetes-related outcomes we investigated. However, only the association between glutamate and waist circumference remained significant after adjustment for the other OCM metabolites and arsenic metabolism.

The positive associations between glutamate and both HOMA2-IR and waist circumference are consistent with several previous prospective and cross-sectional studies, reporting a positive relationship between glutamate and diabetes,[99-103] including prospective metabolomic analyses.[54, 56, 104] Further, elevated cerebral glutamate measured via proton magnetic resonance spectroscopy in occipitoparietal grey matter has been reported in adults with metabolic syndrome.[105] In vitro studies in human islet cells have shown chronic exposure to glutamate through treated cell media causes dose-dependent increases in insulin secretion as well as β-cell apoptosis.[106] The mechanism behind these associations is likely due to glutamate's role in a regulatory system in which dietary protein ingestion stimulates ß-cells to stimulate insulin secretion: The increase in branched-chain amino acids following a protein-containing meal activates glutamate dehydrogenase in ß-cells which oxidizes glutamate to α-ketoglutarate and there is a consequent increase in the ATP/ADP ratio.[107] Insulin secretion is very sensitive to this because it closes the ATP-gated K+ channel which depolarizes the cell and activates a voltage-gated Ca2+ channel.[107] The increase in Ca2+ then triggers insulin release.[107] Further, excess extracellular levels of glutamate have been shown to inhibit the glutamate/cysteine antiporter system, which depletes the cells of cysteine, in turn limiting the production of glutathione, thus decreasing the antioxidant defense.[106, 108]

Few studies have evaluated the association between SAM and SAH and risk for developing diabetes-related outcomes. In this study, SAM correlated positively with both Hcys and SAH (a potentially more accurate predictor of cardiovascular disease than Hcys).[109] Our finding of a positive relationship between SAH and diabetes-related outcomes is consistent with several studies that have reported an association between

high levels of Hcys and risk for diabetes and diabetes-associated conditions such as impaired beta-cell function and insulin resistance,[110-113] with some null findings.[114, 115] In contrast, there is also evidence that the association between SAM and Hcys with increased risk for diabetes-related outcomes is due to reverse causality. An *in vitro* study found that when hepatic cells were exposed to elevated insulin and glucose, Hcys and SAM concentrations increased.[116] In addition, animal models have shown insulin resistance and diabetes can alter one carbon metabolism-related metabolites and enzymes, including increases in hepatic SAM, and that administration of insulin can prevent these perturbations.[117, 118]

SAM, SAH, glutamate and cysteine all correlated positively with a more efficient arsenic metabolism profile characterized by higher DMA%. This is consistent with observational studies from Bangladesh which reported greater SAM to be associated with lower iAs%[119] and greater cysteine to be associated with lower MMA% and higher DMA%,[120] as well as indirect evidence provided by experimental studies reporting a protective effect of SAM in regard to arsenic toxicity.[121, 122] We found one study that has evaluated the association between glutamate and arsenic metabolism.[123] In contrast to our findings, this study reported glutamate having an inverse correlation with DMA%, however, the study was conducted in pregnant women which may have influenced results. Further, both glutamate and cysteine are critical for glutathione synthesis, and increases in glutathione when glutathione levels are low have been shown to accelerate arsenic metabolism, supporting our findings.[124]

Of the PCs evaluated in this study, one was a diacyl PC (PC aa 38:3), two were acyl-alkyl PCs (PC ae 34:3 and PC ae 40:6) and one was a LPC (LPC 18:2). The diacyl

PC, like the previously discussed metabolites, was associated with a more efficient arsenic metabolism profile (positive correlation with DMA% and negative correlation with iAs% and MMA%). Also, similar to the previously discussed metabolites, this PC was associated with greater HOMA2-IR. The other PCs, LPC 18:2, PC ae 34:3 and PC ae 40:6, were significantly associated with improvements in diabetes-related outcomes (lower HOMA2-IR and waist circumference) and with a less efficient arsenic metabolism profile (inverse correlations with DMA% and positive correlations with iAs% and MMA%). The associations between LPC 18:2 and PC ae 40:6 with waist circumference remained significant in adjusted models.

The findings in our study between PCs and diabetes-related outcomes are consistent with previous studies which have reported inverse associations between ae PCs and LPCs with diabetes risk and BMI, but positive associations between aa PCs and diabetes risk.[125-127] Still, other studies have suggested odd versus even chain PCs explain the difference in findings between PC types and metabolic risk, with odd chain appearing protective and even chain appearing to be a risk factor.[128] The mechanisms behind the contrasting associations between ae PCs and LPCs versus aa PCs are not clear and warrant further investigation. Further, some evidence suggests the association between PCs and diabetes is due to reverse causality. Animal studies have shown significant alterations in both hepatic and blood PC levels in diabetic versus control rats.[118] Regardless, the strong associations between the PCs and LPCs with multiple diabetes-related outcomes in our study are not surprising given the established link between PCs (the most abundant phospholipid), dyslipidemia and obesity-associated insulin resistance.[129]

Some studies have evaluated the association between arsenic exposure and PCs, with inconsistent findings.[130, 131] This is the first study to evaluate the association between arsenic metabolism and PCs. Our particularly strong (meeting Bonferroni correction criteria (p=0.0015)) correlations between LPC and all three arsenic metabolite percentages is intriguing. LPC is formed by the hydrolysis of aaPCs, during which a fatty acid is freed.[52] In turn, LPCs are hydrolyzed to choline.[130] As choline assists in the remethylation of Hcys to methionine in the OCM pathway through conversion to betaine, we might expect LPCs to be associated with enhanced arsenic metabolism. However, our results suggest the opposite and may reflect the fact that the PC/LPC pathway for choline synthesis, one of two choline synthesis pathways, consumes three molecules of SAM per molecule of choline.[98, 132, 133] This is supported by the significant inverse correlation (-0.35) between SAM and LPC 18:2 in this study.

Together, our results provide evidence that OCM metabolites may have an instrumental role in the previously observed associations between arsenic metabolism and diabetes-related outcomes. In our regression analyses, we show strong attenuations and loss of significance for associations between both DMA% and MMA% with HOMA2-IR and waist circumference after adjustment for OCM metabolites. We propose 3 potential pathways for how these OCM metabolites may influence the observed relationships between arsenic metabolism and diabetes-related outcomes. First (DAG 1), OCM metabolites may be acting as confounders. All of the OCM metabolites were positively associated with HOMA2-IR and waist circumference were also positively associated with enhanced arsenic metabolism capacity. Likewise, the metabolites negatively correlated with those two diabetes-related outcomes were also associated with reduced arsenic

metabolism capacity. Further, the previous paragraphs have summarized the consistency of these findings with other studies. In this way, OCM metabolites could cause a spurious association between enhanced arsenic metabolism and higher HOMA2-IR and waist circumference.

Second (DAG 2), the associations seen between both OCM metabolites and arsenic metabolism with diabetes-related outcomes may be a result of reverse causality. Despite multiple metabolomic studies showing an association between most of our metabolites of interest with incident diabetes, it is still possible diabetes converters were in early stages of disease progression, which in turn resulted in early metabolite alterations.

Finally, (DAG 3), OCM may still directly affect diabetes-related outcomes and arsenic metabolism (per DAG 1), however, in this scenario, part of the effect of OCM metabolites on HOMA2-IR and waist circumference is through its influence on arsenic metabolism. The results of this study provide evidence that arsenic metabolism alone is not responsible for the associations reported between arsenic metabolism and diabetes-related outcomes. However, it is still possible arsenic metabolism has some effect on diabetes-related outcomes and is not simply confounded by OCM metabolites (DAG 1) or an outcome of insulin resistance (DAG 2). To this point, in Table 3, we still observe a non-significant reduction in HOMA2-IR with increasing MMA%, despite full adjustment for all OCM metabolites. Robust mechanistic evidence is lacking for this pathway, however, the trivalent forms of DMA (DMAIII) and MMA (MMAIII) have been shown to be potent inhibitors of insulin signal transduction in experimental models,[134, 135] providing some explanation for greater diabetes risk with higher DMA%, although we

are not able to distinguish between DMAIII and DMAV. Still it is unclear how this would lead to lower MMA% being inversely associated with diabetes outcomes, unless it was just an indirect association due to an increased risk with DMA%. Another theory postulates that increased methylation reactions of methyl-consuming xenobiotics, such as arsenic, may lead to a depletion of the endogenous methyl pool and generation of reactive oxygen species, and in turn tissue injury.[136] We feel this to be unlikely, as the methylation of As consumes a very small proportion of SAM (< 4%).[137]

This study was limited by a small sample size and therefore conclusions should be interpreted with caution. Further, due the small sample size, final models did not include other confounders for arsenic metabolism diabetes-related outcomes, such as age and sex. Final models also did not account for family relatedness. However, in sensitivity analyses adjusting for those factors resulted in similar findings. The limited sample size also prevented formal mediation analyses which are needed to confirm direction of associations.

CONCLUSIONS

We found that several OCM-related metabolites were significantly and relatively strongly associated with both arsenic metabolism and diabetes-related outcomes. LPC 18:2 had the strongest association with arsenic metabolism, a novel finding that warrants additional research. In addition, glutamate, LPC 18:2 and PC ae 40:6 remained significantly associated with waist circumference after adjustment for both arsenic metabolism and all other metabolites, highlighting a potential role of these three metabolites in the development or physiological consequences of central adiposity.

Adjusting arsenic metabolism biomarkers for all OCM-related metabolites in models evaluating the association between arsenic metabolism and both HOMA2-IR and waist circumference resulted in significant attenuations and losses in statistical significance. Our findings provide evidence that the OCM pathway may be - either directly or indirectly - influencing the previously reported relationship between arsenic metabolism and diabetes-related outcomes. Further, this pathway may, in part, explain the contrasting associations seen between arsenic metabolism and diabetes-related outcomes versus arsenic metabolism and other arsenic-related health outcomes.

TABLES

Table 1. Participant Characteristics, Pilot versus Full Strong Heart Family Study Cohort

	Pilot (N=59) Median (IQR)	**Full Cohort (N=1577)[a]** Median (IQR)
Age (years)	35.3 (26.4, 46.3)	35.3 (23.6, 46.3)
Sex		
Male, n (%)	28 (47.5)	654 (41.6)
Female, n (%)	31 (52.5)	923 (58.4)
BMI (kg/m^2)	31.6 (25.9, 38.0)	29.8 (25.5, 35.0)
Vitamin B_6 intake (mg)	1.60 (1.05, 2.65)	1.70 (1.10, 2.60)
<RDA[b], n (%)	21 (35.6)	545 (34.6)
≥RDA[b], n (%)	38 (64.4)	1032 (65.4)
Vitamin B_2 intake (mg)	1.60 (1.00, 2.30)	1.70 (1.10, 2.70)
<RDA[c], n (%)	19 (32.2)	358 (22.7)
≥RDA[c], n (%)	40 (67.8)	1219 (77.3)
Folate intake (µg)	348 (226, 518)	360 (234, 559)
<RDA[d], n (%)	37 (62.7)	890 (56.4)
≥RDA[d], n (%)	22 (37.3)	687 (43.6)
Plasma Folate (nmol/L)	51.3 (40.2, 63.9)	---
Plasma Homocysteine (µmol/L)	7.53 (5.85, 8.98)	---
∑As (µg/g)	3.66 (2.64, 8.27)	4.59 (3.02, 7.49)
iAs%	10.7 (7.29, 16.4)	10.0 (6.94, 14.1)
MMA%	15.3 (11.3, 19.9)	14.5 (11.1, 18.2)
DMA%	72.9 (65.9, 78.8)	74.6 (67.8, 80.8)
HOMA2-IR	1.44 (1.06, 2.62)	1.44 (0.97, 1.44)
Fasting Plasma Glucose (mg/dL)	93.0 (89.0, 101.5)	93.0 (87.0, 100.0)
Waist Circumference (cm)	105.0 (91.5, 117.5)	99.0 (88.0, 111.0)

Abbreviations: RDA (recommended dietary allowance); ∑As (sum of inorganic arsenic and its methylated metabolites); iAs (inorganic arsenic); MMA (monomethylarsonic acid); DMA (dimethylarsinic acid)

[a]Excluding participants with missing data on variables in table

[b]RDA for B_6: Males (≤50 years=1.3 mg; >50=1.7 mg); Females (14-18 years=1.2 mg; 19-50 years=1.3 mg; >50 years=1.5 mg)

[c]RDA for B_2: Males (≥14 years =1.3 mg); Females (14-18 years=1.0 mg; >18 years=1.1 mg)

[d]RDA for folate: Males and Females ≥14 years=400 µg

Table2. Associations between MMA%, DMA% and OCM Metabolites with Diabetes-Related Outcomes

	Model 1[a]: Unadjusted	**Model 2[b]:** Metabolite Adjusted	**Model 3[c]:** Metabolite Adjusted +MMA%	**Model 4[d]:** Metabolite Adjusted +DMA%
		Geometric Mean Ratio (95%CI) of HOMA2-IR[e]		
MMA%	0.75 (0.63, 0.89)	0.90 (0.74, 1.09)	----------	----------
DMA%	1.13 (1.03, 1.25)	0.99 (0.89, 1.11)	----------	----------
SAM	1.46 (1.22, 1.76)	1.19 (0.87, 1.63)	1.23 (0.90, 1.70)	1.19 (0.86, 1.64)
SAH	1.41 (1.08, 1.85)	1.16 (0.81, 1.67)	1.10 (0.75, 1.60)	1.17 (0.79, 1.73)
Cysteine	1.10 (0.82, 1.48)	0.76 (0.56, 1.03)	0.74 (0.55, 1.01)	0.76 (0.56, 1.04)
Glutamate	1.45 (1.19, 1.77)	1.13 (0.90, 1.42)	1.11 (0.88, 1.40)	1.13 (0.90, 1.43)
LPC 18:2	0.74 (0.59, 0.91)	0.80 (0.65, 0.99)	0.84 (0.67, 1.06)	0.79 (0.61, 1.03)
PC ae 40:6	0.74 (0.59, 0.93)	0.80 (0.62, 1.03)	0.83 (0.64, 1.09)	0.80 (0.61, 1.04)
PC ae 34:3	0.81 (0.66, 1.00)	1.04 (0.82, 1.31)	1.02 (0.81, 1.29)	1.04 (0.82, 1.31)
PC aa 38:3	1.35 (1.09, 1.67)	1.23 (1.00, 1.52)	1.22 (0.99, 1.50)	1.23 (0.99, 1.53)
		Mean Difference (95%CI) of Waist Circumference[e]		
MMA%	-7.83 (-12.8, -2.90)	0.93 (-4.04, 5.90)	----------	----------
DMA%	3.66 (0.95, 6.38)	-1.16 (-3.99, 1.67)	----------	----------
SAM	10.3 (4.99, 15.6)	-3.70 (-11.8, 4.39)	-4.00 (-12.3, 4.32)	-4.36 (-12.6, 3.92)
SAH	11.3 (3.73, 18.8)	5.84 (-3.49, 15.2)	6.35 (-3.45, 16.1)	7.28 (-2.72, 17.3)
Cysteine	9.22 (1.12, 17.3)	3.46 (-4.43, 11.4)	3.67 (-4.37, 11.7)	3.52 (-4.4, 11.4)
Glutamate	12.8 (7.55, 18)	8.43 (2.52, 14.3)	8.62 (2.57, 14.7)	8.46 (2.53, 14.4)
LPC 18:2	-11.5 (-17.3, -5.81)	-8.52 (-14, -3.05)	-8.97 (-15.0, -2.94)	-10.0 (-16.7, -3.41)
PC ae 40:6	-10.2 (-16.6, -3.9)	-7.28 (-13.9, -0.69)	-7.63 (-14.5, -0.72)	-7.98 (-14.8, -1.15)
PC ae 34:3	-8.9 (-14.4, -3.43)	-1.17 (-7.08, 4.75)	-1.04 (-7.05, 4.96)	-0.76 (-6.78, 5.26)
PC aa 38:3	5.49 (-0.797, 11.8)	2.48 (-2.92, 7.87)	2.56 (-2.90, 8.02)	2.96 (-2.58, 8.50)
		Mean Difference (95%CI) of FPG[e]		
MMA%	-2.08 (-4.35, 0.18)	-1.28 (-4.24, 1.68)	----------	----------
DMA%	0.75 (-0.49, 1.99)	0.14 (-1.56, 1.85)	----------	----------
SAM	2.28 (-0.23, 4.80)	1.75 (-3.1, 6.60)	2.15 (-2.80, 7.11)	1.83 (-3.17, 6.82)
SAH	2.07 (-1.43, 5.57)	1.17 (-4.42, 6.76)	0.47 (-5.37, 6.31)	1.00 (-5.04, 7.03)
Cysteine	0.75 (-2.94, 4.43)	-1.27 (-6.00, 3.46)	-1.56 (-6.35, 3.23)	-1.28 (-6.06, 3.50)
Glutamate	2.08 (-0.57, 4.73)	0.59 (-2.95, 4.13)	0.32 (-3.29, 3.93)	0.59 (-2.99, 4.16)
LPC 18:2	-1.77 (-4.56, 1.02)	-1.61 (-4.89, 1.67)	-0.98 (-4.58, 2.61)	-1.42 (-5.42, 2.57)
PC ae 40:6	-1.84 (-4.81, 1.13)	-1.77 (-5.71, 2.18)	-1.28 (-5.39, 2.84)	-1.68 (-5.8, 2.44)
PC ae 34:3	-0.59 (-3.18, 1.99)	1.46 (-2.08, 5.01)	1.29 (-2.28, 4.87)	1.41 (-2.22, 5.04)
PC aa 38:3	0.32 (-2.49, 3.14)	-0.64 (-3.87, 2.60)	-0.75 (-4.01, 2.50)	-0.70 (-4.04, 2.64)

Abbreviations: DMA (dimethylarsinic acid); FPG (fasting plasma glucose); LPC (lysophosphatidylcholine); MMA (monomethylarsonic acid); PC (phosphatidylcholine); SAH (S-Adenosylhomocysteine); SAM (S-Adenosylmethionine)

[a]Model 1: unadjusted

[b]Model 2: adjusted for the seven OCM-related metabolites (SAM, SAH, cysteine, glutamate, LPC 18:2, PC ae 40:6, PC ae 34:3, PC aa 38:3)

[c]Model 3: Model 2 adjustments plus additional adjustment for MMA%

[d]Model 4: Model 2 adjustments plus additional adjustment for DMA%

[e]Per 5% increases for arsenic metabolites and per IQR increases for OCM metabolites

FIGURES

Figure 1. Correlation matrices between and within metabolite and biomarker groups. Transparent lines reflect correlations ≥ |10|. Solid lines represent correlations with p-values ≤ 0.05. Abbreviations: ∑As (sum of inorganic arsenic and its methylated metabolites); iAs (inorganic arsenic); DMA (dimethylarsinic acid); FPG (fasting plasma glucose); LPC (lysophosphatidylcholine); MMA (monomethylarsonic acid); OCM (one carbon metabolism); PC (phosphatidylcholine); SAH (S-Adenosylhomocysteine); SAM (S-Adenosylmethionine); WC (waist circumference)

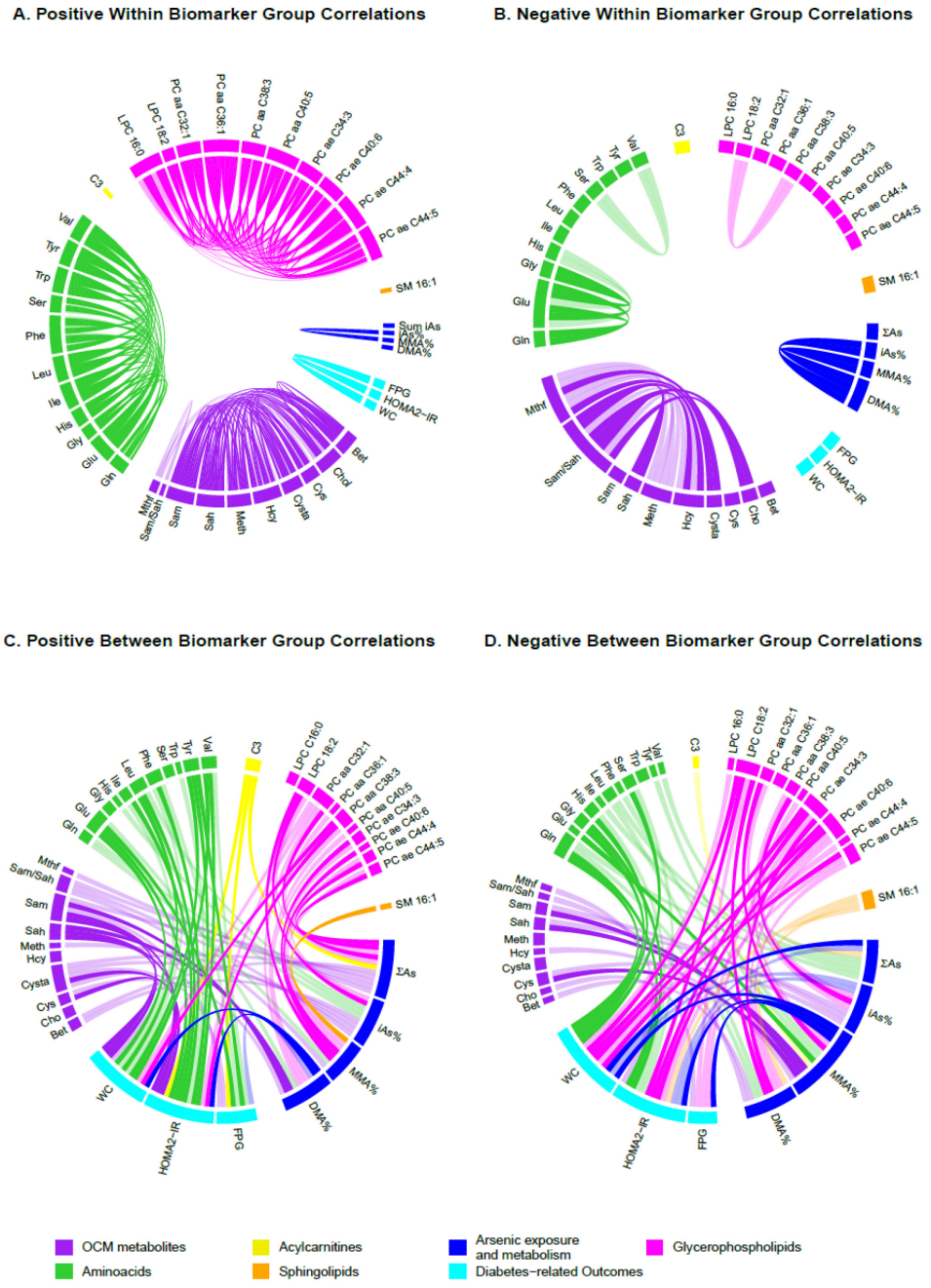


Figure 2. Forrest Plot of Participant Characteristics by Metabolites of Interest. Squares represent the median levels of metabolites and lines represent the interquartile range in each subcategory. Elevated FPG and elevated waist circumference were based on ATP III guideline's metabolic syndrome criteria. Elevated waist circumference: ≥102 cm for men, ≥88 cm for women; FPG ≥100mg/dL (or on medication). Abbreviations: ∑As (sum of inorganic arsenic and its methylated metabolites); BMI (body mass index); DMA (dimethylarsinic acid); FPG (fasting plasma glucose); HOMA (homeostasis model assessment); iAs (inorganic arsenic); LPC (lysophosphatidylcholine); MMA (monomethylarsonic acid); PC (phosphatidylcholine); SAH (S-Adenosylhomocysteine); SAM (S-Adenosylmethionine)

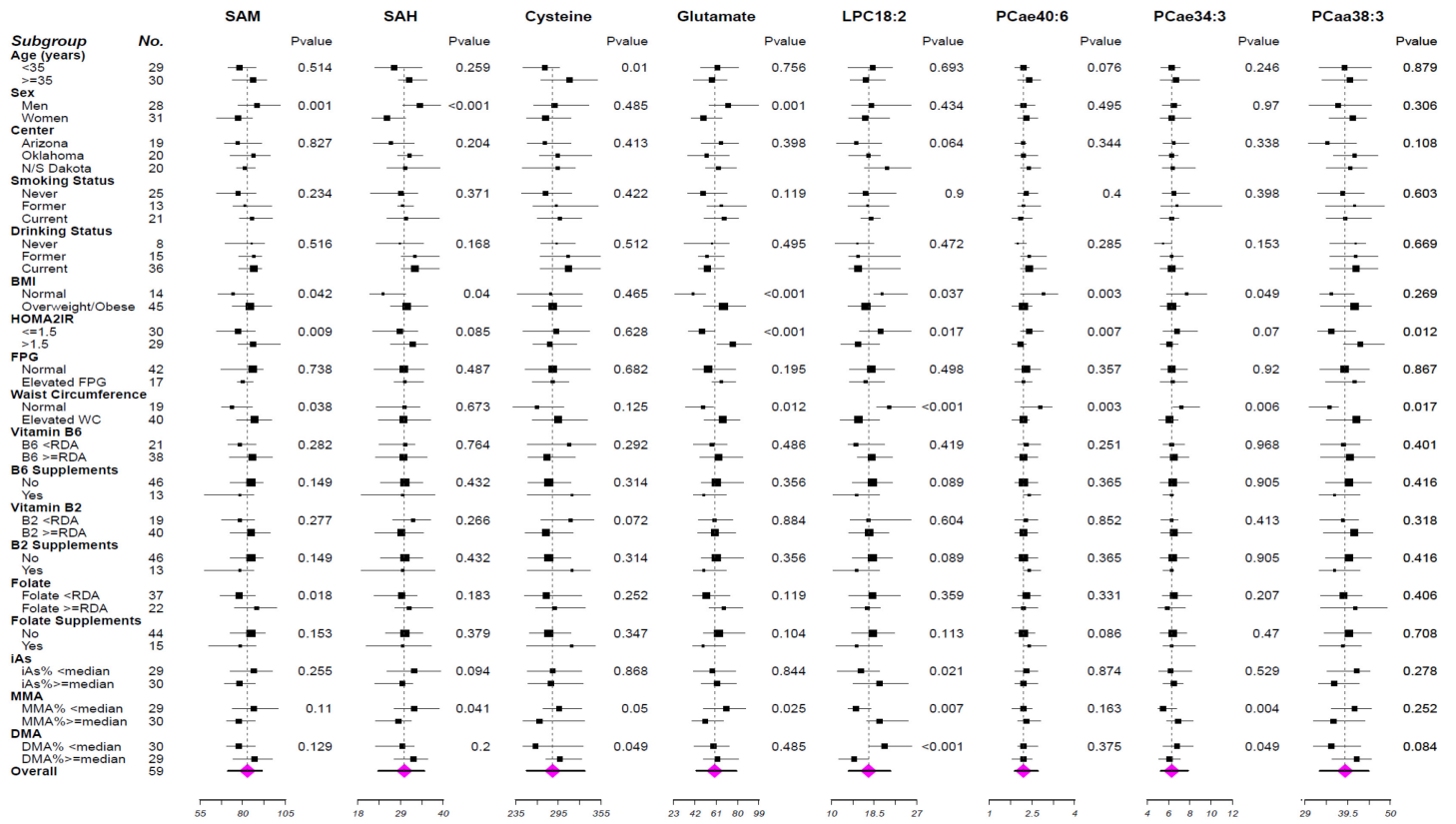


Figure 3. Spearman Correlations between arsenic metabolism biomarkers, diabetes-related outcomes, and metabolites of interest. ∑As and HOMA2-IR are log-transformed. P-value <0.05 for correlations ≥0.26; P-value <0.01 for correlations ≥0.33; P-value <0.001 for correlations ≥0.42. Abbreviations: Sum As (sum of inorganic arsenic and its methylated metabolites); DMA (dimethylarsinic acid); FPG (fasting plasma glucose); HOMA (homeostasis model assessment); iAs (inorganic arsenic); LPC (lysophosphatidylcholine); MMA (monomethylarsonic acid); PC (phosphatidylcholine); SAH (S-Adenosylhomocysteine); SAM (S-Adenosylmethionine); WC (waist circumference)

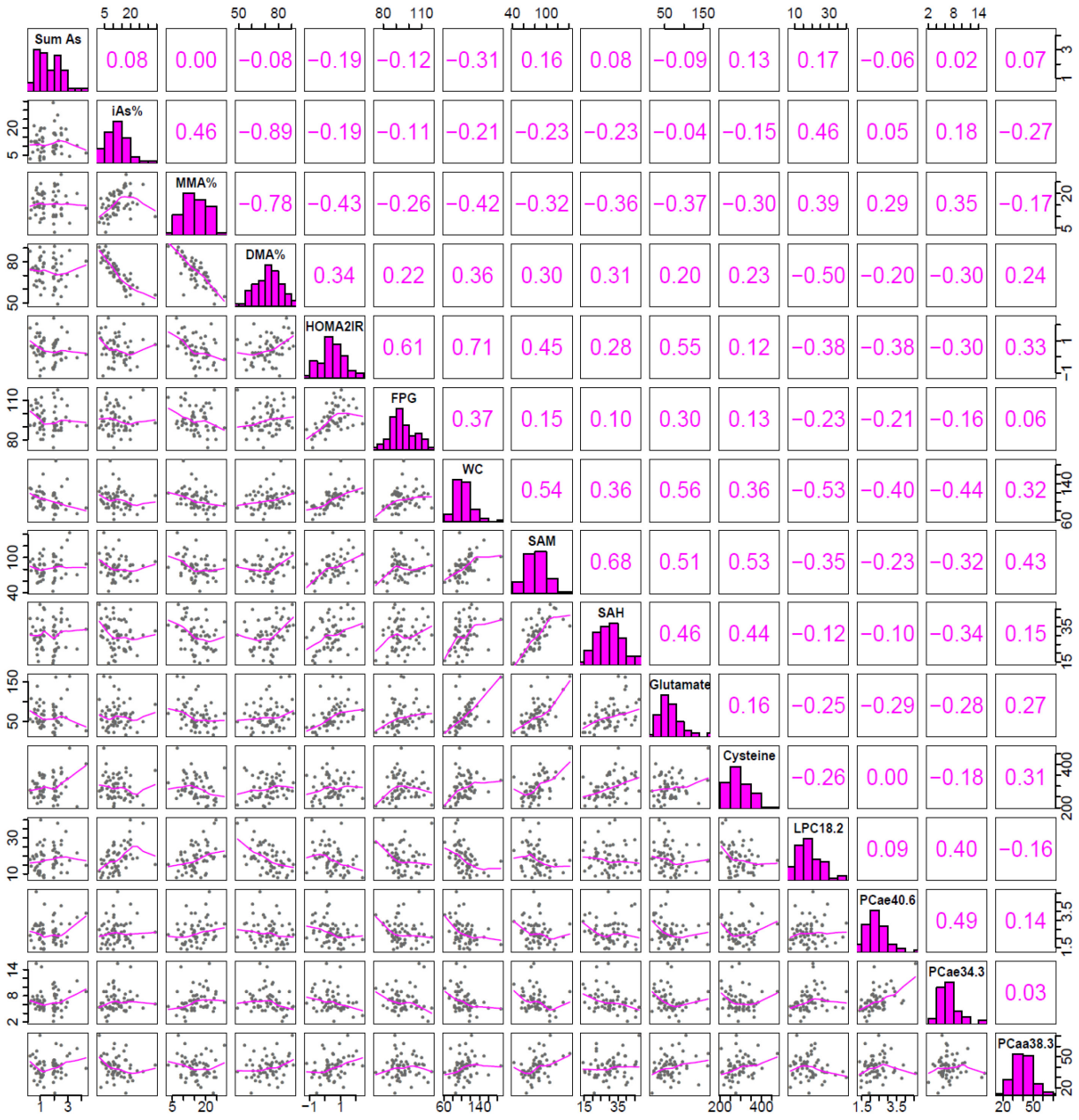


Figure 4. Study Metabolites in the One Carbon Metabolism Pathway. One carbon metabolism metabolites of interest (i.e., metabolites significantly correlated with both arsenic metabolism and a metabolic outcome) are bolded (a-f). One carbon metabolism facilitates the generation of SAM (a), the methyl donor for numerous substrates, which are essential to many biological processes including arsenic metabolism. SAH (b) is a product of SAM-dependent methylation reactions, and is hydrolyzed to Hcys, which can be remethylated to form methionine and activated to regenerate SAM. SAH is a potent inhibitor of most transmethylation reactions, including arsenic, through its binding with methyltransferases. SAH is only removed from methyltransferases if Hcys is pulled forward for the remethylation of Hcys to form methionine or diverted to the transsulfuration pathway for glutathione synthesis. Glutathione is a tripeptide consisting of cysteine (c), glutamate (d), and glycine and is critical to the body's antioxidant response (in its reduced form as GSH), including detoxification of heavy metals. Choline can provide methyl groups for the remethylation of Hcys through conversion to betaine. Choline is also used in the synthesis of phosphatidylcholine (PC) (e), an abundant phospholipid involved in maintenance of hepatic lipid metabolism. PCs may also be synthesized through the methylation of phosphatidylethanolamine using SAM as the methyl donor. aaPCs consist of glycerol linked to phosphocholine and two fatty acid residues, and removal of one fatty acid produces LPCs (f). aePC's comprise an ether linkage to one alkyl chain and one polyunsaturated fatty acid. Abbreviations: As (inorganic arsenic); DAG (diacylglycerols); DMA (dimethylarsinic acid); GSH (glutathione); Hcys (homocysteine); LysoPCs (lysophosphatidilycholines); MMA (monomethylarsonic acid); MTHFD1 (Methylenetetrahydrofolate dehydrogenase); MTHFR (Methylenetetrahydrofolate reductase); MTR (Methionine synthase); MTRR (Methionine synthase reductase); SAH (S- adenosylhomocysteine); SAM (S-adenosylmethionine); SHMT1 (Serine hydroxymethyltransferase 1)

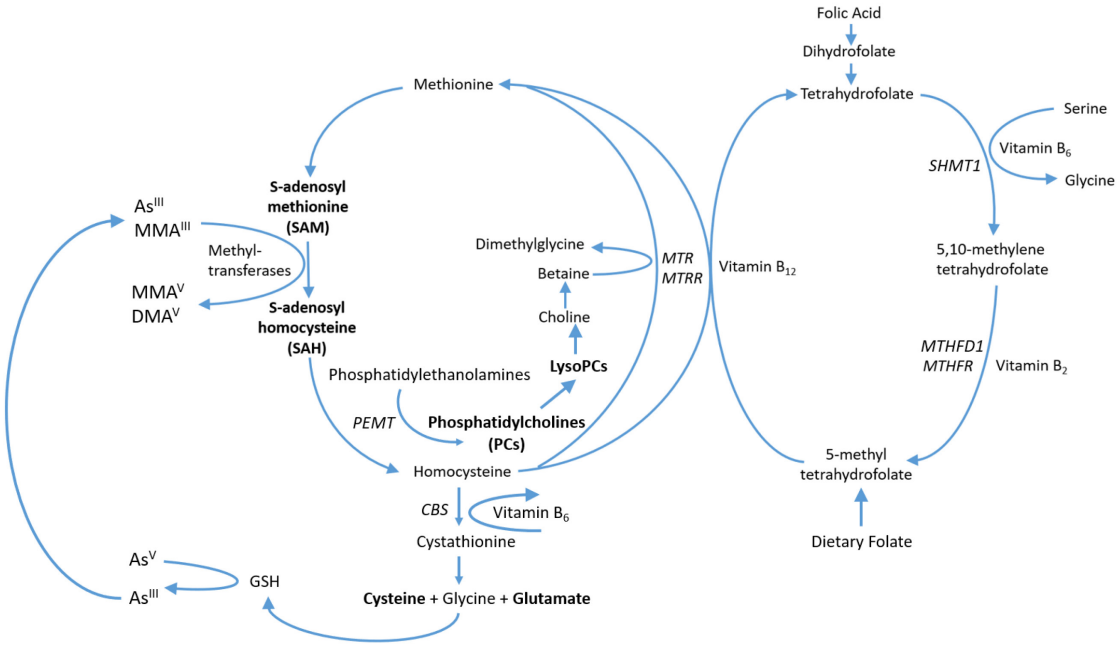
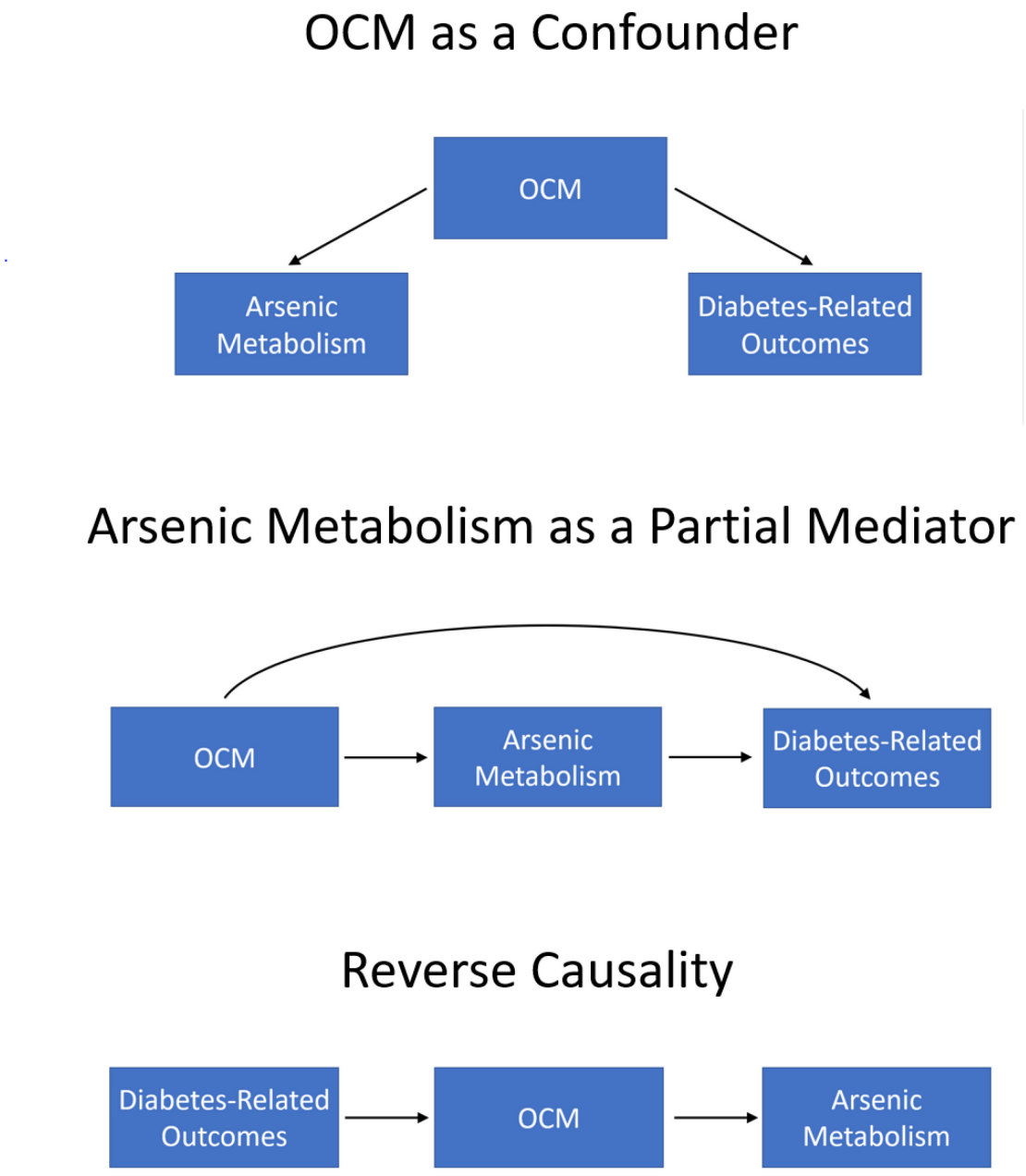

Folic Acid
Dihydrofolate
Tetrahydrofolate
Serine
Vitamin B6
SHMT1
Glycine
Methionine
S-adenosyl methionine (SAM)
AsIII
MMAIII
Methyl-transferases
MMAV
DMAV
S-adenosyl homocysteine (SAH)
Dimethylglycine
Betaine
Choline
MTR
MTRR
Vitamin B12
5,10-methylene tetrahydrofolate
MTHFD1
MTHFR
Vitamin B2
Phosphatidylethanolamines
LysoPCs
PEMT
Phosphatidylcholines (PCs)
Homocysteine
5-methyl tetrahydrofolate
CBS
Vitamin B6
Cystathionine
Dietary Folate
AsV
AsIII
GSH
Cysteine + Glycine + Glutamate

Figure 5. Directed acyclic graphs of proposed pathways between arsenic metabolism, one carbon metabolism and diabetes-related outcomes



CHAPTER 4

Arsenic, one-carbon metabolism and diabetes-related outcomes in the Strong Heart Family Study

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ABSTRACT

Background: Inorganic arsenic exposure and inter-individual differences in its metabolism have been associated with cardiometabolic risk. A more efficient arsenic metabolism profile (lower MMA%, higher DMA%) has been associated with reduced risk for arsenic-related health outcomes; however, this profile has also been associated with increased risk for diabetes-related outcomes. The mechanism behind these contrasting associations is equivocal.

Objective: Evaluate whether one carbon metabolism (OCM) may influence the relationship between arsenic metabolism and diabetes-related outcomes

Methods: We evaluated the association between OCM-related variables (B vitamin intake and genetic variants) and arsenic metabolism variables (iAs%, MMA% and DMA%) in 935 participants free of diabetes and metabolic syndrome at baseline from the Strong Heart Family Study, a family-based prospective cohort comprised of American Indian tribal members aged 14+ years. The association between OCM-related variables and diabetes-related outcomes (metabolic syndrome, diabetes, HOMA2-IR and waist circumference) was also assessed. We then evaluated the effect OCM status has on the association between previously reported associations between arsenic metabolism and diabetes-related outcomes.

Results: Of the 935 participants free of both diabetes and metabolic syndrome at baseline,

279 (29.8%) developed metabolic syndrome over a median of 5.3 years of follow-up and of the 1,458 participants free of diabetes at baseline, 167 (11.3%) developed diabetes over follow-up. OCM-related nutrients were not associated with arsenic metabolism, however, higher vitamin B_6 was consistently associated with three of the four diabetes-related outcomes studied (higher HOMA2-IR and increased risk for diabetes and metabolic syndrome). Adjustment for arsenic metabolism in these models did not affect the associations between vitamin B_6 and diabetes-related outcomes. Further, previously reported associations between arsenic metabolism and diabetes-related outcomes were not affected by adjustment for any of our OCM (nutrient or genetic) variables. However, a polymorphism in methionine synthase (*MTR*) was associated with both higher MMA% 2.57 (95% CI: 0.22, 4.92) and lower HOMA2-IR (GMR=0.79, 95% CI=0.66, 0.93 per 5 years of follow-up). After adjustment for MMA% the association between the *MTR* SNP and all diabetes-related outcomes were attenuated or reversed direction.

Conclusions: Additional research is needed to determine whether excess B vitamin intake is associated with increased risk for diabetes-related outcomes. Our findings suggest MMA% may be a partial mediator in the association between OCM and diabetes-related outcomes. Formal mediation analyses are needed to confirm this finding.

INTRODUCTION

One carbon metabolism (OCM) is a network of interrelated biochemical reactions critical to the biosynthesis of purines and thymidylate as well as the generation of methyl groups.[1] The transfer of a methyl group to substrates is essential to multiple biological processes including arsenic metabolism.[1] OCM functioning, and adequate methyl group availability, is dependent on essential nutrients that support this pathway including folate, vitamin B_{12}, vitamin B_6, vitamin B_2, and methionine. Therefore, evaluation of OCM status in epidemiologic and *in vivo* studies has been evaluated through the measurement of both circulating levels and dietary intake of these nutrients. Low OCM nutrient status in observational studies has been associated with numerous adverse health effects[2-8] including diabetes-related outcomes.[9-12] However, findings on the efficacy of OCM nutrient supplementation clinical trials to reduce these outcomes have been mixed. [13-15] Just two trials have evaluated the effect of OCM supplementation (one evaluating folic acid alone,[16] one evaluating a B vitamin combination (folic acid, B_6 and B_{12}) pill[17]) on diabetes: results were null except in one high risk group (hypertensive obese participants) sub-analysis.[16, 17] Smaller supplementation trials focused on folic acid, on the other hand, have suggested supplementation may improve diabetes indicators and complications among diabetes cases.[18-22] Still, some observational studies have suggested high circulating folate levels may actually have deleterious health effects on diabetes-related outcomes, including the offspring of mothers with high folate during pregnancy.[23-27] Studies evaluating the effect of intake of folate from foods or other OCM nutrients on diabetes-related outcomes are limited, mostly cross-sectional in design and findings are even more conflicting,[9, 12, 28-35] highlighting the need for more research on this

relationship. Single nucleotide polymorphisms (SNPs) in genes encoding enzymes involved in OCM have also been associated with diabetes-related outcomes[36-44] and provide a useful, hypothetically unbiased alternative assessment of OCM status.

Evidence suggests arsenic metabolism may also be affected by OCM status. Methylation reactions involved in the metabolism of inorganic arsenic (arsenate and arsenite (iAs)) into mono- and di-methylated arsenicals (MMA and DMA) require the transfer of a methyl group generated by the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) in the OCM pathway.[1] Typically, arsenic metabolism in epidemiologic studies is measured as each relative percentage of iAs (iAs%), MMA (MMA%) and DMA (DMA%) out of the three urinary concentrations summed together. DMA has a shorter circulating half-life and is more rapidly excreted than iAs or MMA,[45-50] thus, a urine profile reflecting higher percentages of DMA is generally considered a more efficient arsenic metabolism profile. Randomized clinical trials conducted in highly arsenic-exposed Bangladeshi populations have successfully showed folic acid supplementation can enhance arsenic metabolism, reflected in increases in DMA% and decreases in MMA%, likely through supporting efficiency of the OCM pathway.[51, 52] Observational cross-sectional studies also suggest OCM nutrients may enhance arsenic metabolism.[53-55] Further, OCM SNPs have been found to influence arsenic metabolism as well.[56-59] This is highly important as arsenic metabolism, specifically higher MMA%, has been identified as a risk factor for arsenic-related health outcomes, including cardiovascular disease, skin lesions and cancer.[60-67] However, higher MMA% has also been associated with a reduced risk for diabetes-related outcomes. Indeed, in the Strong Heart Study and Strong Heart Family Study (SHFS), higher MMA% has been associated

with lower risk for diabetes,[68] metabolic syndrome[69] and elevated waist circumference, as well as lower BMI[70] and HOMA2-IR[71]. The contrasting association between arsenic metabolism and diabetes-related outcomes versus other arsenic-related health outcomes is unclear.

The goal of this study was to characterize the role OCM plays in the association between arsenic metabolism and diabetes-related outcomes in an attempt to better understand the contrasting relationships between arsenic metabolism and these outcomes versus other arsenic-related health outcomes. Previous research suggests OCM is strongly tied to both arsenic metabolism and diabetes-related outcomes, making it an ideal candidate to potentially provide a mechanistic pathway between the two variables. In this study we first evaluated the associations between OCM variables (SNPs: *PEMT, MTR, MTRR, MTHFR, MTHFD1, SHMT1* and *CBS*; and nutrient intake: vitamin B_6, vitamin B_2 and folate) and both arsenic metabolism (iAs%, MMA% and DMA%) as well as four diabetes-related outcomes (metabolic syndrome, waist circumference, diabetes, insulin resistance [measured with HOMA2-IR] and waist circumference). Diabetes-related outcomes were selected based on previously reported associations between arsenic metabolism and these outcomes in the Strong Heart Study and SHFS.[68, 71, 72] We then evaluated whether OCM influences previously reported associations between arsenic metabolism and diabetes-related outcomes in this study population. We used data from participants in the Strong Heart Family Study, a prospective family-based cohort study of cardiometabolic disease in American Indian tribal members from Arizona, Oklahoma, and North/South Dakota.

METHODS

Study population

The SHFS recruited 2,919 participants in 1998-1999 (n=428) and 2001-2003 (n=2,491) with follow-up visits conducted in 2006-2009. For this study, we did not include baseline visits conducted in 1998-1999 as OCM nutritional information was only conducted in during visits in 2001-2003. We included participants with sufficient urine for arsenic analyses and who were free of diabetes at baseline (n=1,661). We used urinary arsenic and OCM nutrient data measured from visits conducted in 2001-2003, and diabetes-related outcome (metabolic syndrome, waist circumference, HOMA2-IR, diabetes) data from follow-up visits in 2006-2009. We excluded participants missing information on outcome data (n=91), OCM genetic variant data (n=19) or OCM nutrient status (n=82). We also excluded participants with missing data on education, smoking, alcohol intake, BMI, and estimated glomerular filtration rate (eGFR) (n=9), resulting in 1,458 participants available for analyses evaluating HOMA2-IR, waist circumference or incident diabetes. For incident metabolic syndrome analyses, we further excluded participants with prevalent cases of metabolic syndrome (n=935) (**Figure 1**). All participants provided informed consent and study protocols were approved by multiple institutional review boards, participating communities and The Indian Health Service.

Data Collection

Baseline and follow-up visits included bio-specimen collection, physical exam, food frequency questionnaire and an interview-administered questionnaire (age, sex, education, smoking history, alcohol use, medical history).[73] Exam measures (waist

circumference, blood pressure, height, weight) and collection of urine and fasting blood samples were performed by centrally trained nurses following a standardized protocol.[74]

Urine arsenic determinations

Morning spot urine samples were collected in polypropylene tubes, frozen within 1 to 2 hours of collection, shipped buried in dry ice and stored at -70°C in the Penn Medical Laboratory, MedStar Research Institute, Washington, DC for up to 18 years. For arsenic analyses, urine samples were thawed and up to 1.0 mL from each urine sample was transferred to a small vial, transported on dry ice to the Trace Element Laboratory at Graz University, Austria and stored at -80°C until analyses.

Total urine arsenic concentrations were measured using inductively coupled plasma-mass spectrometry (ICPMS). The urine concentrations of arsenite, arsenate, MMA and DMA were measured using high performance liquid chromatography/-ICPMS (HPLC/ICPMS). The inter-assay coefficients of variation for arsenite, arsenate, MMA, DMA and total arsenic were 14.7%, 6.9%, 6.4%, 6.0% and 4.7% respectively. Limits of detection were 0.1 µg/L for all four species. Arsenic species concentrations below the limit of detection (<5% for all species) were imputed as the limit of detection divided by √2.

OCM Nutrient Collection

Dietary intake of OCM-related micronutrients was measured during the baseline visit through estimated daily averages of dietary intake of vitamins B_6 and B_2 and folate in the past-year. These variables, as well as total caloric intake, were measured through

an interviewer-administered Block 119-item food frequency questionnaire (FFQ). The Block questionnaire is one of the most widely used questionnaires with demonstrated reliability and validity.[75] To enhance accuracy of the questionnaire in this cohort, additional questions relating to foods commonly consumed by American Indians were added.[75]

OCM SNP Selection and Genotyping

DNA was extracted from blood specimens obtained at the baseline visit using organic solvents and was genotyped according to Illumina protocol[76] using the Illumina Cardio-Metabo DNA Analysis BeadChip (MetaboChip), which contains 196,725 markers. These markers were selected based on a large-scale meta-analysis for cardiometabolic traits such as coronary artery disease and type 2 diabetes. Markers included in this analysis have been genotyped previously using strict QC methods[77] and were selected based on their role in OCM. Single nucleotide polymorphisms (SNPs) were only included if minor allele frequencies were >2%. For genes with multiple SNPs available, SNPs that had been previously associated with arsenic metabolism or diabetes-related outcomes (or SNPs in perfect LD with these SNPs) were selected. SNPs in the following genes were included: rs4646371 in phosphatidylethanolamine N-methyltransferase (*PEMT*), rs3818239 and rs17751556 in methylenetetrahydrofolate dehydrogenase (*MTHFD1*), rs12952556 and rs2273027 in serine hydroxymethyltransferase 1 (*SHMT1*), rs1801131, rs1801133 and rs2274976 in methylenetetrahydrofolate reductase (*MTHFR*), rs10495387 in methionine synthase

(*MTR*), rs3776464 and rs16879334 in methionine synthase reductase (*MTRR*) and rs12482221 in cystathionine β-synthase (*CBS*).

Diabetes-Related Variable Collection and Definitions

Incident type 2 diabetes was defined as fasting plasma glucose ≥ 126 mg/dL, self-reported physician diagnosis or self-reported use of insulin or oral diabetes treatment. Baseline and follow-up HOMA2-IR values were calculated with the computed solved model for HOMA2-IR[78] using fasting glucose and insulin values. Metabolic syndrome was defined according to the National Cholesterol Education Program ATP III guidelines, the most accepted guidelines for the US.[79, 80] To have the metabolic syndrome, participants had to have at least three of the following criteria: elevated waist circumference (≥40 inches in men and ≥35 inches in women); triglycerides ≥150 mg/dL (or on medication); fasting glucose ≥100 mg/dL (or on medication); HDL cholesterol <40 mg/dL for men and <50 mg/dL for women (or on medication); systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg (or on medication). Metabolic syndrome status was measured at baseline and follow-up visits. Waist circumference was measured at the umbilicus while the participant was in a supine position.[74] Other measurements for metabolic syndrome status were measured as follows. For systolic and diastolic blood pressure, three measurements were taken on the right arm in seated position after 5 minutes of rest with an appropriately sized cuff using a Baum mercury sphygmomanometer; the average of the last two measurements was used or analyses.[74] Blood samples collected after 12-hour fasting were used to measure triglycerides,

glucose, and cholesterol using enzymatic methods.[74] High-density lipoprotein cholesterol levels were measured by precipitation with heparin and manganese chloride.[74]

Sociodemographic and Diabetes-Related Risk Factor Data

The standardized questionnaire included sociodemographic data (age, sex, education, income), smoking history, alcohol use, physical activity, and medical history.[73] Physical exam measures were performed by centrally trained nurses following a standardized protocol and included measurements of height, weight and body fat.[74] eGFR was measured from age, sex and recalibrated plasma creatinine using the Chronic Kidney Disease – Epidemiology Collaboration formula.[81]

Statistical Analyses

Arsenic metabolism was computed by dividing each inorganic and methylated arsenic metabolite concentration over the sum of those species x 100 (iAs%, MMA% and DMA%). Arsenic exposure, calculated as the sum of inorganic and methylated arsenic species ($\sum$As) in urine and creatinine-corrected to account for urine dilution, was right-skewed and log-transformed in analyses. OCM nutrients (folate, vitamin B_6 and vitamin B_2) were adjusted for total caloric intake using a residual analysis approach. This approach has been suggested over including caloric intake as a variable in regression models, as the nutrient and total caloric intake variables are measured through the same questionnaire, and therefore, their errors are correlated.[82] For the residual analysis approach, we regressed each log-transformed vitamin intake on log-transformed total caloric intake. We then added the mean log-transformed value of each nutrient to the

nutrient residuals to create calorie-corrected nutrient variables. OCM SNPs were included in analyses using co-dominant inheritance models. For the SNPs *MTHFD1* (rs3818239), *MTHFR* (rs2274976), *MTHFD1* (rs17751556) and *MTR* (rs10495387), the homogenous variant genotype was <10 and therefore grouped with the heterogenous genotype in analyses, resulting in just one comparison group to the wild-type genotype.

The association between OCM status (genetic- and nutrient-related) and outcome (arsenic metabolism and diabetes-related) variables were measured in participants free of prevalent metabolic syndrome and diabetes (n=935) to remove the potential for the effect metabolic state may have on arsenic metabolism. In OCM nutrient analyses, the mean difference (95% CI) of each arsenic species percentage (iAs%, MMA%, DMA%) per interquartile range (IQR) increase in each calorie-corrected nutrient variable (vitamin B_6, vitamin B_2 and folate) was estimated using mixed linear regression models to account for family-relatedness. In OCM SNP analyses, mixed linear regression models were used to estimate the mean difference (95% CI) of each arsenic species percentage comparing the heterogenous and homogenous variant genotypes to the wild type genotype (reference) for each OCM-related SNP. OCM nutrient and genetic models with arsenic metabolism were adjusted for log ∑As, age (continuous), sex, region (Arizona, Oklahoma, North/South Dakota), education (<12 years, 12+ years (or above/below appropriate level of schooling if aged <18 years)), BMI (<25, 25-<30, ≥30 kg/m2), smoking status (never, former, current), alcohol use (never, former, current), and eGFR (continuous). In models evaluating waist circumference, BMI was not included as an adjustment for consistency with previous literature.[83]

The association between OCM status (genetic- and nutrient-related) was also evaluated with each diabetes-related outcome. For diabetes, HOMA2-IR and waist circumference outcomes, analyses were conducted in participants free of baseline diabetes (n=1458). In metabolic syndrome analyses, analyses were conducted in participants free of baseline diabetes and baseline metabolic syndrome (n=935). For diabetes and metabolic syndrome, our dichotomous outcomes, we used modified poisson regression with robust variance[84] using generalized estimating equations with an independence working correlation structure to account for family clustering. Results were reported as relative risk (RR) and 95% confidence intervals (95% CI) of incident diabetes and metabolic syndrome per IQR and quartile increase in calorie-corrected OCM nutrients as well as comparing the heterogenous and homogenous variant genotypes to the wild-type genotype (reference) for each OCM SNP. For our continuous outcomes, HOMA2-IR and waist circumference, we conducted multi-level models (MLM) in which both HOMA2-IR (log-transformed) and waist circumference values at baseline and at follow-up were treated as the outcome and the linear predictor included the interaction of our OCM variable and time since baseline (in years). Specifically, the time variable included two values for each participant: time=0 and time=follow-up duration (in years). In this way, we were able to estimate the GMR of HOMA2-IR and mean difference of waist circumference and 95% CIs by OCM status variables at follow-up (considering 5 years of follow-up, i.e. time=5). Again, we used mixed effects linear regression models to account for family clustering.

Models evaluating the association between OCM SNPs and diabetes-related outcomes were adjusted for age, sex and 4 population stratification principal components.

Models evaluating the association between OCM nutrients and diabetes-related outcomes were adjusted for age, sex, center, education, BMI, kidney function, smoking status and drinking status.

In previous analyses in this cohort, arsenic metabolism has been associated with risk for diabetes, metabolic syndrome and elevated waist circumference, as well as higher HOMA2-IR. We re-ran these analyses in this study before and after adjustment for OCM variables to evaluate whether OCM status might be confounding these associations. We also re-ran final models evaluating the association between OCM nutrients and SNPS with diabetes-related outcomes after adjusting for arsenic metabolism (i.e., MMA%) and ∑As, to evaluate whether arsenic metabolism may act as a partial mediator in these associations.

Multiple sensitivity analyses were conducted to better understand the association between vitamin B_6 and diabetes-related outcomes. First, we ran spearman correlations between our calorie-corrected B_6 variable and dietary food groups to determine if there were any high correlations that might potentially be serving as a proxy for vitamin B_6. We then attempted to understand whether associations between vitamin B_6 and diabetes-related outcomes were confounded by specific food sources by additional adjustments for intake of processed meat, red meat, fried chicken, vegetables and fruits separately in the final model. We also ran models using raw vitamin values with separate adjustment for total caloric intake instead of calorie-corrected vitamins. Finally, we stratified analyses by sex.

RESULTS

Participant Characteristics

Of the 1,458 participants free of diabetes at baseline, 167 (11.3%) developed diabetes over a median of 5.3 years of follow-up, and of the 935 participants free of both diabetes and metabolic syndrome at baseline, 279 (29.8%) developed metabolic syndrome over follow-up. Participants who developed diabetes or metabolic syndrome at follow-up were older, had higher DMA%, BMI, HOMA2-IR and waist circumference and lower MMA% ($p \leq 0.05$) compared with participants that did not develop diabetes or metabolic syndrome (**Table 1**). Participants who developed diabetes were also exposed to higher levels of ∑As than those that did not develop diabetes. Participants who developed diabetes or metabolic syndrome did not differ by vitamin intake or other sociodemographic variables.

Association between OCM Variables and Arsenic Metabolism

In mixed linear regression models, calorie-corrected OCM vitamins (B_6, B_2 and folate), were not associated with any arsenic metabolism variables (iAs%, MMA%, DMA%) (**Table 2**). In mixed linear regression models evaluating the association between OCM SNPs and arsenic metabolism, carriers of one or two A alleles in the *MTR* rs10495387 polymorphism was associated with 2.57 (95% CI: 0.22, 4.92) higher MMA% and non-significantly associated with 4.08 (95% CI -8.32, 0.17) lower DMA% (**Table 3**). No other OCM SNPs were associated with arsenic metabolism.

Association between OCM Variables and Diabetes-Related Outcomes

In mixed modified poisson regression models, IQR increases in calorie-corrected vitamin B_6 were associated with greater risk for incident metabolic syndrome (RR=1.15; 95% CI=1.02, 1.29) and incident diabetes (RR=1.28; 95% CI=1.10, 1.48) (**Table 4**). Vitamin B_6 was also borderline associated with higher HOMA2-IR in mixed multi-level models (GMR=1.04; 95% CI=1.00, 1.08 per 5-years of follow-up). Results were similar categorizing vitamin B_6 into quartiles. Results were consistent in sensitivity analyses stratifying by sex; using non-calorie-corrected vitamin B_6 values and adjusting separately for caloric intake in models; and further adjusting final models separately for intake of fried chicken, processed meat, red meat, vegetables and fruit (data not shown). Calorie corrected vitamin B_2 was also associated with increased risk for incident diabetes (RR per IQR increase in vitamin B_2=1.21; 95% CI=1.03, 1.41). Folate was not significantly associated with any diabetes-related outcome.

Three OCM SNPs in *CBS*, *PEMT* and *MTR* genes had significant associations with diabetes-related outcomes. Each addition of the variant A allele in the *CBS* SNP was associated with an allele-dependent decrease in waist circumference per 5 years of follow-up (β_{AG}= -0.51, 95% CI=-2.36, 1.33; β_{GG}= -0.3.28; 95% CI=-5.82, -0.74) (**Table 5**). *CBS* was not associated with any other diabetes-related outcome. For *PEMT*, each addition of the variant A allele was associated with a allele-dependent increase in both metabolic syndrome (RR_{AG}=1.12, 95% CI= 0.88, 1.43; RR_{GG}=1.37; 95% CI=1.02, 1.85) and waist circumference (β_{AG}= 1.92, 95% CI=-0.13, 3.71; β_{GG}= 2.51, 95% CI=-0.14, 5.17 per 5 years of follow-up). A similar trend was observed for diabetes, however, the association was not significant. For *MTR*, one or two copies of the variant A allele was associated with lower HOMA2-IR (GMR=0.79, 95% CI=0.66, 0.93 per 5 years of

follow-up). The trend was consistent for metabolic syndrome and diabetes, but not significant.

Of the three OCM nutrients and twelve OCM SNPs, *MTR* was the only variable associated with both a diabetes-related outcome (HOMA2-IR) and an arsenic metabolism variable (MMA%). Therefore, we ran additional models evaluating the effect the *MTR* SNP had on diabetes-related outcomes after adjustment for MMA% and ∑As. Adjustment for arsenic variables attenuated associations, with the association for HOMA2-IR almost losing significance (GMR=0.83, 95% CI=0.71, 0.99). Further, the very slight negative association between *MTR* and waist circumference before MMA% adjustment reversed to a slightly positive association after adjustment (β=1.35, 95% CI=-2.51, 5.21) (**Table 6**). We also attempted to understand the potential role of OCM as a confounder in the relationship between the previously reported associations with arsenic metabolism (lower MMA% and higher DMA%) and increased risk for diabetes-related outcomes by adjusting separately for OCM nutrients and SNPs. Associations remained consistent before and after adjustments (data not shown).

DISCUSSION

In this study of American Indian men and women aged ≥14 years, OCM nutrients were not associated with arsenic metabolism, however, higher vitamin B_6 was consistently associated with three of the four diabetes-related outcomes studied (higher HOMA2-IR and increased risk for diabetes and metabolic syndrome). Adjustment for arsenic metabolism in these models did not affect the associations between vitamin B_6 and diabetes related outcomes. Further, previously reported associations between arsenic

metabolism and diabetes-related outcomes were not affected by adjustment for any of our OCM (nutrient or genetic) variables. However, the *MTR* rs10495387 polymorphism was associated with both lower MMA% and higher HOMA2-IR per 5 years of follow-up. After adjustment for MMA% the association between the *MTR* SNP and all diabetes-related outcomes were attenuated or reversed direction, suggesting MMA% may be a partial mediator in the association between this OCM SNP and diabetes-related outcomes.

Arsenic metabolism has been identified in epidemiological studies as a risk factor for health outcomes associated with arsenic exposure. Indeed, higher MMA% has consistently been associated with greater risk for skin lesions,[85-88] cancer[67, 89-93] and cardiovascular disease.[94-96] As a result of these findings, clinical trials have been conducted in highly arsenic-exposed regions of the world in order to enhance arsenic metabolism (i.e., increase DMA% and decrease MMA%). In contrast, lower MMA% has been identified as protective of diabetes-related outcomes, including diabetes, increased BMI, elevated waist circumference, higher HOMA2-IR and metabolic syndrome.[68, 70-72, 97] The mechanism behind these contrasting associations is unclear, but essential to uncover in order to determine if the link between arsenic metabolism and diabetes-related outcomes is an epidemiological artifact or a true association that should be considered in future arsenic health effects prevention trials. Both arsenic metabolism and diabetes-related outcomes are tightly connected to OCM, providing an intriguing potential pathway to explain this equivocal relationship.

We characterized OCM in our study through two different metrics: OCM SNPs and intake of OCM nutrients. Despite inherent limitations to these variables as proxies to

true OCM status that can be obtained through direct measurement of circulating OCM metabolites, they do have a distinct advantage: they are not influenced by a participant's metabolic state allowing for a better understanding of relationship directions. OCM nutrients including vitamin B_6, vitamin B_2 and folate were selected for this study based on their role in OCM as outlined in Figure 2. OCM facilitates the generation of methyl groups, through the activation of methionine to S-adenosylmethionine (SAM), which provides the methyl group for most methylation reactions in the body.[1] S-adenosylhomocysteine (SAH) is formed as a byproduct of these reactions and inhibits OCM by tightly binding to most methylation transferase enzymes.[1] SAH is only removed from these enzymes and hydrolyzed to homocysteine if existing homocysteine is either regenerated into methionine or rerouted to the transsulfuration pathway.[98] Vitamins B_6 and B_2 are co-enzymes in the conversion of tetrahydrofolate (THF) to 5,10-methylene-THF and the subsequent conversion of 5,10-methylene-THF to 5-methyl tetrahydrofolate (THF), respectively. Dietary folate can enter the OCM pathway as 5-methyl tetrahydrofolate (THF), which can then transfer a methyl donor to homocysteine to generate either THF or methionine. Vitamin B_6 also plays a role in the transsulfuration pathway acting as a co-factor in the conversion of homocysteine to cystathionine, which is used in the generation of glutathione.[98]

Our null results on the association between OCM nutrients and arsenic metabolism contrasts with findings from randomized supplementation trials conducted in highly exposed regions in Bangladesh which have shown folic acid supplementation can enhance arsenic metabolism (increase DMA% and reduce MMA% in urine).[51, 52] Further, our group has previously reported an association between higher intake of vitamins B_6

and B_2 and enhanced arsenic metabolism in the Strong Heart Study, the parent study to the SHFS comprised of older American Indian tribal members recruited in 1989-1991.[55] It's possible the lack of association in this study is in part due to the relatively high intake levels of these OCM nutrients compared to the parent study, which occurred prior to mandatory folic acid fortification. Some evidence suggests increasing OCM nutrient intake only has an effect on arsenic metabolism when OCM status is low.[53, 99] Indeed, the only other US-based study evaluating this relationship also reported null findings.[100]

We did, however, see strong consistent positive associations between vitamin B_6 and our diabetes-related outcomes, including increased risk for metabolic syndrome and diabetes, as well as higher HOMA2-IR. These findings conflict with numerous observational studies which have found low OCM nutrient status to be associated with adverse health effects[5-7] including cardiometabolic outcomes.[2-4, 8-11] Low levels of OCM nutrients resulting in impaired OCM functioning, in turn causing elevations in homocysteine, has been identified as a potential mechanism behind these findings.[101] Still, causality has been questioned as folic acid and B-vitamin combination pill supplementation trials have had inconsistent results for reducing cardiovascular-related outcomes,[13, 14] with mostly no significant benefits reported for diabetes incidence.[16, 17] Further, some evidence suggests high folate may actually increase mortality in diabetes patients.[24] Finally, observational associations may also be a result of reverse causality. Animal models have shown diabetes can alter OCM functioning and metabolite levels, including reductions in vitamin B_6.[102, 103] This could explain the difference in findings, as metabolic state would not necessarily affect intake. Still, it's unclear why higher B_6 intake would be associated with greater risk for diabetes-related outcomes. We conducted

several sensitivity analyses to evaluate whether confounding might explain the association. Our calorie-corrected B_6 vitamin was not associated with caloric intake or other food patterns that have been associated with diabetes related outcomes (e.g., processed meat[75], cereals/carbohydrates) (**Supplemental Figure 1**). Models evaluating vitamin B_6 and diabetes-related outcomes remained consistent after adjustment for these food patterns as well as stratifying by sex. Although residual confounding cannot be disregarded, it is also possible the association is not spurious. Some studies suggest mandatory B vitamin fortification, resulting in excess intake, has contributed to the obesity epidemic.[104-106] In support of this hypothesis, The National Academies *Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline* has reported that there is some evidence that the B vitamin niacin, when used to treat patients with hypercholesterolemia, can cause impaired glucose tolerance.[107] Additional research in large prospective cohorts is needed to better understand this relationship.

As with OCM nutrients, twelve OCM SNPs in seven genes were selected based on their role in OCM as outlined in Figure 2, as well as their availability in the MetaboChip illumina, a custom genotyping array of over 200,000 SNPs related to metabolic, cardiovascular and anthropometric traits.[76] In our study, *MTR* was the only gene with a SNP significantly associated with arsenic metabolism. One or two of the variant alleles was associated with higher MMA% and non-significantly associated with higher iAs% and lower DMA% compared to the wild-type genotype, among participants free of baseline metabolic syndrome and diabetes. This is consistent with a previous study conducted in Argentina which found the variant genotype of a different *MTR* SNP

to be significantly associated with arsenic metabolism in the same direction (higher MMA% and lower DMA%).[56] *MTR* plays a key role in OCM by catalyzing the remethylation of homocysteine to methionine and variants in the gene have been associated with differences in homocysteine levels.[108] The *MTR* SNP in our study was associated with lower HOMA2-IR over 5 years of follow-up and with non-significant decreases in all three other diabetes-related outcomes. Consistently, a common *MTR* SNP, A2756G, has also been associated with diabetes-related outcomes, including obesity[39] and hypertriglyceridemia.[40] Because the *MTR* rs10495387 SNP was associated with both higher MMA% and reduced risk for diabetes-related outcomes, it served as an ideal OCM variable to explore whether OCM influences the association between arsenic metabolism and diabetes-related outcomes. However, adjusting for *MTR* rs10495387 did not affect any of these previously reported associations. Adjustment for other OCM SNPs as well as OCM nutrients also did not affect any of the previously reported associations, suggesting that in this study population, our measures of OCM were not confounding the relationship between arsenic metabolism and diabetes-related outcomes. In contrast, when we adjusted models evaluating the association between *MTR* rs10495387 and diabetes-related outcomes for MMA% and ∑As, all associations were attenuated and reversed direction for waist circumference, providing some evidence MMA% may serve as a partial mediator in the association between OCM and diabetes-related outcomes. Formal mediation analyses are needed to confirm these findings, such as use of structural equation models to create latent variables for OCM, as a single OCM variable cannot fully represent the pathway functioning. Further, utilizing Mendelian randomization would provide enhanced understanding of the role OCM SNPs play in these complex

intersecting relationships. Finally, we cannot discount the possibility of reverse causality in the observed associations between arsenic metabolism and diabetes-related outcomes. Studies with multiple urine arsenic measurements are needed to understand the effect metabolic state may have on arsenic metabolism.

This study was limited by a reduced sample size for metabolic syndrome analyses due high prevalence of baseline metabolic syndrome. Our evaluation of dietary OCM intake was limited by lack of measurement of choline, methionine and vitamin B_{12}, all important nutrients in OCM functioning that were not available in the SHFS. Further, despite the strength of FFQ data in its ability to provide OCM nutrient estimates not affected by metabolic state, FFQs are a self-reported dietary assessment tool that is associated with underestimates of intake, particularly energy and proteins.[109, 110] Assessment of nutritional biomarkers and OCM metabolites as a third measure of OCM function would have enhanced analyses and study interpretations. Finally, multiple urine arsenic measurements would have allowed for better evaluation of direction of associations.

CONCLUSIONS

Our study identified vitamin B_6 intake as a strong predictor of development of diabetes-related outcomes, a finding that remained consistent in multiple sensitivity analyses. Additional research is needed to better understand these results and determine whether chronic high intake of vitamin B_6 has the potential to disturb metabolic activity. We also identified a SNP in the *MTR* gene that was associated with both arsenic metabolism and HOMA2-IR. The association between this SNP and diabetes-related

outcomes were attenuated after adjustment for arsenic metabolism. Additional research is needed to confirm directions of association; however, these findings add to the evidence that a complex and interconnected relationship exists between arsenic metabolism, OCM and diabetes-related outcomes.

TABLES

Table 1. Baseline Participant Characteristics of American Indians in the Strong Heart Family Study by Incident Metabolic Syndrome and Diabetes Status at Follow-up, 1998-2009

	No MetS at Follow-up	MetS at Follow-up	P-Value	No Diabetes at Follow-up	Diabetes at Follow-up	P-Value
Total	656 (70.2)	279 (29.8)		1314 (88.7)	167 (11.3)	
Age (years)	28.6 (19.4, 41)	33.7 (24.9, 44.1)	<0.001	34.3 (22.4, 45.8)	39.5 (29.6, 50.9)	<0.001
Sex						
Female	285 (43.4)	114 (40.9)	0.51	540 (41.1)	66 (39.5)	0.76
Male	371 (56.6)	165 (59.1)		774 (58.9)	101 (60.5)	
Education						
<12 years	171 (26.1)	70 (25.1)	0.82	321 (24.4)	47 (28.1)	0.34
12+ years	485 (73.9)	209 (74.9)		993 (75.6)	120 (71.9)	
Smoking Status						
Never	282 (43)	120 (43)	0.94	534 (40.6)	65 (38.9)	0.77
Ever	119 (18.1)	53 (19)		268 (20.4)	38 (22.8)	
Current	255 (38.9)	106 (38)		512 (39)	64 (38.3)	
Alcohol Intake						
Never	85 (13)	27 (9.7)	0.21	141 (10.7)	20 (12)	0.08
Ever	137 (20.9)	69 (24.7)		327 (24.9)	54 (32.3)	
Current	434 (66.2)	183 (65.6)		846 (64.4)	93 (55.7)	
Caloric Intake	2228.1 (1537, 3470)	2271 (1426, 3252)	0.32	2215 (1511, 3347)	2373 (1561, 3348)	0.99
Vitamin B_6						
Intake (mg)	1.7 (1.1, 2.8)	1.7 (1.1, 2.5)	0.40	1.7 (1.1-2.7)	1.9 (1.2-2.7)	0.39
Supplement Use	149 (22.7)	77 (27.6)	0.13	319 (24.3)	39 (23.4)	0.87
Vitamin B_2						
Intake (mg)	1.7 (1.1, 2.6)	1.6 (1.1, 2.5)	0.85	1.6 (1.1-2.6)	1.9 (1.1-2.7)	0.22
Supplement Use	149 (22.7)	77 (27.6)	0.13	319 (24.3)	39 (23.4)	0.87
Folate						
Intake (μg)	356 (226, 550)	355 (222, 514)	0.49	355 (231, 550)	412 (248, 610)	0.20
Supplement Use	160 (24.4)	85 (30.5)	0.06	345 (26.3)	42 (25.1)	0.83
∑As (μg/L)	4.4 (2.9, 7.1)	4.7 (3.1, 7.5)	0.21	4.5 (3.0, 7.3)	5.3 (3.4, 9.4)	0.003
iAs%	10.9 (7.5, 15.5)	9.9 (7.3, 13.5)	0.04	10.3 (7.1, 14.2)	9.1 (6.7, 13.3)	0.05
MMA%	15.9 (12.6, 19.9)	14 (10.9, 17.8)	<0.001	14.8 (11.3, 18.4)	13.1 (10.4, 16.8)	<0.001
DMA%	72 (65.5, 78.5)	74.6 (68.9, 80.8)	<0.001	74.2 (67.3-80.4)	77.5 (70.7-82)	0.001
BMI (kg/m^2)						

Normal	276 (42.1)	46 (16.5)	<0.001	338 (25.7)	9 (5.4)	<0.001
Overweight	205 (31.2)	92 (33)		389 (29.6)	28 (16.8)	
Obese	175 (26.7)	141 (50.5)		587 (44.7)	130 (77.8)	
HOMA2-IR	1.1 (0.8, 1.6)	1.5 (1.0-2.2)	<0.001	1.3 (0.9-2.1)	2.3 (1.5-3.7)	<0.001
Waist Circumference (cm)	90 (80, 99)	99 (91, 111)	<0.001	97 (87, 109)	111 (100, 123)	<0.001

Abbreviations: ∑As (iAs+MMA+DMA); BMI (body mass index); dimethylarsinic acid (DMA); eGFR (estimated glomerular filtration rate); HOMA2-IR (Homeostatic Model Assessment of Insulin Resistance); inorganic arsenic (iAs); IQR (interquartile range); metabolic syndrome (MetS); monomethylarsonic acid (MMA)

Table 2. Mean Difference in Arsenic Metabolism by IQR Increase in One Carbon Metabolism-Related Nutrient Intake (N=935)

Nutrient[a]	Model 1[b]	Model 2[c]	Model 3[d]
		iAs%	
Vitamin B_6	0.06 (-0.50 0.61)	0.05 (-0.48 0.59)	0.16 (-0.36 0.68)
Vitamin B_2	0.23 (-0.32 0.78)	0.07 (-0.48 0.61)	0.15 (-0.41 0.70)
Folate	-0.52 (-0.98 -0.06)	-0.21 (-0.65 0.24)	-0.12 (-0.56 0.33)
		MMA%	
Vitamin B_6	-0.03 (-0.52 0.46)	-0.09 (-0.56 0.38)	-0.08 (-0.53 0.37)
Vitamin B_2	0.47 (-0.01 0.94)	0.26 (-0.20 0.72)	0.26 (-0.19 0.70)
Folate	-0.43 (-0.85 0.00)	-0.15 (-0.56 0.25)	-0.14 (-0.52 0.24)
		DMA%	
Vitamin B_6	-0.03 (-0.92 0.87)	0.04 (-0.80 0.88)	-0.08 (-0.89 0.73)
Vitamin B_2	-0.70 (-1.55 0.16)	-0.33 (-1.16 0.51)	-0.40 (-1.23 0.42)
Folate	0.95 (0.19 1.70)	0.36 (-0.35 1.07)	0.26 (-0.43 0.94)

[a]Nutrient intake is calorie-corrected using a residual analysis method
[b]Model 1: adjusted for log creatine-adjusted urinary total arsenic
[b]Model 2: Model 1 adjustments plus age, sex, center
[d]Model 3: Model 2 adjustments plus education, kidney function, smoking status, drinking status, BMI

Table 3. Relationship Between One Carbon Metabolism-Related Single Nucleotide Polymorphisms and Arsenic Metabolism (N=935)

Variable	iAs%	MMA%	DMA%
CBS			
AA	0	0	0
AG	0.15 (-0.7, 0.99)	-0.18 (-0.89, 0.52)	0.04 (-1.28, 1.36)
GG	-0.42 (-1.62, 0.79)	-0.29 (-1.23, 0.66)	0.70 (-1.12, 2.52)
SHMT1 (rs12952556)			
AA	0	0	0
AG	0.42 (-0.51, 1.36)	0.03 (-0.67, 0.72)	-0.45 (-1.75, 0.84)
GG	0.80 (-1.63, 3.23)	1.54 (-0.64, 3.73)	-2.35 (-6.56, 1.87)
SHMT1 (rs2273027)			
AA	0	0	0
AG	0.51 (-0.42, 1.45)	-0.21 (-1.04, 0.62)	-0.31 (-1.82, 1.21)
GG	0.00 (-1.18, 1.19)	-0.33 (-1.31, 0.64)	0.33 (-1.50, 2.15)
PEMT			
GG	0	0	0
GA	-0.44 (-1.35, 0.47)	0.18 (-0.56, 0.92)	0.26 (-1.13, 1.65)
AA	-0.47 (-1.89, 0.94)	0.08 (-0.99, 1.15)	0.39 (-1.74, 2.52)
MTRR (rs3776464)			
AA	0	0	0
AT	0.13 (-0.79, 1.06)	0.25 (-0.43, 0.93)	-0.38 (-1.75, 0.98)
TT	0.01 (-1.72, 1.75)	0.25 (-1.29, 1.79)	-0.26 (-3.09, 2.57)
MTRR (rs16879334)			
CC	0	0	0
CG	0.13 (-0.79, 1.06)	0.28 (-0.41, 0.96)	-0.41 (-1.78, 0.95)
GG	0.01 (-1.72, 1.75)	0.26 (-1.28, 1.80)	-0.27 (-3.10, 2.55)
MTHFR (1801131)			
CC	0	0	0
CT	0.07 (-0.85, 0.98)	-0.28 (-1.02, 0.46)	0.21 (-1.17, 1.60)
TT	-1 (-2.84, 0.85)	-0.97 (-2.46, 0.52)	1.97 (-0.46, 4.39)
MTHFR (1801133)			
AA	0	0	0
AC	0.07 (-0.73, 0.87)	0.42 (-0.29, 1.13)	-0.49 (-1.79, 0.81)
CC	1.18 (-0.62, 2.98)	1.02 (-0.42, 2.46)	-2.20 (-4.82, 0.43)
MTHFR (2274976)			
GG	0	0	0
GA/AA	-0.89 (-2.03, 0.25)	-0.75 (-1.68, 0.18)	1.64 (-0.10, 3.39)
MTHFD1 (rs3818239)			
AA	0	0	0
AG/GG	-1.08 (-2.32, 0.15)	-0.40 (-1.42, 0.62)	1.48 (-0.38, 3.34)
MTHFD1 (rs17751556)			
AA	0	0	0
AG	-0.32 (-2.00, 1.35)	0.65 (-0.76, 2.06)	-0.32 (-3.05, 2.40)
MTR			
CC	0	0	0
CA/AA	1.51 (-0.64, 3.66)	2.57 (0.22, 4.92)	-4.08 (-8.32, 0.17)

Models adjusted for age, sex, ∑As, 4 genetic PCs

Table 4. Association between One Carbon Metabolism-Related Nutrient Intake and Diabetes-related Outcomes

Variable	Relative Risk for Metabolic Syndrome (n=935)[a]	Mean Difference in Waist Circumference (N=1458)[a]	Relative Risk for Diabetes (n=1458)[a]	Geometric Mean Ratio for HOMA2-IR (n=1458)[a]
Vitamin B_6[b]				
<1.2	1 (Reference)	(Reference)	1 (Reference)	1 (Reference)
1.2 – <1.8	1.17 (0.89 1.53)	1.18 (-1.21, 3.57)	1.28 (0.82 2.00)	1.07 (0.98, 1.17)
1.8 – <2.7	1.08 (0.81 1.43)	1.33 (-1.08, 3.74)	1.00 (0.62 1.63)	0.98 (0.89, 1.07)
≥2.7	1.40 (1.06 1.85)	0.47 (-1.94, 2.88)	1.72 (1.13 2.61)	1.07 (0.98, 1.18)
IQR	1.15 (1.02 1.29)	0.03 (-1.01, 1.07)	1.28 (1.10 1.48)	1.04 (1.00, 1.08)
Vitamin B_2[b]				
<1.2	1 (Reference)	(Reference)	1 (Reference)	1 (Reference)
1.2 – <1.8	1.08 (0.80 1.44)	0.13 (-2.26, 2.51)	1.23 (0.80 1.90)	0.97 (0.88, 1.06)
1.8 – <2.8	1.26 (0.96 1.65)	0.16 (-2.25, 2.57)	1.17 (0.75 1.82)	1.00 (0.91, 1.09)
≥2.8	0.93 (0.69 1.27)	-1.83 (-4.24, 0.58)	1.53 (1.00 2.34)	1.02 (0.93, 1.12)
IQR	1.01 (0.89 1.14)	-0.95 (-2.01, 0.12)	1.21 (1.03 1.41)	1.01 (0.97, 1.05)
Folate[b]				
<234	1 (Reference)	(Reference)	1 (Reference)	1 (Reference)
234 - <355	0.83 (0.63 1.10)	1.48 (-0.89, 3.86)	1.08 (0.71 1.63)	0.95 (0.87, 1.04)
355 - <560	1.03 (0.78 1.35)	2.11 (-0.27, 4.50)	1.35 (0.88 2.05)	1.00 (0.91, 1.09)
≥560	0.97 (0.74 1.26)	-0.70 (-3.13, 1.73)	1.27 (0.83 1.94)	1.05 (0.96, 1.15)
IQR	1.02 (0.90 1.15)	-0.93 (-1.96, 0.11)	1.15 (0.97 1.36)	1.01 (0.97, 1.05)

[a]Models adjusted for age, gender, center, education, BMI, kidney function, smoking status, drinking status
[b]Nutrient intake is calorie-corrected using a residual analysis method

Table 5. Association between One Carbon Metabolism-Related Single Nucleotide Polymorphisms and Diabetes-Related Outcomes

Variable	n	Relative Risk for Metabolic Syndrome (N=935)	n	Mean Difference in Waist Circumference (N=1458)	Relative Risk for Diabetes (N=1458)	Geometric Mean Ratio for HOMA2-IR (N=1458)
Total	935		1458			
CBS						
AA	365	1	558	Reference	1	1
AG	409	1.02 (0.81, 1.29)	658	-0.51 (-2.36, 1.33)	1.10 (0.81, 1.51)	0.99 (0.91, 1.07)
GG	161	1.24 (0.93, 1.66)	242	-3.28 (-5.82, -0.74)	1.03 (0.67, 1.57)	0.99 (0.89, 1.11)
SHMT1 (rs12952556)						
AA	637	1	1002	Reference	1	1
AG	269	1.02 (0.81, 1.28)	410	0.97 (-0.91, 2.86)	1.03 (0.75, 1.43)	1.03 (0.95, 1.12)
GG	29	1.07 (0.59, 1.93)	46	2.08 (-2.72, 6.87)	0.64 (0.22, 1.82)	1.01 (0.82, 1.24)
SHMT1 (rs2273027)						
AA	286	1	454	Reference	1	1
AG	463	0.89 (0.71, 1.11)	732	0.24 (-1.68, 2.16)	1.24 (0.87, 1.78)	1.05 (0.97, 1.14)
GG	186	1.06 (0.79, 1.42)	272	1.19 (-1.33, 3.71)	0.93 (0.57, 1.51)	1.08 (0.96, 1.20)
MTHFD1 (rs3818239)						
AA	838	1	1311	Reference	1	1
AG/GG	77	1.00 (0.73,1.37)	147	0.95 (-1.85, 3.75)	0.94 (0.50, 1.76)	1.03 (0.91, 1.16)
PEMT						
GG	360	1	584	Reference	1	1
GA	452	1.12 (0.88, 1.43)	681	1.92 (0.13, 3.71)	1.07 (0.80, 1.44)	1.00 (0.92, 1.08)
AA	123	1.37 (1.02, 1.85)	193	2.51 (-0.14, 5.17)	1.21 (0.80, 1.84)	1.01 (0.90, 1.14)
MTRR (rs3776464)						
AA	513	1	775	Reference	1	1
AT	358	0.96 (0.78, 1.19)	579	-0.09 (-1.91, 1.74)	1.11 (0.79, 1.55)	1.01 (0.93, 1.09)
TT	64	0.86 (0.54, 1.36)	104	-0.43 (-3.89, 3.04)	1.13 (0.61, 2.09)	0.90 (0.77, 1.04)
MTRR (rs16879334)						
CC	514	1	776	Reference	1	1
CG	357	0.97 (0.79, 1.19)	578	-0.22 (-2.05, 1.60)	1.11 (0.79, 1.56)	1.01 (0.93, 1.09)
GG	64	0.86 (0.54, 1.36)	104	-0.50 (-3.96, 2.96)	1.13 (0.61, 2.09)	0.90 (0.77, 1.04)
MTHFR (rs1801131)						
CC	586	1	943	Reference	1	1
CT	306	0.99 (0.80, 1.24)	449	0.73 (-1.14, 2.59)	0.80 (0.57, 1.13)	1.01 (0.93, 1.09)

TT	43	0.71 (0.37, 1.37)	66	2.03 (-2.16, 6.22)	0.72 (0.28, 1.80)	1.12 (0.93, 1.34)
MTHFR (rs1801133)						
AA	473	1	748	Reference	1	1
AC	394	1.08 (0.89, 1.33)	599	-1.03 (-2.79, 0.73)	0.94 (0.69, 1.28)	0.95 (0.88, 1.03)
CC	68	0.87 (0.56, 1.34)	111	-2.34 (-5.61, 0.93)	0.83 (0.47, 1.46)	0.98 (0.85, 1.13)
MTHFR (rs2274976)						
GG	790	1	1235	Reference	1	1
GA/AA	145	1.07 (0.82, 1.40)	223	1.21 (-1.11, 3.52)	0.91 (0.60, 1.37)	1.08 (0.98, 1.20)
MTHFD1 (rs17751556)						
AA	870	1	1362	Reference	1	1
AG/GG	97	0.68 (0.40, 1.17)	96	-1.90 (-5.33, 1.53)	0.91 (0.48, 1.70)	0.94 (0.81, 1.09)
MTR						
CC	892	1	1390	Reference	1	1
CA/AA	43	0.84 (0.54, 1.32)	68	-0.03 (-4.01, 3.94)	0.67 (0.30, 1.50)	0.79 (0.66, 0.93)

Models adjusted for age, sex and 4 population stratification PCs

Table 6. Association Between *MTR* and Diabetes Related Outcomes Before and After Adjustment for Arsenic Metabolism

Model	Relative Risk for Diabetes (N=1458)	Geometric Mean Ratio for HOMA2-IR (N=1458)	Mean Difference in Waist Circumference (N=1458)	Relative Risk for Metabolic Syndrome (N=935)
Fully Adjusted Model				
MTR				
CC	1.00 (Reference)	1.00 (Reference)	0.00 (Reference)	1.00 (Reference)
CA/AA	0.67 (0.30, 1.50)	0.79 (0.66, 0.93)	-0.03 (-4.01, 3.94)	0.84 (0.54, 1.32)
Fully Adjusted Model + MMA% & ∑As				
MTR				
CC	1.00 (Reference)	1.00 (Reference)	0.00 (Reference)	1.00 (Reference)
CA/AA	0.70 (0.31, 1.60)	0.83 (0.71, 0.99)	1.35 (-2.51, 5.21)	0.93 (0.58, 1.48)

All models adjusted for age, sex and 4 population stratification PCs

FIGURES

Figure 1. Study Flow Diagram

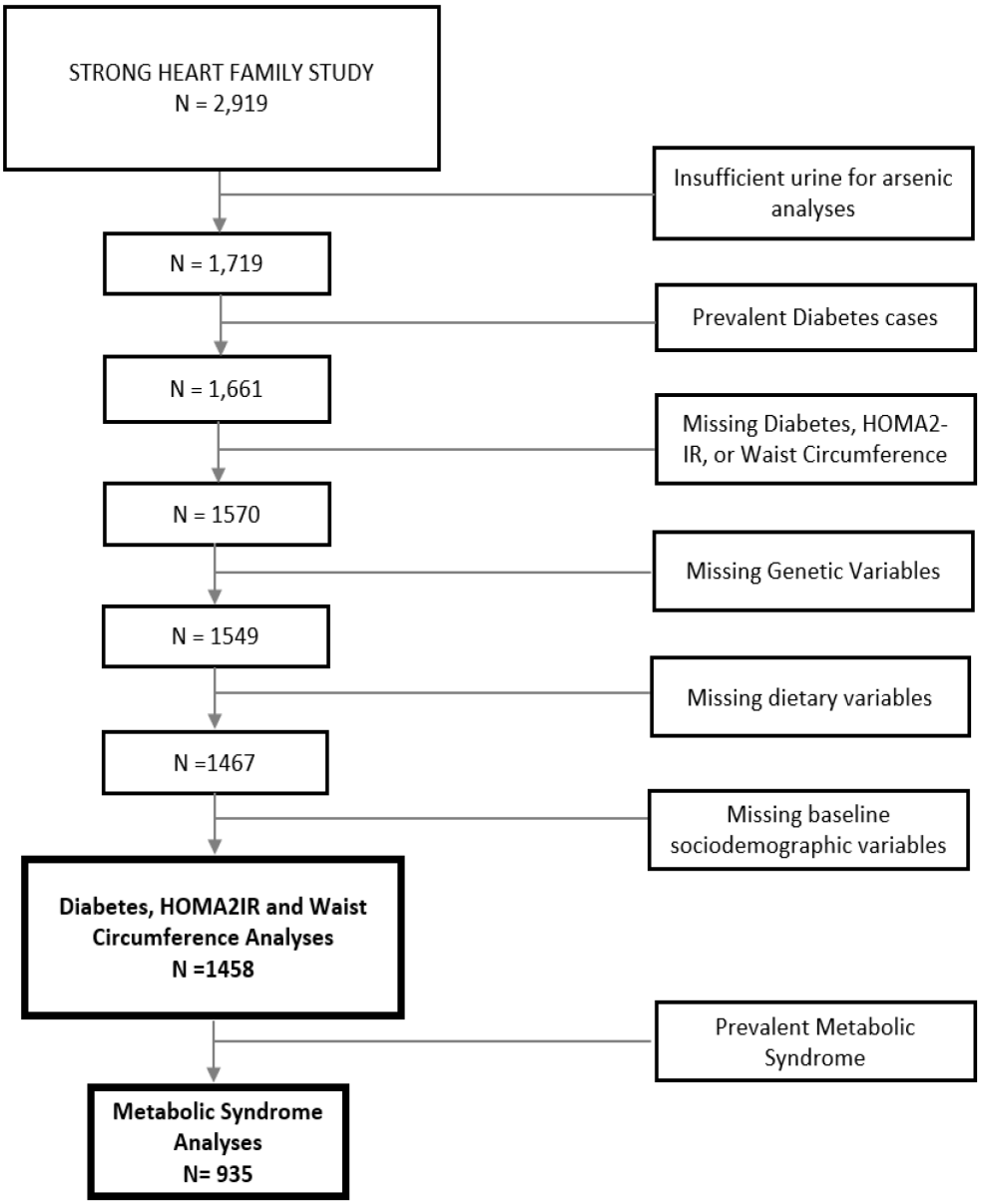
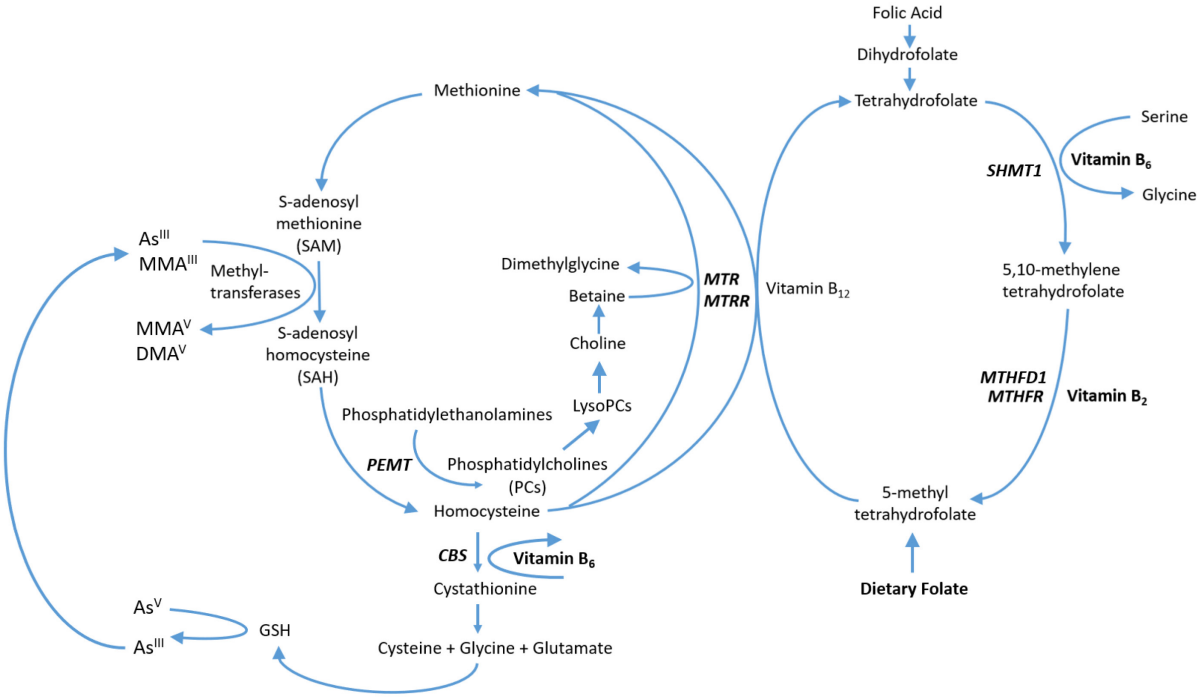
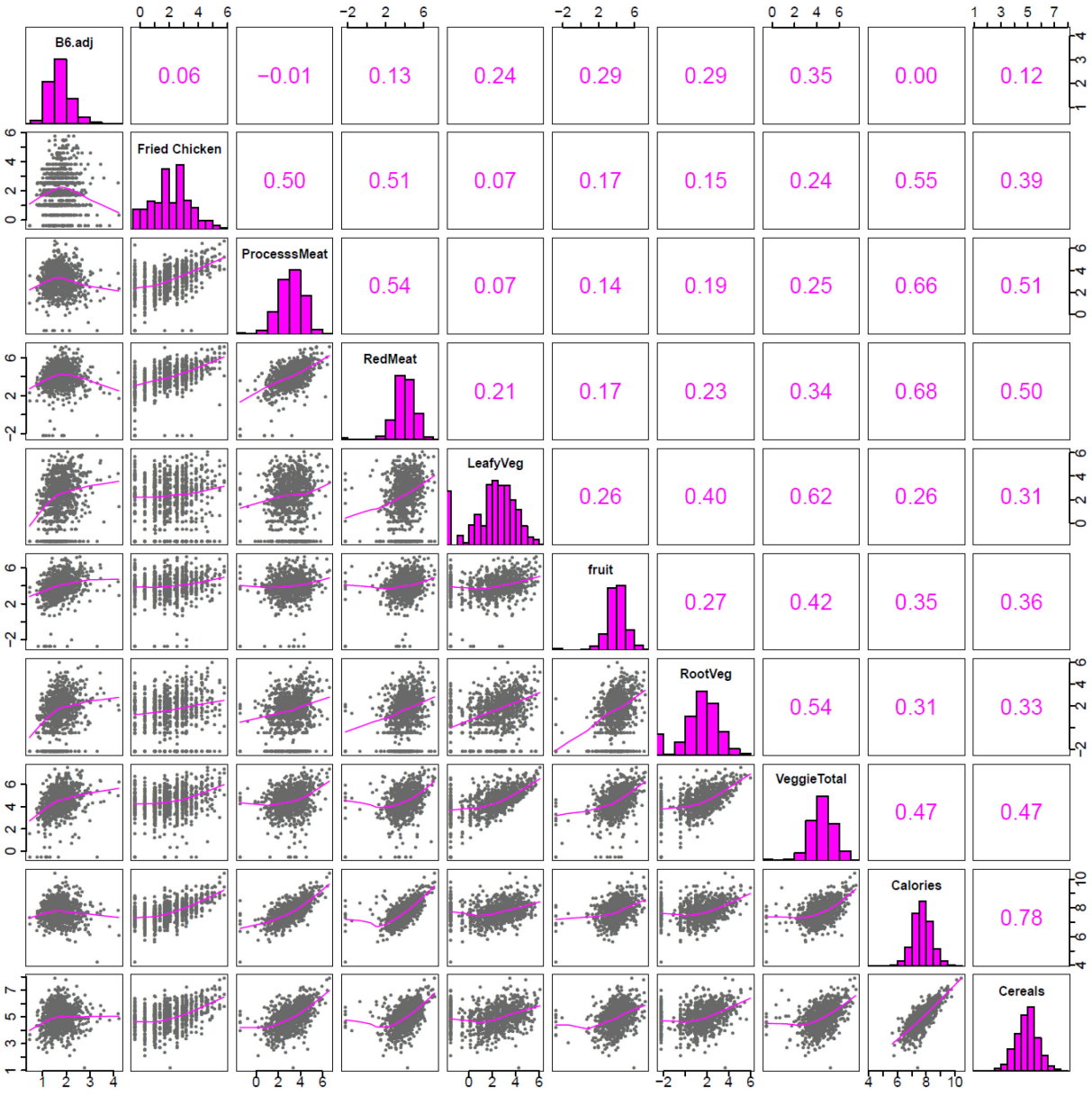


Figure 2. Role of Study's One Carbon Metabolism Nutrients and Genes in One Carbon Metabolism Pathway. One carbon metabolism-related nutrients are bolded. One carbon metabolism-related genes are bolded and italicized.



Supplemental Figure 1. Spearman Correlations Between Intake of Calorie-Corrected Vitamin B_6 and Food Groups



DISCUSSION

Summary of Findings

An arsenic metabolism profile reflecting higher percentages of DMA and lower percentages of MMA, characterized as more efficient, has been associated with reduced risk for numerous arsenic-related adverse health outcomes, including cardiovascular disease, skin lesions and cancer. [1-8] Conversely, this profile, has also been associated with increased risk for diabetes-related outcomes.[9-14] The mechanism behind this contrasting relationship in not clear. Evidence suggests one carbon metabolism (OCM) plays an important role in both arsenic metabolism[15-21] and diabetes-related outcomes,[22-31] providing one potential mechanistic link. This dissertation attempted to better characterize these intersecting relationships and understand the influence of OCM on the unique and abstruse association between arsenic metabolism and diabetes-related outcomes.

In Chapter 1, we conducted a cross-sectional analysis evaluating the association of dietary intake of OCM nutrients (folate and vitamins B_2, B_6 and B_{12}) with urinary arsenic methylation patterns (iAs%, MMA% and DMA%) in a subset (n=405) of participants from the Strong Heart Study (SHS) a population-based cohort of American Indian adult men and women aged 45-74. Participants were recruited pre-mandatory folic acid fortification (1989-1991) and exposed to low-moderate levels of inorganic arsenic from drinking water and food. In general, higher intake of B vitamins, in particular B_2 and B_6, was associated with lower percentages of iAs and MMA and higher percentages of DMA, a profile suggested to reflect enhanced arsenic metabolism. These associations persisted for vitamins B_2 and B_6 after adjustment for sociodemographic factors, smoking,

alcohol intake, BMI, and kidney function, as well as all one carbon metabolism (OCM) nutrients. In joint analyses, an antagonistic association was found between folate and vitamin B_6, with higher folate being associated with higher DMA% and lower iAs% only in the presence of high vitamin B_6.

In Chapter 2, we conducted a prospective analysis evaluating the effect of arsenic exposure and arsenic metabolism on incident metabolic syndrome and each of its individual components (elevated waist circumference, elevated triglycerides, reduced HDL, hypertension and elevated fasting plasma glucose (FPG)). We evaluated these relationships in 1,047 participants free of prevalent metabolic syndrome or diabetes from the Strong Heart Family Study (SHFS) which included American Indian men and women, aged ≥14 years from Arizona, Oklahoma and North/South Dakota recruited for baseline visits in 1998-1999 and 2001-2003 with follow-ups in 2001-2003 and 2006-2009. Arsenic exposure was associated with increased risk for elevated FPG but not with metabolic syndrome or other individual components. Arsenic metabolism patterns, independent of arsenic exposure, were associated with both incident metabolic syndrome and elevated waist circumference, but not with other components of the syndrome. The relative percentages of MMA appeared to be the main driver behind these associations. The distinct and independent associations between arsenic exposure and arsenic metabolism with metabolic syndrome and its individual components suggest these are unrelated phenomena that could be contributing to overall diabetes risk at low-levels of arsenic exposure. For arsenic exposure, the association appears to be predominately with hyperglycemia. For arsenic metabolism, the association appears to be mainly with central adiposity. The possible effect of arsenic exposure on hyperglycemia and of arsenic

metabolism on central adiposity, potentially through insulin resistance, could be underlying mechanisms for the observed associations between arsenic and diabetes in multiple populations.

In Chapter 3, we conducted a cross-sectional analysis using data from a pilot targeted metabolomic study (n=59) in the SHFS. We observed significant correlations between eight metabolites (S-adenosylmethionine, S-adenosylhomocysteine, cysteine, glutamate, lysophosphatidylcholine (LPC) 18:2, phosphatidylcholine (PC) ae 34:3, PC ae 40:6 and PC aa 38:3) and both a diabetes-related outcome (HOMA2-IR, FPG, waist circumference) and at least one arsenic metabolism biomarker (iAs%, MMA% or DMA%). These eight metabolites of interest all play a role in the OCM pathway. Before adjustment, higher MMA% was associated with lower HOMA2-IR and waist circumference, and higher DMA% was associated with higher HOMA2-IR and waist circumference. After adjustment for the eight OCM-related metabolites, associations between arsenic metabolism and diabetes-related outcomes were substantially attenuated and no longer significant. Of the eight OCM-related metabolites, glutamate, LPC 18:2 and PC ae 40:6 remained statistically significantly associated with waist circumference even after adjustment for other metabolites and for arsenic metabolism biomarkers. Together, our results suggest that these metabolites play a key role in the relationship between arsenic metabolism and diabetes-related outcomes and may explain the difference in association seen between arsenic metabolism and diabetes-related outcomes compared to the associations reported for arsenic metabolism and other health outcomes. Further, these findings support our hypothesis that OCM is an important link in the relationship between arsenic metabolism and diabetes-related outcomes.

In Chapter 4, we conducted multiple analyses using data from the SHFS, in order to better understand the role OCM status plays in arsenic metabolism, diabetes-related outcomes and the relationship between the two. We first conducted a cross-sectional analysis among participants free of prevalent metabolic syndrome and diabetes evaluating the association between intake of OCM nutrients (folate, vitamin B_6 and vitamin B_2) and arsenic metabolism (iAs%, MMA% and DMA%). We also ran these analyses evaluating the associations between OCM-related genetic variants and arsenic metabolism. Next, we evaluated the associations between both OCM nutrients and OCM-related genetic variants with diabetes, metabolic syndrome, waist circumference and HOMA2-IR. OCM nutrients were not associated with arsenic metabolism, however, higher vitamin B_6 was consistently associated with three of the four diabetes-related outcomes studied (higher HOMA2-IR and increased risk for diabetes and metabolic syndrome). Adjustment for arsenic metabolism in these models did not affect the associations between vitamin B_6 and diabetes-related outcomes. Further, previously reported associations between arsenic metabolism and diabetes-related outcomes were not affected by adjustment for any of our OCM (nutrient or genetic) variables. However, the *MTR* rs10495387 genetic variant was associated with both higher MMA% and lower HOMA2-IR per 5 years of follow-up. After adjustment for MMA%, the association between the *MTR* variant and all diabetes-related outcomes were attenuated or reversed direction, suggesting MMA% may be a partial mediator in the association between this OCM genetic variant and diabetes-related outcomes, again supporting our hypothesis that OCM is an important component in the relationship between arsenic metabolism and diabetes-related outcomes.

Unexpected Findings

Our finding of a significant association between higher vitamin B_6 and increased risk for several diabetes-related outcomes in Chapter 4 was unexpected. Low OCM status has generally been associated with adverse health outcomes, including diabetes, so we anticipated the same to be true with our diabetes-related outcomes in this study.[22-24, 32-38] We conducted several sensitivity analyses to try to determine if any food patterns were confounding the association, with consistent results. It is possible the previously reported associations between low OCM and adverse health outcomes was a result of reverse causality. Animal models have shown diabetes can alter OCM functioning and metabolite levels, including reductions in vitamin B_6.[39, 40] This could explain the difference in findings, as metabolic state would not necessarily affect intake. It is also possible there is a U-shaped relationship between OCM nutrients and health, particularly in regard to metabolic health, with adverse effects seen at very low and very high levels. Indeed, some studies have suggested mandatory B vitamin fortification, resulting in excess intake, has contributed to the obesity epidemic.[41-43] Additional research in large prospective cohorts is needed to better understand this relationship and determine whether excess OCM intake has potential adverse effects in certain populations.

Our lack of association between OCM nutrient intake and arsenic metabolism in Chapter 4 was also unexpected and contrasted with our findings from Chapter 1, where we observed significant associations between increasing vitamin B_6 and B_2 intake with lower MMA% and higher DMA%. It is possible this difference in finding is due to underlying differences in the health and behavior of the two populations evaluated. The SHS (the population evaluated in Chapter 1) was older (aged 45-74), likely had different

dietary patterns than participants in the SHFS (the population evaluated in Chapter 4, which were recruited at least 10 years after the SHS participants), and reported OCM intake markedly lower (also due to the SHS intake measured prior to mandatory folic acid fortification) than the SHFS (see **Table 1**). Indeed, other studies have observed significant associations between OCM nutrients and arsenic metabolism only in populations deficient in that nutrient.[17, 21] It is also possible the food frequency questionnaire was able to more accurately ascertain true OCM intake in the SHS due to participants likely having more traditional diets that were less variable than those in the SHFS, which could have led to greater measurement error in the SHFS, in turn biasing results to the null.

Finally, in Chapter 2 we observed a significant association between fasting plasma glucose and arsenic exposure but not with arsenic metabolism. Further, we observed a significant association between metabolic syndrome and waist circumference with arsenic metabolism but not with arsenic exposure. We had expected to see significant associations between both arsenic exposure and arsenic metabolism with these outcomes. Instead, the distinct and independent associations between arsenic exposure and arsenic metabolism with metabolic syndrome and its individual components suggest these are unrelated phenomena that could be contributing to overall diabetes risk at low-levels of arsenic exposure.

Table 1. Comparison of OCM intake between the SHS and SHFS

	SHS **N = 405**	**SHFS visit 4** **N = 967**
Vitamin B_{12}	Median: 3.04 (1.74, 5.67) Mean: 4.88 % Supplements: *	Median: * Mean: * % Supplements: 24%
Vitamin B_6	Median: 1.23 (0.75, 1.90) Mean: 1.45 % Supplements: *	Median: 1.70 (1.10, 2.60) Mean: 2.17 % Supplements: 24%
Vitamin B_2	Median: 1.37 (0.89, 1.95) Mean: 1.57 % Supplements: *	Median: 1.70 (1.10, 2.70) Mean: 2.26 % Supplements: 24%
Folate	Median: 207.6 (120.4, 336.7) Mean: 262.2 % Supplement: 4%	Median: 355.4 (225.2, 548.0) Mean:473.8 % Supplements: 26%
Calories	Median:1602 (1090, 2134) Mean: 1743	Median: 2235 (1504, 3368) Mean: 2846

*Data not available

Strengths and Limitations

This dissertation benefitted from several strengths. The SHS and SHFS are well-established cohorts with high-quality laboratory methods, high participant retention, consistent variable collection, careful outcome determination and strong support from the communities involved. Further, we were able to employ a multi-faceted approach in our evaluation of two of our main variables of interest, metabolic state and OCM status. Metabolic state was measured through multiple diabetes-related outcomes, including diabetes, HOMA2-IR, metabolic syndrome, waist circumference, triglycerides, HDL, hypertension and fasting plasma glucose. Multiple measurements of metabolic state allowed for enhanced understanding of specific mechanistic pathways behind observed associations. OCM status was measured through B vitamin intake, OCM-related

metabolites and OCM-related genes. Multiple measurements of OCM status enabled improved interpretation of direction of associations. In addition, leveraging evaluation of OCM status and arsenic metabolism in two distinct cohorts, provided the ability to better understand how a population's nutritional baseline may affect associations. Finally, the prospective nature of the cohort allowed for evaluation of incident outcomes or changes in outcomes over time leading to more robust conclusions in regard to directions of association.

As in all epidemiological studies, several limitations affected the results and interpretations of this thesis. Our evaluation of OCM intake in both Chapter 1 and Chapter 4 was not comprehensive. Important missing nutrients included methionine, choline and betaine in Chapters 1 and 4, in addition to vitamin B_{12} in Chapter 4. Inclusion of these nutrients would have provided a fuller picture of OCM status. Further, dietary folate equivalents, which incorporate both folic acid and dietary folate, were not available for our analyses, likely leading to underestimates of folate intake. In addition, our evaluation of OCM metabolites was limited to a very small subset of the SHFS, making broad conclusions from these results difficult and prohibiting formal mediation analyses. We also were unable to evaluate any biomarkers of OCM status in Chapters 1 and 4. Although, the prospective design was a clear strength, longer follow-up time would have made these analyses and their interpretations stronger. Finally, while urine arsenic metabolites have been shown to be consistent over time,[44] we cannot discount the potential for some changes over time that may be related to our other variables of interest and which may have impacted findings. Urine arsenic collected at multiple visits would have made our results more robust.

Implications and Future Research

One important finding from our evaluation of the association between OCM nutrient intake and arsenic metabolism in two populations (SHS and SHFS), was that the effect nutrient intake has on arsenic metabolism may be specific to the underlying nutritional status of that population. This is critical information as it suggests supplementation trials, such as those that have successfully enhanced arsenic metabolism and lowered blood arsenic in Bangladeshi populations, may have no effect in more OCM sufficient populations, as in regions with prevalent vitamin fortification of food products.

Our finding regarding the significant association between arsenic exposure and elevated fasting plasma glucose adds evidence to the diabetogenic effects of inorganic arsenic even at low levels of exposure, and supports a mechanism of hyperglycemia as opposed to increased adiposity. Studies evaluating the effect of low level arsenic exposure are critical to assist in accurately characterizing the total health risk posed by this ubiquitous toxicant, particularly as countries are reassessing exposure level standards in both food and drinking water. As the diabetes epidemic continues to expand, identifying and preventing the contribution of modifiable risk factors, such as environmental exposures like inorganic arsenic, is essential.

Our unexpected finding between increasing vitamin B_6 intake and increased risk for diabetes-related outcomes is intriguing and warrants further research to better understand this relationship. Longitudinal studies, with data on both intake and biomarkers of B vitamins, as well as participants with a broad spectrum of B vitamin

values to allow for stratified analyses at both low and high extremes of values would provide a better understanding of potential risks to inappropriate B vitamin status. Further, experimental studies are needed to help explain potential mechanisms behind the association.

Finally, this dissertation provides novel evidence that OCM status may, at least in part, explain the association between arsenic metabolism and diabetes-related outcomes. Arsenic metabolism has been identified as a risk factor for diabetes-related outcomes; if this is true it should be considered in any future trials intended to enhance arsenic metabolism. Additional efforts are needed to determine if this is a true association or if it is instead an artifact due to confounding by OCM, as our findings suggest, or reverse causality. Larger, longitudinal metabolomic studies of OCM-related metabolites that allow for mediation analyses are essential to fully disentangle the complex relationship between OCM, arsenic metabolism and diabetes-related outcomes.

Conclusions

Diabetes is among the leading causes of death worldwide, making identification of modifiable risk factors as well as high risk populations, a public health priority. Previous studies have reported arsenic exposure and arsenic metabolism to be risk factors for diabetes and related outcomes; however, the arsenic metabolism profile indicating increased risk for diabetes is also associated with reduced risk for other arsenic-related health outcomes. The mechanism behind these contrasting associations is equivocal. This dissertation adds evidence that arsenic is associated with hyperglycemia, even at low

levels of exposure, and that arsenic metabolism is associated with increased risk for metabolic syndrome and elevated waist circumference (two outcomes understudied in regard to arsenic) in the same direction as other diabetes-related outcomes. Further, we present novel support for the hypothesis that the association reported between arsenic metabolism and diabetes-related outcomes may be influenced by OCM status, providing incentive for additional research to better understand these intersecting relationships.

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Specific Aims

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Chapter 1

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Chapter 4

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Discussion

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CURRICULUM VITAE

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EDUCATION

PhD	Johns Hopkins Bloomberg School of Public Health Environmental Health and Engineering Thesis Advisor: Dr. Ana Navas-Acien	January 2018
MHS	Johns Hopkins Bloomberg School of Public Health Environmental Health Science	May 2007
BA	Johns Hopkins University Public Health	May 2006

RESEARCH AND PROFESSIONAL EXPERIENCE

Research Assistant, Columbia University Mailman School of Public Health, Department of Environmental Health Sciences — 2017 – 2018

- Assisted in Departmental research activities

Graduate Student, Johns Hopkins Bloomberg School of Public Health, Department of Environmental Health and Engineering — 2014 – 2018

- Dissertation: evaluation of the complex interplay between one carbon metabolism, arsenic exposure and metabolism, and diabetes related outcomes

Program Coordinator, Northwell Health, Department of Occupational Medicine, Epidemiology & Prevention — 2010 – 2014

- Involved in employee health program development and evaluation, occupational and environmental health research activities, and coordination of large departmental grant writing and submissions

Water Quality and Health Analyst, New York City Department of Environmental Protection — 2007 – 2010

- Ran the Waterborne Disease Risk Assessment Program (WDRAP), a joint-agency syndromic surveillance program between NYCDEP and NYC Department of Health and Mental Hygiene

Research Assistant, Johns Hopkins Bloomberg School of Public Health — 2006 – 2007

- Assisted in the development and completion of several projects conducted by the Center for Injury Research and Policy

TEACHING EXPERIENCE

Teaching Assistant	Johns Hopkins Bloomberg School of Public Health Introduction to Environmental and Occupational Law	March-June 20
Teaching Assistant	Johns Hopkins Bloomberg School of Public Health Environmental and Occupational Epidemiology	March-June 20

ACADEMIC HONORS, PROFESSIONAL SOCIETIES AND CERTIFICATIONS

Poster Award, Honorable Mention, Environmental Health & Engineering Annual Departmental Retreat, Johns Hopkins Bloomberg School of Public Health	2018
Poster Award, Judges' Choice, Environmental Health & Engineering Annual Departmental Retreat, Johns Hopkins Bloomberg School of Public Health	2017
Healthy Nail Salons Coalition, New York Committee for Occupational Safety & Health	2012-2014
Certification in Risk Assessment and Public Policy, Johns Hopkins Bloomberg School of Public Health	2007
BA awarded with general honors, Johns Hopkins University	2006
Kenneth Yamoaka Music Scholarship, John Jay High School	2002

PEER REVIEW ACTIVITIES

Journal of Exposure Science and Environmental Epidemiology	2017
Environmental Research	2016, 2017
Toxicological Sciences	2015

PEER REVIEWED PUBLICATIONS

Spratlen M, Grau M, Best LG, Yracheta J, Lazo M, Vaidya D, Balakrishnan P, Gamble MV, Francesconi K, Goessler W, Cole S, Umans JG, Howard BV, Navas-Acien A. The Association of Arsenic Exposure and Arsenic Metabolism with the Metabolic Syndrome: Prospective Evidence from the Strong Heart Family Study. American Journal of Epidemiology (*In press*).

Grau-Perez M, Kuo CC, Gribble MO, Balakrishnan P, **Jones Spratlen M**, Vaidya D, Francesconi KA, Goessler W, Guallar E, Silbergeld EK, Umans JG, Best LG, Lee ET, Howard BV, Cole SA, Navas-Acien A. Association of Low-Moderate Arsenic Exposure and Arsenic Metabolism with Incident Diabetes and Insulin Resistance in the Strong Heart Family Study. Environ Health Perspect. 2017 Dec 20;125(12):127004. doi: 10.1289/EHP2566.

Grau-Perez M, Kuo CC, **Spratlen M,** Thayer KA, Mendez MA, Hamman R, Dabelea D, Adgate JL, Knowler WC, Bell RA, Miller FW, Liese AD, Zhang C, Douiller C, Drobna Z, Mayer-Davis E, Styblo M, Navas-Acien A. The Association of Arsenic Exposure and Metabolism With Type 1 and Type 2 Diabetes in Youth: The SEARCH Case-Control Study. *Diabetes Care*. 2017 Jan;40(1):46-53. doi: 10.2337/dc16-0810. Epub 2016 Nov 3.

Spratlen, M, Gamble M.V. Grau, M, Best, LG, Yracheta J, Francesconi K, Goessler W, Umans JG, Howard BV, Navas-Acien A. Association between arsenic metabolism and one-carbon metabolism: evidence from the Strong Heart Study. *Food Chem Toxicol. 2017 Jul;105:387-397.*

OTHER PUBLICATIONS

NYC Dept. of Health and Mental Hygiene & NYC Department of Environmental Protection. (2009). Waterborne Disease Risk Assessment Program: 2008 Annual Report. http://www.nyc.gov/html/dep/pdf/wdrap08.pdf

NYC Dept. of Health and Mental Hygiene & NYC Department of Environmental Protection. (2010). Waterborne Disease Risk Assessment Program: 2009 Annual Report. http://www.nyc.gov/html/dep/pdf/wdrap09.pdf

ONGOING RESEARCH SUPPORT

Title of grant: "Arsenic, metabolism and metabolic syndrome in the Strong Heart Study"
Dates: 07/01/17 – 06/30/20
Sponsoring agency: NIEHS F31 ES027796-01
Principal investigator: Miranda Jones Spratlen
The goal of this study is to gain a better understanding of the mechanisms behind arsenic toxicity and its possible role in the development of the Metabolic Syndrome, as well as important information on a potentially modifiable risk factor for Metabolic Syndrome development, nutrient levels of specific vitamins involved in one-carbon metabolism and their connection with arsenic metabolism.
Role: Principal Investigator

POSTER PRESENTATIONS

Spratlen M, Grau-Perez M, Umans JG, Yracheta J, Best LG, Francesconi K, Goessler W, Bottiglieri T, Gamble MV, Cole SA, Zhao J, Navas-Acien A. Targeted Metabolomics to Understand the Association between Arsenic Metabolism and Diabetes-Related Outcomes: Preliminary Evidence from the Strong Heart Family Study. Environmental Health & Engineering Annual Departmental Retreat, Johns Hopkins Bloomberg School of Public Health. Baltimore, MD. January 2018

Spratlen M, Grau-Perez M, Umans JG, Yracheta J, Best LG, Francesconi K, Goessler W, Bottiglieri T, Gamble MV, Cole SA, Zhao J, Navas-Acien A. Targeted Metabolomics to Understand the Association between Arsenic Metabolism and Diabetes-Related Outcomes: Preliminary Evidence from the Strong Heart Family Study. Superfund Research Program Annual Meeting. Philadelphia, PA. December 2017.

Spratlen M, Grau M, Best LG, Yracheta J, Lazo M, Vaidya D, Balakrishnan P, Gamble MV, Francesconi K, Goessler W, Cole S, Umans JG, Howard BV, Navas-Acien A. The Association of Arsenic Exposure and Arsenic Metabolism with the Metabolic Syndrome: Prospective Evidence from the Strong Heart Family Study. Environmental Health & Engineering Annual Departmental Retreat, Johns Hopkins Bloomberg School of Public Health. Baltimore, MD. January 2017

Spratlen, M, Gamble M.V. Grau, M, Best, LG, Yracheta J, Francesconi K, Goessler, Umans JG, Howard BV, Navas-Acien A. Association between arsenic metabolism and one-carbon metabolism: evidence from the Strong Heart Study. International Society for Environmental Epidemiology Annual Conference. Rome, Italy. August 2016