ASSOCIATIONS BETWEEN INORGANIC ARSENIC EXPOSURE AND THE DEVELOPMENT OF TYPE 2 DIABETES: DIETARY AND GENETIC SUSCEPTIBILITY

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ABSTRACT

Xiaofan Xu: Associations between Inorganic Arsenic Exposure and the Development of Type 2 Diabetes: Dietary and Genetic Susceptibility
(Under the direction of Penny Gordon-Larsen)

Compelling evidence has linked high exposure to inorganic arsenic (iAs) with increased risk of Type 2 diabetes (T2D). There is growing concern that low-to-moderate level of iAs exposure may contribute substantially to the epidemic of T2D. Nevertheless, the results of the current perspective studies are inconsistent, which could be attributable to varied susceptibility due for example to differences in intake of beneficial nutrients and existence of genetic variants of enzymes involved in iAs metabolism.

We capitalized on China Health and Nutrition Survey with measured baseline (i.e. 2009) iAs exposure using toenail; Mg and Zn intake at baseline; fasting glucose and insulin at follow-up (i.e. 2015). Using multivariable adjusted regression models, we investigated the associations between baseline toenail arsenic and T2D incidence and indicators of glucose homeostasis at follow-up. We also examined potential effect modification by Mg and Zn intake at baseline on iAs-associated diabetes. In addition, we determined the gene-environment interaction using data from Mexico.

We found a positive association between baseline iAs exposure and fasting glucose as well as odds of incident T2D. In addition, our findings suggest that instead of insulin resistance, pancreatic β-cell dysfunction is primarily involved in iAs-associated T2D. Moreover, though the association between baseline iAs exposure and pancreatic β-cell dysfunction at follow-up was

stronger among participants with adequate Zn intake, the joint association between iAs exposure and dietary intake of Mg and Zn supports the beneficial effects of adequate Mg and Zn intake. In addition, our findings confirm that several genetic variants of arsenic methyl transferase (*AS3MT*) are in part responsible for the inter-individual differences in iAs metabolism, and roles of the variants may differ among populations with different levels of iAs exposure.

Our study adds to the research on iAs and T2D by determining how, in the population with low-to-moderate iAs exposure, baseline iAs exposure relates to the development of T2D over 6 years. The proposed study also informs efforts to maximize the effectiveness of Mg and Zn intake to combat the diabetogenic effects of iAs and identifies genetically susceptible subgroups due to impaired iAs metabolism.

To my parents and grandparents,
Thank you for your unconditional love and support.
This work would not be possible without you.

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LIST OF ABBREVIATIONS

AS3MT	Arsenic (+3 Oxidation State) Methyltransferase
BMI	Body Mass Index
CHNS	China Health and Nutrition Survey
DMAs	Dimethylated Arsenic
FG	Fasting glucose
FI	Fasting insulin
HbA1c	Hemoglobin A1c
HOMA	Homeostasis model assessment
iAs	Inorganic Arsenic
MAs	Monomethylated Arsenic
Mg	Magnesium
RDA	Recommended Daily Allowance
SNP	Single nucleotide polymorphism
T2D	Type 2 Diabetes
UL	Tolerable upper intake level
Zn	Zinc

CHAPTER 1. INTRODUCTION

Background

The prevalence of type 2 diabetes (T2D), which is a well-recognized cause of premature death and disability, has increased globally over the past two decades, especially in China[1-5]. It has become abundantly clear that both pancreatic β-cell dysfunction and insulin resistance are involved in the development of T2D, but the interplay between pancreatic β-cell dysfunction and insulin resistance remains highly complex and somewhat undefined[6,7]. Though obesity is a well-known risk factor for T2D and is associated with insulin resistance, it has been suggested that obesity alone cannot fully explain the escalating epidemic of T2D, especially in regions where the prevalence of obesity is low[3,8-12]. Recent studies stress on the importance of pancreatic β-cell dysfunction in the development of diabetes as participants with insulin resistance are still be able to maintain normal glucose tolerance as pancreatic β-cells with normal function are able to compensate for insulin resistance[7,13,14]. Thus, there is urgent need to identify and understand other T2D risk factors beyond obesity and insulin resistance, especially factors involved in pancreatic β-cell dysfunction[15].

Inorganic arsenic (iAs) is widely distributed in the environment, such as drinking water, contaminated food, rice, air, dust, and soil[16]. Growing literature has linked exposure to high levels of iAs with increased risk of T2D. There is growing concern that low-to-moderate levels of iAs exposure, which is much more common worldwide, may contribute substantially to the epidemic of T2D. However, few epidemiological studies have been conducted in low-to-

moderately exposed populations, and most studies have been cross-sectional[19,22-24]. This is of concern as T2D has been shown to affect the metabolism of iAs, posing the risk that altered iAs biomarkers in diabetic individuals may be a consequence—vs. a cause—of T2D in cross-sectional studies[17]. Thus, longitudinal studies are urgently needed in the low-to-moderately iAs exposed population, such as China and Mexico, to examine the association between iAs exposure and development of diabetes[3,8-12].

Effective strategies to counter the adverse effects of low-to-moderate iAs exposure are critical as it is challenging to eliminate iAs from daily life[18,19]. Recently, Petriello et al.[20] advocated identifying nutritional strategies to mitigate cardio-metabolic effects of toxic metals, such as iAs. Laboratory data also suggests that the adverse health effects of toxic metals could be reduced with optimal intake of dietary essential metals, such as magnesium (Mg) and zinc (Zn), which may decrease uptake or counter adverse metabolic effects of toxic metals[21-27]. However, human evidence on the effectiveness of such dietary strategies to reduce the adverse health effects of iAs is limited, and epidemiological studies have yet to access whether susceptibility to the diabetogenic effects of iAs may vary depending on levels of Mg and Zn intake[28-30].

Arsenic (+3 oxidation state) methyltransferase (AS3MT) is a key enzyme in the pathway for the methylation of iAs[31]. Previous studies have linked several polymorphic sites in *AS3MT* to significant differences in urinary measures of iAs metabolism—namely proportions of the methylated metabolites—in various populations[32-40]. However, to our knowledge, no population study has formally explored to what extent associations between AS3MT variants and measures of iAs metabolism may vary depending on levels of iAs exposure. Such heterogeneity, if present, could lead to inconsistencies across populations with varying iAs exposure in the extent to which genetic variants either relate to measures of iAs metabolism, or modify health risks associated

with environmental iAs exposure.

To sum up, the literature is lacking how, in a population with low-to-moderate iAs exposure, baseline (i.e.2009) iAs exposure relates to the development of T2D during follow-up (i.e.2015), and potential effect modification by Mg and Zn intake at baseline on the iAs-associated T2D. In the proposed study, we took advantage of the genetic data from Mexico as well as rich longitudinal data from the ongoing longitudinal China Health and Nutrition Survey (CHNS), which include: toenail concentration of arsenic that reflects iAs exposure level in recent months at baseline; baseline dietary Mg and Zn intake captured using 3-day 24 hour recall; fasting glucose and insulin at follow-up (i.e. 2015). Using these unique data, we sought to 1) determine how, in a population with low-to-moderate iAs exposure, iAs exposure at baseline relates to the T2D incidence and indicators of glucose homeostasis at follow-up; 2) determine whether adequate intake of Mg and Zn at baseline may mitigate the diabetogenic effects of iAs; 3) determine gene-environment interactions between genetic variants of *AS3MT* and iAs exposure.

Research Aims

The primary goal of this dissertation was to determine the associations between baseline (i.e.2009) iAs exposure, which was captured using toenail arsenic concentration, and T2D incidence during 6-years follow-up as well as the indicators of glucose homeostasis, which was characterized by fasting glucose, fasting insulin, glycated hemoglobin, pancreatic β-cell dysfunction, and insulin resistance at follow-up (i.e. 2015). We also aimed to examine potential effect modification by Mg and Zn intake at baseline on the associations between baseline iAs exposure and development of T2D over 6 years. In addition, we also aimed to determine the gene-environment interactions between genetic variants of *AS3MT* and iAs exposure.

Aim 1: Determine the associations between baseline iAs exposure and the development of T2D over 6 years. Measures of T2D development include a) indicators of glucose homeostasis at follow-up: fasting glucose (FG), fasting insulin (FI), pancreatic β-cell function and insulin resistance, which were assessed using the updated homeostasis model assessment (HOMA2)[41-43], and b) incidence of pre-diabetes (FG≥110mg/dL and <126mg/dL) and T2D (FG≥126mg/dL) over 6 years of follow-up.

We hypothesize that among adults without diabetes at baseline, higher toenail arsenic at baseline will be associated with elevated FG and decreased pancreatic β-cell function at follow-up. We hypothesize null association between baseline toenail arsenic and insulin resistance at follow-up since β-cell dysfunction, rather than insulin resistance, is thought to be primarily involved in iAs-associated T2D. We also hypothesize positive associations between toenail arsenic at baseline and odds of incident T2D and pre-diabetes over 6 years.

Aim 2: Determine the associations between intake of Mg and Zn at baseline and iAs-associated T2D. We prospectively assess whether the susceptibility to the diabetogenic effects of low-to-moderate iAs exposure may vary depending on levels of Mg and Zn intake at baseline. We also examine the associations between Mg and Zn intake at baseline and the T2D incidence and indicators of glucose homeostasis at follow-up.

We hypothesize that the fasting glucose and odds of incident T2D are lower among participants with adequate intake of Mg and Zn at baseline, and there are significant interactions between toenail arsenic and dietary intake of Mg and Zn at baseline for the development of T2D. The associations between iAs exposure and measures of T2D development are attenuated among participants with adequate intake of Mg and Zn at baseline (intake ≥ recommended dietary

allowance (RDA) and < tolerable upper intake level) comparing with people have Mg and Zn deficiencies (intake <RDA).

Aim 3: Examine the consistency of previously established associations between multiple *AS3MT* variants and the profiles of urinary arsenic metabolites and determine evidence of heterogenic in the magnitude of these associations depending on exposure level.

We hypothesize *AS3MT* SNPs previously reported to be associated with higher DMAs% are consistently associated with higher DMAs% in our study. We also hypothesize the magnitudes of the associations between *AS3MT* genetic variants and urinary arsenic profiles are stronger among participants with relatively high exposure level.

CHAPTER 2. LITERATURE REVIEW

Inorganic arsenic is widely distributed in our environment and thought to contribute to the global epidemic of Type 2 diabetes

Type 2 diabetes (T2D) which is a well-recognized cause of premature death and disability, is a complex disease with multiple contributing factors, such as obesity, environmental pollution, diet, and genetic components[1-5,44-48]. China has the greatest number of diabetes cases—an estimated 98.4 million—worldwide, and the prevalence of T2D doubles during the last two decades[4]. Though obesity is a well-known risk factor for T2D and is associated with insulin resistance, it has been suggested that obesity alone cannot fully explain the escalating epidemic of T2D, especially in regions where the prevalence of obesity is low[3,8-12]. Recent studies stress on the importance of pancreatic β-cell dysfunction in the development of diabetes as participants with insulin resistance are still be able to maintain normal glucose tolerance as pancreatic β-cells with normal function are able to compensate for insulin resistance[7,13,14]. Thus, there is a urgent need to identify and understand other T2D risk factors beyond obesity and insulin resistance, especially factors involved in pancreatic β-cell dysfunction[15].

iAs is widely distributed in our environment, such as in drinking water, contaminated food, rice, air, dust, and soil[16]. Previous studies reported that more than 100 million people worldwide are exposed to unsafe levels of iAs in drinking water[16]. Compelling laboratory data and growing epidemiological literature indicates that high iAs exposure, normally defined as ≥150μg/L iAs in drinking water, is associated with increased risk of T2D, and the 2011 National

Institute of Environmental Health Science-National Toxicity Program panel has agreed that existing data support an association between high exposure of iAs and increased risk of T2D[49-54]. There is growing concern that low-to-moderate level of iAs exposure, which is the exposure level much more common worldwide, may contribute substantially to the epidemic of T2D. Nevertheless, the results of the few prospective studies conducted in the low-to-moderately exposed populations are inconsistent[51,55-57]. The mixed results could be due in part to factors that include: 1) varied susceptibility due for example to differences in intake of beneficial nutrients, such as magnesium and zinc[21,58-60]; 2) misclassification of disease: laboratory studies have indicated the association between iAs exposure and hemoglobin concentration[61,62], which raises concern about using HbA1c to diagnose diabetes among participants exposed to iAs[63,64]. Furthermore, the established association between biomarkers of iAs exposure and T2D is subject to criticism as it comes largely from cross-sectional studies. Yet diabetes has been shown to affect iAs metabolism and excretion[53], posing the risk that altered iAs biomarker in diabetic individuals may be a consequence—vs. a cause—of disease[17].

Adequate intake of essential metals—notably Magnesium (Mg) and Zinc (Zn)—may mitigate iAs uptake and improve the glucose homeostasis

It is challenging to eliminate iAs from daily life due to the diverse sources of iAs exposure and limited awareness of being exposed[18,19]. Thus, effective strategies to counter the adverse health effects of low-to-moderate iAs exposure are critical. Recently, Petriello et al.[20] has advocated identifying nutritional strategies to mitigate cardio-metabolic effects of toxic metals, such as iAs. Laboratory data also suggests that the adverse health effects of toxic metals could be reduced with optimal intake of dietary essential metals, such as magnesium (Mg) and zinc (Zn), which may decrease uptake or counter adverse metabolic effects of toxic metals[21-27].

However, human evidence on the effectiveness of such dietary strategies to reduce the adverse health effects of iAs is limited, and epidemiological studies have yet to access whether susceptibility to the diabetogenic effects of iAs may vary depending on levels of Mg and Zn intake[28-30]. Indeed, population differences in intake of Mg and Zn may contribute to the heterogeneous findings on iAs-associated diabetes to date, especially among the population low-to-moderately exposed to iAs[21,26,30,58,59].

In addition, deficiencies of Mg and Zn intake, which is defined as intake < recommended dietary allowance (RDA), are common worldwide, including China and the US[54,65-69]. The existing evidence thus indicates that promoting adequate intake of Mg and Zn, defined as intake ≥RDA and < tolerable upper intake level (UL), could potentially be an effective strategy to counter the adverse effects of iAs, such as T2D. However, human evidence that such dietary strategies effectively counter iAs-associated T2D is limited.

Toenail arsenic better captures chronic exposure to iAs than spot urine especially in low-to-moderately exposed populations

There is now increasing interest in using of nails as a routine biomarker of iAs exposure[70-72]. On ingestion, soluble iAs is absorbed from the gastro-intestinal tract and distributed to all bodily systems in the blood. iAs is thought to accumulate in hair and nails as a result of its affinity for sulfhydryl groups and remains isolated from the body's metabolic processes after nail formation[73]. In addition, researchers found toenails preferentially sequester inorganic arsenic species, which is a more toxic form than organic arsenic[70-72].

Though urinary arsenic was widely used in studies as the biomarker of iAs exposure, the long-term accumulation of iAs in nails makes the toenail arsenic more useful as the biomarker of chronic iAs exposure[70-72,74]. A previous study conducted by Meharg et al showed considerable

variation (up to 13-fold) in an individual's total iAs urine content throughout the day[75], calling into question the robustness of the routinely used spot urine sampling for iAs analysis, especially in the population that is low-to-modernly exposed to iAs.

Collectively, studies indicate toenail arsenic is a better biomarker for chronic iAs exposure comparing with spot urine, which is frequently used, especially among people low-to-moderately exposed to iAs.

China provides a special opportunity to conduct this study

First, Chinese are at higher risk of T2D at relatively lower body mass index, which highlight the need to better understand the T2D risk factors beyond obesity[3,4,47,76]. Secondly, to determine the association between low-to-moderate iAs exposure and development of T2D, studies are largely needed to be conducted among populations with low-to-moderate iAs exposure, which is the exposure level in most part of China[77]. Thirdly, the prevalence of Mg and Zn deficiency are high in China[54,65,67]. The Chinese then provide a great opportunity for us to determine whether adequate intake of Mg and Zn could be an effective strategy to counteract the adverse metabolism effects of iAs. Lastly, the China Health and Nutrition Survey (CHNS) with rich longitudinal data, especially high-quality dietary data and toenail concentration of arsenic that reflects iAs exposure level in recent months at baseline (i.e. 2009), provides a special opportunity for us to conduct this study. All these factors make China a unique model to conduct the proposed study.

The role of *AS3MT* gene in iAs metabolism

In humans, the primary pathway for metabolism of iAs involves sequential methylation to form monomethylated As (MAs) and dimethylated As (DMAs) metabolites, which are excreted in the urine [31]. Higher percentages of total urinary As represented by DMAs (DMAs%),

and lower percentages of MAs or the unmethylated iAs (MAs% and iAs%, respectively) in urine have been hypothesized to be indicators of higher capacity to metabolize iAs [31,78,79]. The ratios of MAs to iAs (MAs/iAs) and of DMAs to MAs (DMAs/MAs) in urine have also been widely used as indicators of capacity for the first and second methylation steps. However, the measures of iAs metabolism most predictive of increased health risks remain to be established, given the conflicting associations reported in recent studies [31,80-83].

Arsenic (+3 oxidation state) methyltrasferase (AS3MT) is a key enzyme in the pathway for the methylation of iAs, and variants in the *AS3MT* have been shown to be associated with inter-individual differences in iAs metabolism [32-35,84,85]. Previous studies have linked polymorphic sites in this gene to significant differences in urinary measures of iAs metabolism in various populations [32-35,84,86]. It has been suggested that iAs exposure level may modify iAs metabolism, as reflected by changes in urinary As methylation profiles, with a shift in the proportions of urinary metabolites among persons exposed to levels approximately >50ppb vs. ≤50ppb [85,87-89]. We have previously reported based on laboratory experiments that levels and proportions of the methylated products, including DMAs/MAs ratio, differ between recombinant variants of human *AS3MT* and depend on the substrate concentration [36]. However, to our knowledge, no population study has formally explored to what extent associations between *AS3MT* variants and measures of iAs metabolism may vary depending on levels of iAs exposure. Such heterogeneity, if present, could lead to inconsistencies across populations with varying iAs exposure in the extent to which genetic variants either relate to measures of iAs metabolism, or modify health risks associated with environmental iAs exposure.

Summary

The proposed study better informs how, in a population low-to-moderately exposed to iAs, baseline dietary Mg and Zn intake relates to the association between baseline iAs exposure and development of T2D during six years of follow-up. In addition, we examined whether the influence of genetic variants in *AS3MT* on iAs metabolism varied by level of iAs exposure. In this dissertation, we used geographically–diverse longitudinal CHNS data to address these questions. First, we determined the association between baseline iAs exposure, which was captured by toenail arsenic at baseline, and T2D incidence as well as indicators of glucose homeostasis, which were characterized by fasting glucose, fasting insulin, glycated hemoglobin, pancreatic β-cell function, and insulin resistance at follow-up (i.e.2015). Second, we determined whether adequate intake of Mg and Zn at baseline, which was defined as intake≥ RDA and <UL, might protect against the development of T2D associated with iAs exposure. We used the updated homeostasis model assessment (HOMA2)[41-43] to estimate the pancreatic β-cell dysfunction and insulin resistance at follow-up. By determining the associations between iAs exposure and pancreatic β-cell dysfunction vs. insulin resistance, our study can better inform the underlying mechanism of iAs-associated T2D.

CHAPTER 3. EXPOSURE TO INORGANIC ARSENIC AND DEVELOPMENT OF TYPE 2 DIABETES: A LONGITUDINAL STUDY IN A POPULATION OF LOW TO MODERATE INORGANIC ARSENIC EXPOSURE

Overview

Compelling evidence has linked high inorganic arsenic (iAs) exposure with increased risk of type 2 diabetes (T2D), however, inconsistent results were seen among populations low-to-moderately exposed to iAs. The inconsistent results could due in part to most studies are cross-sectional, whereas longitudinal study should be conducted to better capture the association between baseline iAs exposure and development of T2D. Though the underlying mechanism for iAs-associated T2D is not well established, laboratory studies indicate β-cell dysfunction, rather than insulin resistance, is predominately involved.

In this population with low-to-moderate level of iAs exposure, we determined how toenail arsenic at baseline (i.e.2009) related to T2D incidence and indicators of glucose homeostasis at follow-up (i.e.2015). We found a positive association between baseline iAs exposure and development of diabetes, as defined by higher FG, lower pancreatic β-cell function, as well as higher odds of incident prediabetes and diabetes. Our results further confirm the diabetogenic effects of low-to-moderate iAs exposure, which may contribute greatly to the current epidemic of T2D especially in populations with low prevalence of obesity. Moreover, our

findings also suggest that instead of insulin resistance, pancreatic β-cell dysfunction is primarily involved in iAs-associated diabetes.

In addition, we also found that men, younger adults, participants who consumed alcohol, and participants from North China were more susceptible to the diabetogenic effects of iAs. In addition, though previous studies suggested reduced susceptibility of iAs-associated adverse health outcomes among obese individuals, our findings support that iAs exposure promoted rapid progression to more severe diabetes for obese individuals.

Introduction:

The prevalence of T2D, which is a well-recognized cause of premature death and disability, has increased globally over the past two decades, especially in China[1-5]. It has become abundantly clear that both pancreatic β-cell dysfunction and insulin resistance are involved in the development of T2D, but the interplay between pancreatic β-cell dysfunction and insulin resistance remains highly complex and somewhat undefined[6,7]. Though obesity is a well-known risk factor for T2D and is associated with insulin resistance, it has been suggested that obesity alone cannot fully explain the escalating epidemic of T2D, especially in regions where the prevalence of obesity is low[3,8-12]. Recent studies stress on the importance of pancreatic β-cell dysfunction in the development of diabetes as participants with insulin resistance are still be able to maintain normal glucose tolerance as pancreatic β-cells with normal function are able to compensate for insulin resistance[7,13,14]. Thus, there is urgent need to identify and understand other T2D risk factors beyond obesity and insulin resistance, especially factors involved in pancreatic β-cell dysfunction[15].

Compelling evidence has linked exposure to high levels of inorganic arsenic (iAs), a common contaminant in drinking water and foods such as rice, with increased risk of T2D[16,49-52].

Recent reviews and meta-analyses of the epidemiological literature also suggest that exposure to levels of iAs in drinking water >150μg/L may increase the risk of T2D[55,57,90]. There is growing concern that low-to-moderate level of iAs exposure, which is much more common worldwide, may contribute substantially to the current epidemic of T2D, especially in countries with low prevalence of obesity. However, evidence of an association between iAs and development of T2D at low to moderate level of iAs exposure is limited and inconsistent[51,55-57]. In addition, most previous studies are cross-sectional, which is problematic since diabetes has been shown to affect iAs metabolism[53], posing the risk that altered iAs biomarker in diabetic individuals may be a consequence—as opposed to a cause—of disease[17]. Thus, longitudinal studies are urgently needed in the low-to-moderately iAs exposed population, such as China, to examine the association between iAs exposure and development of diabetes[3,8-12].

In vitro studies of the pathogenesis of iAs-associated T2D have implicated the involvement of several biological processes, including impairments in the cellular adaptive response of oxidative stress and inhibition of glucose-dependent insulin secretion as a consequence of pancreatic β-cell dysfunction[53,91-96]. However, the pathway (i.e. insulin resistance vs. β-cell dysfunction) primarily involved in diabetogenic effects of iAs remains unclear. To date, few epidemiological studies have examined associations between low-to-moderate iAs exposure and pancreatic β-cell function vs. insulin resistance. Such studies are especially needed to provide insight into the potential pathogenesis of iAs-associated T2D.

To address this gap, we examined the association between baseline (i.e.2009) iAs exposure and measures of T2D development, characterized by fasting glucose, fasting insulin, glycated hemoglobin, and odds of incident prediabetes and diabetes six years later, in a population-based cohort with low-to-moderately exposure to iAs. In addition, we also aimed to

examine the associations between iAs exposure and insulin resistance vs. pancreatic β-cell function, which were assessed using the updated homeostasis model assessment (HOMA2)[41-43], to inform the understanding of iAs-associated T2D.

Method

China Health and Nutrition Survey. The China Health and Nutrition Survey (CHNS) is an ongoing longitudinal survey, which collected health, economic, sociological, and demographic data in nine diverse provinces throughout China from 1989-2015. Using a multistage random cluster design, a stratified probability sample was used to select counties and cities stratified by income using State Statistical Office definitions, and then communities and households were selected from these strata. The CHNS cohort initially mirrored national age-gender-education profiles and the nine provinces in the CHNS constituted 44% of China's population in 2009 (according to 2009 census). Questionnaires were used to collect demographic, socioeconomic, anthropometric, behavioral and health information. Biomarker data, including fasting glucose (FG), fasting insulin (FI), and glycated hemoglobin (HbA1c), were collected at baseline (i.e. 2009) and follow-up (i.e. 2015). Toenail clippings were collected at baseline and analyzed for arsenic concentration. Survey procedures have been described elsewhere[97,98]. The study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill, the China-Japan Friendship Hospital, National Institute for Nutrition and Health in China, and the Chinese Center for Disease Control and Prevention.

Analysis sample. We restricted analyses to non-pregnant adults (≥18y) at time of measurement (i.e. baseline and follow-up) with toenail arsenic data available at baseline (N=6843). We excluded participants who missed baseline data on body mass index (N=511), smoking or drinking status (N=5), diet (N=80), urbanization index (N=96), physical activity

(N=34), province (N=7), any blood biomarkers (FG, FI, and HbA1c, N=478). For the complete case analysis, we further excluded participants missed any blood biomarkers (i.e. FG, FI, and HbA1c) at follow-up (N=3479). Participants with extreme biomarkers, defined as 6 standard deviation above or below the mean, were also excluded (N=15). We further excluded individuals with diabetes at baseline to reduce the possibility of reverse causality (N=220), resulting in final analytic samples for the complete case analysis N=1918.

Ascertainment of diabetes. Blood samples of all participants were collected by venipuncture after an overnight fasting. Whole blood was immediately centrifuged, serum was aliquoted and frozen at -70 degrees. Aliquots were collected in EDTA-coated vacutainers containing sodium fluoride to prevent glucose degradation. All blood samples were analyzed in a national central lab in Beijing (medical laboratory accreditation certificate ISO15189:2007). Both baseline and follow-up FG were measured by colorimetric assay methods (GOD-PAP; Randox Laboratories Ltd, UK) using a Hitachi 7600 analyzer (Hitachi Inc., Tokyo, Japan). Both baseline and follow-up HbA1c was measured with high performance liquid chromatography (HPLC) system (model HLC-723 G7; Tosoh Corporation, Tokyo, Japan). FI at baseline was measured using a radioimmunoassay (North Institute of Bio-Tech,China) and a gamma counter (XH-6020), while FI at follow-up was measured using a chemiluminescent immunoassay.

Diabetes was defined by FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Pre-diabetes was defined by FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. Measured FI and FG were used to calculate steady state pancreatic β-cell function and insulin resistance (IR) using the Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php). The HOMA2 calculator is an

updated version (computer model) of the original HOMA model (HOMA1). HOMA2 is suggested as a more accurate method than the original HOMA1, since HOMA2 accounts for variations in hepatic and peripheral glucose resistance and the reduction of peripheral glucose-stimulated glucose uptake[41,42]. Regular HOMA2, including regular HOMA2-IR and regular HOMA2-β, were calculated for baseline as it is suggested for insulin measured using radioimmunoassay. Specific HOMA2s (i.e. HOMA2-IRs and HOMA2-βs) were calculated for follow-up since specific HOMA2s were suggested to be used when insulin were measured using a chemiluminescent immunoassay[41,42].

Measurement of toenail arsenic. Participants were asked to let toenails grow for at least two weeks, and stainless-steel nail scissors were used to collect toenail clippings from all ten toes. Toenail clippings were washed and sonicated for 30 minutes and dried at 80℃. After microwave-assisted digestion, toenails were analyzed using inductively coupled plasma mass spectroscopy[99]. The limit of detection (LOD) for arsenic by this method is 0.01μg/g. Toenail arsenic concentrations lower than the LOD were imputed at half of the LOD as 0.005μg/g (N=82/1918).

Dietary assessment. Dietary intake was assessed using three consecutive days 24-hour recalls (two weekdays and one weekend day) at the individual level and a food inventory at the household level during the same 3-day period. Trained investigators interviewed the participants to collect detailed types and amounts (in grams) of all foods and beverages consumed during the preceding 24 hours with the assistance of food pictures to aid quantification. Daily intake was calculated by the mean of three days 24-hour recalls. China Food Composition Table was used to calculate the daily total energy intake, which was validated with the doubly labeled water method in the Human Nutrition Research Center, Tufts University (correlation coefficients

between the two methods were 0.56 for men and 0.60 for women)[100,101]. Daily total energy and rice intake were calculated and categorized into tertiles. After combining all kinds of fish, we characterized participants as consumers vs. non-consumers of fish during the survey period. We used the tailored Alternate Healthy Eating Index (tAHEI), which was generated by tailoring the Harvard AHEI 2010, to assess diet quality in our study[191]. Details of tAHEI have been described elsewhere[191].

Other variables. Standard height (in meters) and weight (in kilograms) were measured using standard protocol by trained nurses. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). We generated weight status based on BMI according to the World Health Organization cutoffs for Asian adults ($<18kg/m^2$ for underweight, 23.0-27.4kg/m^2 for overweight, and $\geq 27.5kg/m^2$ for obese)[102,103]. Information on age (in years), sex, smoking (current, former, never smokers), drinking status (< vs. ≥once per month) was collected using questionnaires.

We used a validated urbanization index to measure degrees of urbanization[104]. The index contains 10 validated components including communication, economic, housing, transportation infrastructure, the availability of schools, markets, and health care, environmental sanitation, and population size and density. The urbanization index, which was positively correlated with degree of urbanization, ranged from 10 to 100 and was categorized into tertiles. We categorized the nine provinces in CHNS into three geographic regions (North: Heilongjiang, Liaoning; Central: Shandong, Henan, Jiangsu; South: Hunan, Hubei, Guangxi, Guizhou). Individual physical activity data was collected through an in-depth interview on time (hours) spent on domestic, occupational, transportation, and leisure-time activity. Separate questions were asked for each of the four domains. We calculated energy expenditure from each activity domain by multiplying

time (hours) spent on each type of physical activities by its specific metabolic equivalent (MET) intensity value, resulting in measures of total MET-hours/week for each domain. We calculated the overall energy expenditure for each individual by summing MET-hours spent in each domain and categorizing into tertiles.

Statistical analyses. We used natural log-transformed toenail arsenic to account for the non-normal distribution of toenail arsenic. ANOVA (continuous variables) and chi-square tests (categorical variables) were used to determine the significance of differences in baseline characteristics across tertiles of iAs exposure.

Multinomial (diabetes, prediabetes, vs. neither) logistic regression models were used to determine the association between baseline toenail arsenic and odds of incident prediabetes and diabetes during follow-up. Multivariable-adjusted linear regression models were used to examine the association between baseline toenail arsenic and FG, FI, HbA1c, HOMA2-IRs, and HOMA2-βs for both cross-sectional and longitudinal complete case analysis.

As shown in the directed acyclic graphs (DAG) (Figure 3.1a), all potential confounders for the association between baseline iAs exposure and development of T2D during over 6-years follow-up were adjusted in the full model (Model 1). These variables included age, sex, drinking status, smoking status, urbanization index, dietary quality index, baseline weight status (weight status may influence the excretion and storage of iAs[105-107] and it is a well-known risk factor for T2D), fish intake (an important source of non-toxic organic arsenic compounds (arsenobetaine and arsenosugars)[108,109] and is associated with T2D risk[110,111]), rice intake (an important source of iAs exposure in the study population[112] and is also associated with risk of T2D[113]). We included daily total energy intake and physical activity level in the model since they were associated with

risk of T2D. We also adjusted for the geographic region due to substantial regional variation in exposure sources, diet, cooking method, and lifestyle factors across China.

In the model 2, we adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$) in our study. The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, and region (Figure 3.1b). Gribble et al.[114] found there was substantial change in the associations between iAs exposure and development of T2D before vs. after adjusting for region. To better understand how region is related to iAs-induced T2D, we examined the associations between iAs exposure and development of T2D adjusting for all identified confounders in our study except region (Model 3).

Gribble et al.[114] found several characteristics of the population were associated with the risk of adverse health effects of iAs exposure. To better compare with previous studies and examine the consistency of the associations between iAs exposure and development of T2D by participant characteristics, we tested the interactions between iAs exposure and characteristics of the population that were suggested to be associated with iAs-associated adverse health outcomes. We also stratified our analyses by the characteristics, i.e. age (<55, 55-64, or ≥65 years), sex, body mass index (<18, 23.0-27.4, or ≥27.5kg/m^2), smoking status (current, former, or never), drinking status (<, or ≥once/month), and geographic region (North, Central, South).

We conducted sensitivity analysis to account for the impact of aging on the metabolism of iAs and risk of T2D by comparing the associations between iAs exposure and development of T2D before vs. after excluding participants age>70; results did not differ meaningfully (data not shown). Rice intake was identified as a confounder in our study (p for stepwise backward regression model<0.10) and was adjusted in the models. Since rice is an important source of iAs

exposure in our study population[112], we conducted sensitivity analysis by including all identified confounders in our study except rice intake to demine the impact of adjusting source of iAs exposure in our study. We also compared the associations between baseline toenail arsenic and development of diabetes (i.e. FG, FI, HbA1c, and HOMA2s) before vs. after excluding participants with pre-diagnosed diabetes at follow-up (N=18), who might have adjusted their behaviors (e.g. reduced rice consumption); results did not differ meaningfully. In addition to complete case analysis, we conducted multiple imputation using predictive mean matching (five closest observations was drawn) to account for the impact of missing data.

Statistical significance of main effects was set at $p<0.05$ with $p<0.10$ indicating marginal significance. All the statistical analyses were performed in Stata, version 13.1 (StataCorp LP, College station, Texas).

Results

Baseline (i.e. 2009) sociodemographic and anthropometric characteristics, as well as biomarkers at both baseline and follow-up (i.e. 2015), arsenic exposure level at baseline characterized by toenail arsenic are provided in Table 3.1. The concentrations of baseline toenail arsenic ranged from below the limit of detection (LOD) to 115.6μg/g, with a median of 0.32μg/g. Participants from Southern China had significantly higher toenail arsenic compared to participants from North and Central China (Supplemental Table 3.1).

For the participants involved in the complete case analysis, baseline FG was significantly lower than FG at follow-up (92.1±10.9 vs. 96.8±16.0 mg/dL), whereas we found no statistically significant difference between baseline vs. follow-up HbA1c (5.4±0.4 vs. 5.6±0.6%). Due to different methods were used to measure FI at baseline vs. follow-up (i.e. radioimmunoassay vs. chemiluminescent immunoassay), differences in FI and HOMAs at baseline vs. follow-up cannot

be estimated. The mean FI at baseline was 12.1μU/mL, mean baseline HOMA2-IR and HOMA2-β were 1.5 and 119.5%, respectively. The mean follow-up FI was 7.1μU/mL, mean HOMA2-IRs and HOMA2-βs at follow-up were 1.1 and 86.5%, respectively.

Overall, 6.7% and 46.6% of the study participants developed diabetes and prediabetes during the six years of follow-up. Compared to participants in the lower tertiles of baseline toenail arsenic distribution, participants in the highest tertile of toenail arsenic exposure (toenail arsenic concentration>0.44μg/g) had marginally higher incidence of prediabetes during follow-up (Tertile 3, Tertile 2, Tertile 1: 49.3, 45.5, 44.8%, p=0.06).

Baseline characteristics of participants across tertiles of toenail arsenic concentration were shown in Table 3.1. Toenail arsenic concentration at baseline was positively associated with rice intake as well as physical activity, and negatively associated with weight status and urbanization index. There was no significant difference in age, sex, smoking status, drinking status, and total energy intake for participants across tertiles of toenail arsenic.

Participants with lower toenail arsenic at baseline had significantly higher baseline FI (T1 vs. T2 vs. T3: 12.9± 10.5 vs. 11.8± 9.5 vs. 11.7±7.7μU/mL), HbA1c (5.5±0.4 vs. 5.4±0.4 vs. 5.3±0.4%), and HOMA2-β (123.8±47.5 vs. 117.0± 43.5 vs. 117.7± 44.7%), while we found no significant difference in baseline FG and HOMA2-IR. In contrast, we found no significant difference in follow-up FI and HbA1c among participants in different tertiles of baseline toenail arsenic, while participants with higher baseline toenail arsenic had significantly higher FG (T1 vs. T2 vs. T3: 95.1±15.8 vs. 96.7±14.4 vs. 98.7±17.4 mg/dL) and lower HOMA2-βs (89.9±32.8 vs. 86.1±32.3 vs. 83.7±32.9%) at follow-up.

In the cross-sectional analyses for the full analytic sample, natural log transformed baseline toenail arsenic was associated with significantly lower baseline FI (-0.32±0.18μU/mL)

and HbA1c (-0.05±0.01%), and the associations remained statistically significant after adjusting for region (Supplemental Table 3.2). We also found statistically significant negative association between baseline toenail arsenic and odds of diabetes at baseline (OR: 0.85, 95% CI: 0.74 - 0.97), and the association remained statistically significant after adjusting for region. In contrast, we found no significant association between baseline toenail arsenic and baseline FG, HOMA2-β, HOMA2-IR, as well as odds of prediabetes at baseline.

Our findings for the prospective analyses were different from the cross-sectional analyses. In multivariable-adjusted models (Model 3, Table 3.2), natural log transformed baseline toenail arsenic was associated with significantly higher FG (1.44±0.30mg/dL) and lower HOMA2-βs (-1.98±0.60%) at follow-up. Moreover, we found baseline ln-toenail arsenic was positively associated with odds of incident prediabetes (OR:1.14, 95% CI:1.05-1.24) and diabetes (OR:1.24, 95% CI: 1.10-1.46) during follow-up. The associations remained statistically significant but were attenuated after further adjustment for regions (Model 2, Table 3.2). We found no significant association between baseline toenail arsenic and FI, HbA1c, and HOMA2-IRs at follow up before and after adjusting for region.

We found statistically significant interactions between baseline toenail arsenic and sex for FG at follow-up (Figure 3.2). The positive association between ln-toenail arsenic and FG at follow-up was statistically significantly stronger among men vs. women (2.07±0.54 vs. 0.92±0.34mg/dL), and it remained statistically significant after adjusting for region. We also found statistically significant modification effect of region on the association between baseline iAs exposure and FG at follow-up. The association between ln-toenail arsenic and FG was strongest among participants from Northern China (1.31±0.47mg/dL).

For the HOMA2-βs at follow-up, we noticed marginally significant interactions between ln-toenail arsenic and age group as well as drinking status before adjusting for regions (Figure 3.2). The negative association between ln-toenail arsenic at baseline and HOMA2-βs at follow-up was stronger and only statistically significant among participants age<55 (-3.20±0.78%, P<0.05) compared with participants with age ≥55. The magnitude of the negative association between ln-toenail arsenic at baseline and HOMA2-βs at follow-up was stronger among participants who drank alcohol vs. non-drinkers (-3.59±1.09 vs. -1.29±0.72%, respectively). We also found marginally significant interaction between ln-toenail arsenic and region for HOMA2-βs at follow-up, participants from North had significantly stronger negative association between ln-toenanil arsenic and HOMA2-βs at follow-up (North vs. Central vs. South: -3.14±1.11 vs. 0.00±1.25 vs. -0.95±1.12%).

For the odds of incident prediabetes, we found a statistically significant interaction between toenail arsenic and sex, and it remained significant after adjusting for region. The association was significantly stronger in men versus women (OR:1.17 vs. 1.03). We also found significant interaction between drinking status and ln-toenail arsenic for odds of incident diabetes, the association was significantly stronger among alcohol consumers (OR: 1.30 vs. 1.01).

We compared baseline characteristics of participants included in the complete case analysis and those excluded due to missing data. As shown in Supplemental Table 3.3, those who were younger, with normal weight status (BMI≤23.0), current smokers, and drinkers were more likely to be excluded due to missing data. In addition, we found no significant difference in the association between iAs exposure and development of T2D using the multiple imputed dataset (Supplemental Table 3.4). The results further implicate that the data analysis samples used in our

study reflect the full population. Moreover, there was no significant change of the associations between iAs exposure and development of T2D before vs. after adjusting for rice intake, which is an important source of iAs exposure in our study population and was identified as one of the confounders in our study. However, the magnitude of the associations between iAs exposure and fasting glucose as well as HOMA2-βs attenuated after adjusting for rice intake (Supplemental Table 3.5).

Discussion

The results of our study support the association between chronic low-to-moderate iAs exposure and development of diabetes which supports the previous literature, although our study is the first to use longitudinal study data in the context of low-to-moderate iAs exposure[55,57,90,115,116].

Toenail arsenic was used as a measurement of individual chronic iAs exposure since toenails have shown to preferentially sequester iAs species, which is a more toxic form than organic arsenic[70-72,74,117]. Most previous studies have estimated iAs exposure level based on iAs concentration in the primary drinking water. However, there is a growing concern that iAs from other sources, such as rice especially among Asians with rice based diet[118,119]. A few studies estimated individual iAs exposure based on urinary arsenic, which is generally regarded as the most reliable indicator of recent exposure to iAs. Nonetheless, a rice-feeding trial conducted by Meharg et al showed considerable variation (up to 13-fold) in an individual's total iAs urine content throughout the day, calling into question the robustness of the routinely used spot urine samples, especially among participants low-to-moderately exposed[75]. The long-term accumulation of iAs in toenails makes the toenail arsenic measure more reliable as a biomarker of chronic iAs exposure [74,117].

Endemic arsenism in China is mainly caused by breathing indoor air contaminated by iAs from coal or charcoal combustion and drinking water contaminated by iAs from natural sources, such as mining[120,121]. Consistent with the patterns of mineral exploitation in China, mining areas and endemic arsenism caused by coal combustion is mainly observed in rural areas of southern China, such as Guizhou[122,123]. Recent studies suggest that rice is another important source of iAs since rice accumulates iAs in the soil and groundwater, and iAs-contaminated rice is an important concern especially in southern China where people are more likely to have a rice-based diets[124-131]. In some areas near mining districts in southern China, arsenic content in rice is around 0.7mg/kg, 2.5 times greater than the acceptable limit proposed by the Minister of Health in China[121,132,133]. As expected, the distribution of toenail arsenic measured in our study is consistent with the distribution of the primary sources of iAs exposure across regions: participants from southern China had higher toenail arsenic.

HbA1c has been proposed as an optional assay for diagnosing diabetes[134-136]. It is widely used, including studies exploring the association between iAs exposure and risk of T2D, due to the convenience and superior technical attributes of HbA1c relative to FG[134-137]. However, the use of HbA1c to diagnose diabetes is inappropriate in some cases, especially for patients with hemoglobinopathies[134-136]. Previous laboratory studies have indicated the association between iAs exposure on hemoglobin concentration[61,62], which raises concern about using HbA1c to diagnose diabetes among population exposed to iAs. In line with several studies indicate null association between iAs exposure and HbA1c[80,138,139], we did not find statistically significant association between baseline (i.e.2009) iAs exposure and HbA1c at follow-up (i.e.2015) though baseline iAs exposure was significantly associated with higher FG at follow-up. Our findings further support the idea that FG is more feasible to capture the change in glucose hemostasis

induced by iAs exposure. The real association between iAs exposure and development of diabetes could be ignored if only HbA1c is used to define diabetes[139]. Additional research is needed to assess the association between iAs exposure and hemoglobin, and the validity of using HbA1c to diagnose diabetes in the population exposed to iAs.

A few studies have examined the association between iAs exposure and insulin resistance, which has been regarded as the mechanism underlying typical T2D[7,140], however, with inconsistent results. In line with our findings, Rotter et al.[83] and Baek et al.[141,142] found null association between iAs exposure and insulin resistance. In contrast with our findings, Lin et al.[143] found higher insulin resistance associated with iAs exposure among obese children and adolescents, and Del Razo et al.[80] found negative association between iAs exposure and insulin resistance. As most of these studies are cross-sectional, the inconsistent results could be partially due to presence of individuals with T2D who may progressively develop insulin resistance over time, even if it is not the primary pathway leading to iAs-associated T2D[144].

We found significantly negative association between baseline iAs exposure and pancreatic β-cell function at follow-up, even after adjusting for region. Notably, In line with our findings, several in vitro and in vivo studies investigating the pathogenesis of iAs-induced T2D have implicated the involvement of β-cell dysfunction, which may be attributed to the inflammatory and oxidative damage induced by iAs[145-149]. Collectively, these results indicate that instead of insulin resistance, which is the mechanism underlying typical T2D, pancreatic β-cell dysfunction is primarily involved in diabetogenic effects of iAs.

There have been growing concerns that cross-sectional study design cannot capture the temporal relationship between iAs exposure and development of diabetes, and diabetes might affect iAs metabolism[53,17]. The results of our study fully agreed with this concern, as we found

different results for the association between toenail arsenic and risk of diabetes using longitudinal vs. cross-sectional analysis. The longitudinal analysis suggests higher FG, aggravated β-cell dysfunction and higher odds of incident diabetes and prediabetes associated with chronic iAs exposure, whereas the cross-sectional analysis suggests lower odds of diabetes associated with iAs exposure. Our findings further suggest that the inconsistent results for the association between iAs exposure and risk of diabetes reported in previous studies could be partially explained by the different study designs.

We found the magnitude of the associations between baseline toenail arsenic and FG as well as odds of incident prediabetes was significantly stronger among men vs. women. Similarly, the magnitude of the association between baseline iAs exposure and HOMA2-βs was stronger among men vs. women, though the interaction was not statistically significant. Consistent with our findings, others have reported that men are more susceptible to toxic effects related to chronic exposure to iAs than women due to their higher capacity for iAs metabolism[150-152].

Consistent with current literature, we found obese individuals tended to have lower toenail arsenic. The reasons for the inverse association between biomarkers of iAs exposure and obesity remain uncertain. Several researchers have hypothesized that the inverse association between biomarkers of iAs exposure and adiposity may ascribe to the improvement in the efficiency of iAs metabolism and secretion among obese individuals[105,106,153]. Alternatively, another possible explanation is that exposure to iAs may itself promote reduce adiposity and adipocyte differentiation[154-159]. Little is known about how obesity may modify the association between iAs exposure and development of diabetes though Fatmi et al.[160] found obese participants were less susceptible to iAs-associated adverse health outcomes. We found the magnitude of the associations between baseline iAs exposure with FG and HOMA2-βs was

stronger among obese participants though the interaction between toenail arsenic and weight status was not statistically significant. The association between baseline toenail arsenic and odds of incident diabetes was only significant among obese participants. In line with our findings, Tseng et al.[161] also found stronger association between iAs exposure and risk of diabetes among the obese participants in the cross-sectional study conducted in Taiwan. Our findings suggest that iAs exposure may promote rapid progression to severe diabetes for those who are obese. Since Chinese are known to have lower BMI but higher percent body fat[162-164], further research is needed to examine the association between obesity with iAs-associated diabetes, especially in the population with double burden from iAs contamination and obesity.

Our analyses were robust to adjustment for several sociodemographic and diabetes risk factors. Consistent with what was observed in the Strong Heart Study, adjustment for region attenuated the associations between iAs exposure and development of diabetes[114]. The associations between iAs exposure and FG, HOMA2-βs, and odds of incident diabetes were stronger among participants from North China, though the interaction was not significant for odds of incident diabetes. Our findings suggest that participants from North may be more susceptible to iAs-associated diabetes, though participants from North had lower baseline iAs exposure. The results could be partially due to other potential confounders highly correlated with region[153], thus further research is needed to examine the association between other region-correlated factors with iAs-associated diabetes, such as intake of macronutrients.

To the best of our knowledge, this is the first prospective longitudinal study designed to understand the association between baseline iAs exposure and development of T2D in a population with the low-to-moderate level of iAs exposure. Our findings contribute to the current literature as there are growing concerns about risk factors involved in the pancreatic β-cell

dysfunction. The consistency of our multivariable-adjusted results and those from sensitivity analysis excluding participants age>70 or pre-diagnosed diabetes at follow-up also supports the positive association between iAs exposure and development of diabetes in the population with low-to-moderate iAs exposure.

Strengths of our study include the standard protocol used to collect data over the follow-up period, high-quality laboratory methods used for individual and direct measurement of chronic iAs exposure. In addition, we excluded participants with diabetes at baseline to better capture the early stage changes of iAs-associated glucose homeostasis indicators. Moreover, we take accounted for different measures of T2D (insulin resistance vs. pancreatic β-cell dysfunction), by testing the associations between iAs exposure and insulin resistance vs. β-cell dysfunction, thus informing understanding of the underlying biological mechanism of iAs-associated diabetes.

Our study has several limitations. First, our study used toenail arsenic as the measurement of iAs exposure. Though toenail arsenic has been suggested as a reliable biomarker for iAs exposure over an integrated period of months, toenail arsenic cannot separate different forms of iAs, such as trivalent vs. pentavalent, which may associate with health outcomes differently[90]. Therefore, we were unable to examine the associations between different forms of iAs exposure and measures of T2D development. Moreover, compared with urinary arsenic profiles, toenail arsenic was not able to capture the information about iAs metabolism, a more expanded knowledge of the correlations between toenail arsenic and existing biomarkers (i.e. urinary arsenic) is needed to better interpret our findings. In addition, though our sample size was larger than many previous studies, we had limited cell sizes for stratified analyses. The small cell sizes may have reduced our power to detect the interactions. Thus, statistical significance

should be interpreted with caution, especially for the interactions and stratified analyses. Moreover, missing data is one of the important concerns in our study. We took into account of missing data by conducting multiple imputation, and our multiple imputation results are highly consistent with the complete case analysis, which ensures that data analysis samples in our study reflect the full population.

Tables and figures

Table 3.1. Characteristics of the Study Participants by Concentration of Toenail Arsenic at baseline (i.e. 2009)

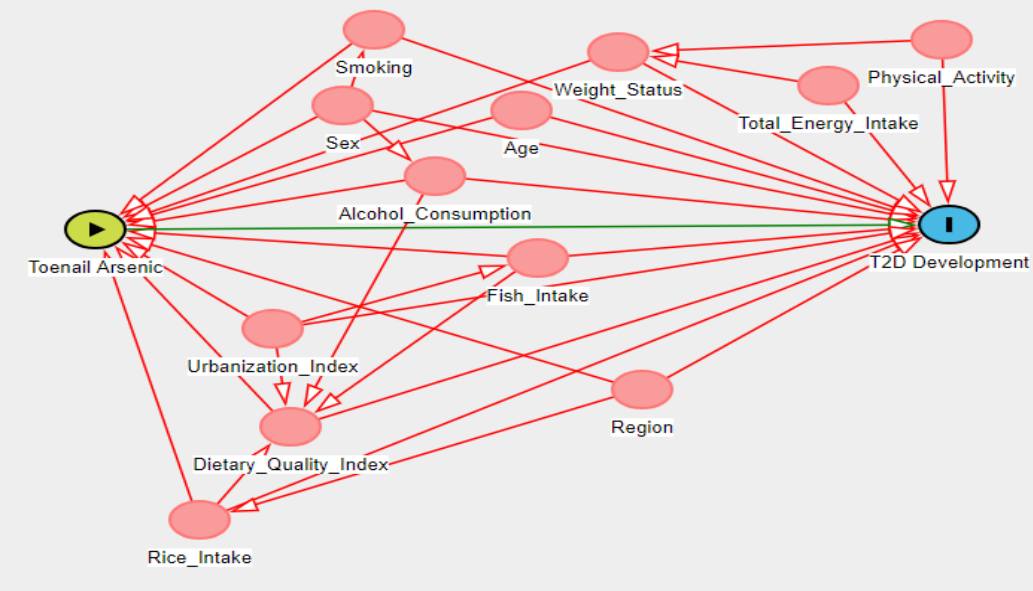
		Toenail arsenic (μg/g) tertiles at 2009		
Characteristics	**All participants**	**<0.23**	**≥0.23 and <0.44**	**>0.44**
Total N	1918	640	639	639
Baseline (i.e.2009) characteristics				
Toenail arsenic, μg/g	0.32 (0.19, 0.54)	0.15 (0.10, 0.18)**	0.31 (0.26, 0.36)	0.68 (0.52, 1.02)
Age, years	51.6±13.0	51.5±13.0	51.5±12.5	52.0±12.9
Male	813 (42.4)	255 (39.8)	273 (42.7)	285 (44.6)
Body mass index, kg/m^2				
23.0-27.4	821 (42.8)	287 (44.8)**	292 (45.7)	242 (37.9)
≥27.5	238 (12.4)	109 (17.0)	66 (10.3)	63 (9.9)
Smoking				
Current	445 (23.2)	138 (21.6)	165 (25.8)	142 (22.2)
Former	61 (3.2)	21 (3.3)	20 (3.1)	20 (3.1)
Drinkers[a]	489 (25.5)	163 (25.5)	172 (26.9)	154 (24.1)
Total energy intake, kcal/day	2167.3±647.0	2156.7±644.1	2188.2±645.8	2157.1±651.7
Rice intake, g/day	640.0±420.2	514.0±380.0**	675.0±428.0	731.2±421.0
Recent fish consumer	752 (39.2)	242 (37.8)*	275 (43.0)	235 (36.8)
Urbanization				
Low	642 (33.5)	154 (24.1)**	251 (39.3)	237 (37.1)
Medium	720 (37.5)	238 (37.2)	205 (32.1)	277 (43.4)
High	556 (29.0)	248 (38.8)	183 (28.6)	125 (19.6)
Total physical activity, METS/week	228.5±221.5	202.5±212.1**	231.9±220.7	251.2±228.9
Region				
North	389 (20.3)	219 (34.2)**	110 (17.2)	60 (9.4)
Central	628 (32.7)	288 (45.0)	217 (34.0)	123 (19.3)
South	901 (47.0)	133 (20.8)	312 (48.8)	456 (71.4)
Baseline Fasting glucose, mg/dL	92.1±10.9	91.7±11.1	91.8±10.8	92.6±10.9
Baseline Fasting insulin, μU/mL	12.1± 9.3	12.9± 10.5*	11.8± 9.5	11.7±7.7
Baseline Hemoglobin A1c, %	5.4±0.4	5.5±0.4**	5.4±0.4	5.3±0.4
Baseline HOMA2-β[b], %	119.5±45.4	123.8±47.5**	117.0± 43.5	117.7± 44.7
Baseline HOMA2-IR[c]	1.5±0.8	1.5±0.9	1.4±0.8	1.5±0.8
Outcome measures at 6-years follow up (i.e.2015)				
Follow-up Fasting glucose, mg/dL	96.8±16.0	95.1±15.8**	96.7±14.4	98.7±17.4
Follow-up Fasting insulin, μU/mL	7.1±4.1	7.1±3.9	7.1±4.0	7.2±4.4
Follow-up Hemoglobin A1c, %	5.6±0.6	5.6±0.5	5.6±0.6	5.6±0.6

Follow-up HOMA2-βs[d], %	86.5±32.8	89.9±32.8**	86.1±32.3	83.7±32.9
Follow-up HOMA2-IRs[e]	1.1±0.6	1.1±0.6	1.1±0.6	1.1±0.7
Follow-up Dysglycemia[f]				
Diabetes	128 (6.7)	32 (5.0)**	49 (7.7)	47 (7.4)
Prediabetes	893 (46.6)	287 (44.8)**	291 (45.5)	315 (49.3)

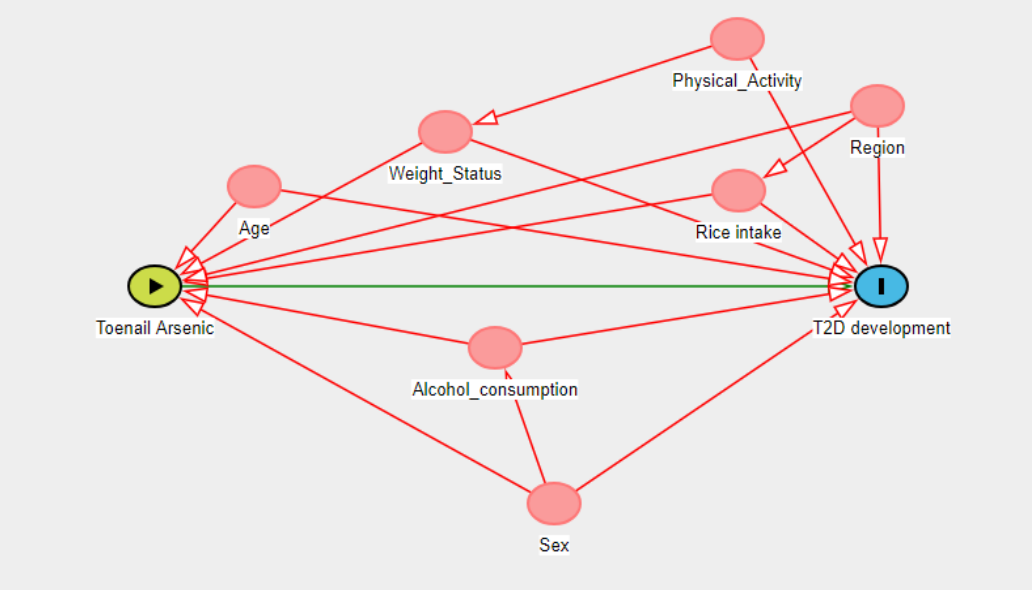
Data are n (%), mean±SD, or median (25th-75th percentile) among individuals involved in the complete case analysis (N=1918). [a] Any alcohol consumption ≥once per month. [b] HOMA2-β: Regular β-cell function calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin, regular HOMA2 is suggested for insulin measured using radioimmunoassay. [c] HOMA2-IR: Regular insulin resistance calculated by HOMA2. [d] HOMA2-βs: Specific β-cell function calculated by HOMA2, specific HOMA2 is suggested when insulin is measured using a chemiluminescent immunoassay. [e] HOMA2-IRs: Specific insulin resistance calculated by HOMA2. [f] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. *p<0.10, **p<0.05 for differences across tertiles of toenail arsenic using one-way analysis of variance (ANOVA), Pearson's chi-square, or Kruskal-Wallis test.

Figure 3.1

a. Potential Confounders Based on Literature Showed in Directed Acyclic Graphs (DAG)



b. Confounders Identified by Stepwise Backward Selection (p<0.10) Showed in DAG



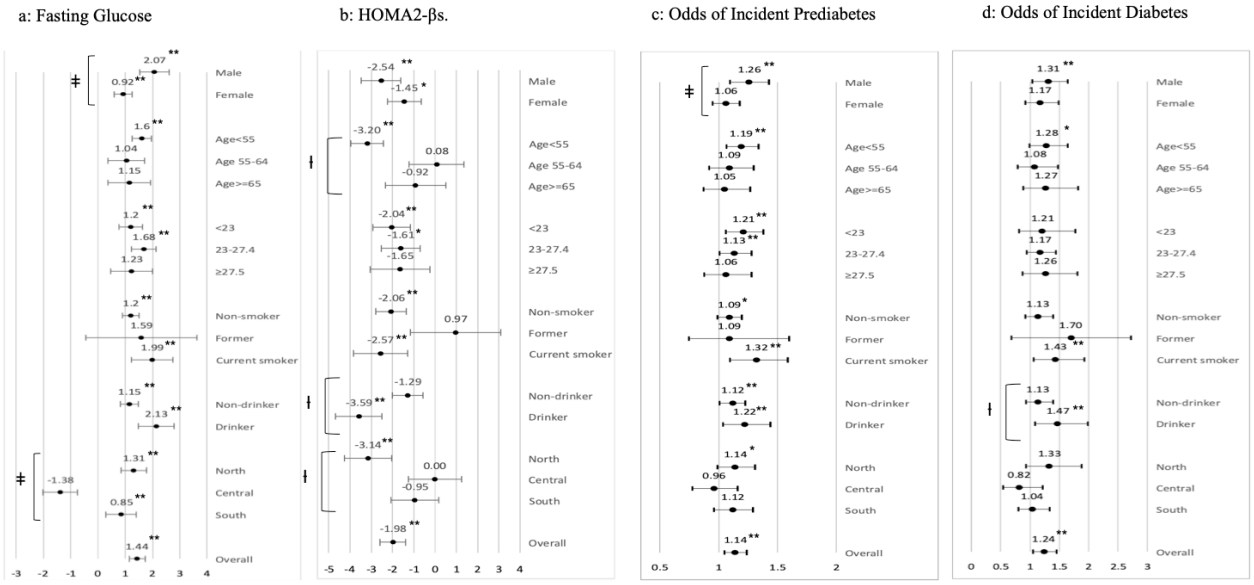
Potential confounders identified based on previous literature in our study included age, sex, drinking status, smoking status, urbanization index, dietary quality index, baseline weight status (weight status may influence the excretion and storage of iAs and it is a well-known risk factor for T2D), fish intake (an important source of non-toxic organic arsenic compounds and is associated with T2D risk), rice intake (an important source of iAs exposure in the study population and is also associated with risk of T2D). We included daily total energy intake and physical activity level in the model since they were associated with risk of T2D. We also adjusted for the geographic region due to substantial regional variation in exposure sources, diet, cooking method, and lifestyle factors across China. Confounders that were identified based on stepwise backward selection (p<0.10) in our study include age, sex, weight status, drinking status, physical activity level, rice intake, and region.

Table 3.2. Associations between Baseline (i.e.2009) Toenail Arsenic and Measures of Diabetes[a] Development during Follow-up, (i.e.2015) Characterized by Follow-up Fasting Glucose, Fasting Insulin, Glycated Hemoglobin, Pancreatic β-cell Function, Insulin Resistance, and Odds of Incident Prediabetes and Diabetes

	ln-toenail arsenic at baseline		
	Model 1: Age, sex, weight status[b], smoking, drinking status, daily total energy intake, dietary quality index, rice intake, fish intake, urbanization index, physical activity level, and region adjusted	Model 2: Age, sex, weight status[b], drinking status, rice intake, physical activity level, and region adjusted	Model 3: Age, sex, weight status[b], drinking status, rice intake, and physical activity level adjusted
Fasting glucose	**0.94±0.33****	**1.01±0.32****	**1.44±0.30****
Fasting insulin	-0.08±0.08	-0.07±0.08	0.06±0.08
Hemoglobin A1c	0.01±0.01	0.01±0.01	0.01±0.01
HOMA2-βs[c]	**-1.3740±0.67****	**-1.59±0.65****	**-1.98±0.60****
HOMA2-IRs[c]	-0.01±0.01	-0.01±0.01	0.01±0.01
Dysglycemia			
Diabetes	**1.18 (0.98 - 1.42)***	**1.19 (0.99, 1.42)***	**1.24 (1.05 - 1.46)****
Prediabetes	**1.08 (0.99 - 1.19)***	**1.10 (0.92 - 1.31)****	**1.14 (1.05 - 1.24)****

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Model 1 adjusted for potential confounders based on literature (Figure 3.1a), including age and sex, weight status (body mass index<18, 23.0-27.4, or ≥27.5), smoking, drinking status, daily total energy intake, dietary quality index, rice intake, fish intake, urbanization index, physical activity level and region. Model 2 adjusted for all the confounders in our study identified by stepwise backward selection ($p<0.10$) (Figure 3.1b). Model 3 adjusted for all identified confounders in our study except region. [a] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. [b] Weight status defined as Underweight: BMI<18kg/m^2; Normal weight: BMI≥23.0 to <27.5kg/m^2; Obese: BMI≥27.5kg/m^2. [c] Specific pancreatic β-cell function (HOMA2-βs) and specific insulin resistance (HOMA2-IRs) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. *$p<0.10$, **$p<0.05$ for coefficients of associations between baseline ln-transformed toenail arsenic and measures of diabetes development at follow-up. **Coefficients were bolded when they reached significance** (P< 0.10).

Figure 3.2. Adjusted Mean (SE) Differences in Indicators of Glucose Homeostasis at Follow-up (i.e.2015) as well as Odds Ratio of Incident Type 2 Diabetes (T2D) Associated with Toenail Arsenic at Baseline (i.e.2009) Stratified by Sex, Age, Weight Status, Smoking and Drinking Status, and Region.



Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models adjusted for all the confounders in our study identified by stepwise backward selection (p<0.10), except region. The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake. [a] HOMA2-βs: Specific β-cell function calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. [b] HOMA2-IRs: Specific insulin resistance calculated by HOMA2. **p*<0.05, ***p*<0.01 for differences in indicators of glucose homeostasis (i.e. FG, FI, HOMA2-IRs, and HOMA2-βs at follow-up) or odds of incident prediabetes and diabetes. ǂ p< .0.05, ł p<0.10 for significance of interaction between ln-transformed toenail arsenic at baseline× sex, age, weight status, smoking and drinking status, and region.

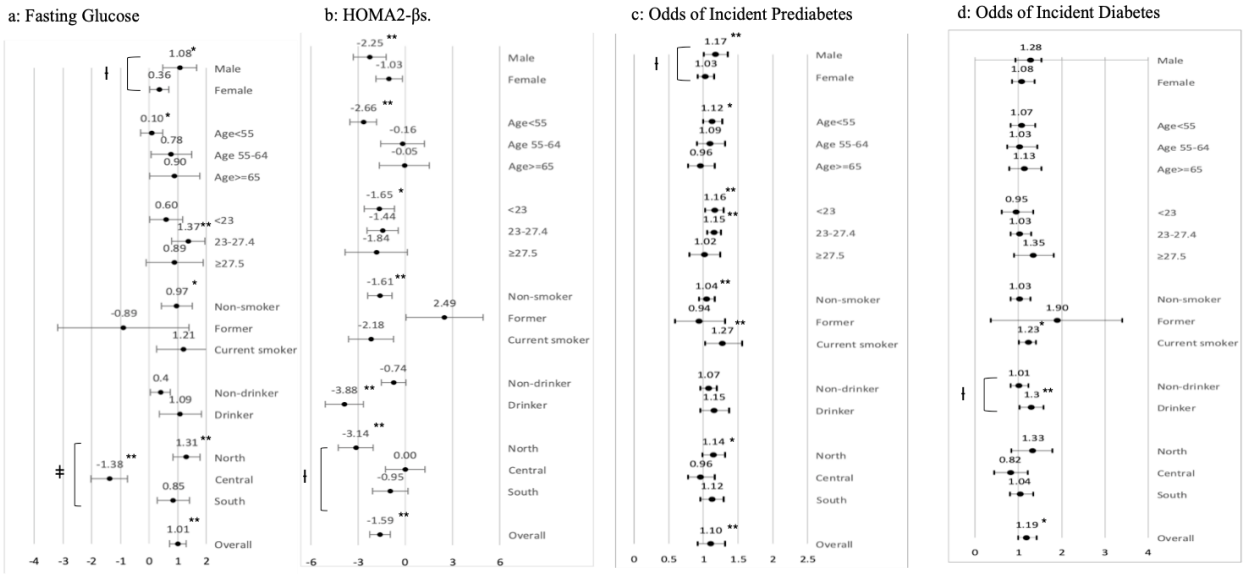
Supplemental Table 3.1. Characteristics of Study Participants by Regions

Characteristics	All participants	Regions		
		North	Central	South
Total N	1918	389	628	901
Baseline (i.e.2009) characteristics				
Toenail arsenic, µg/g	0.32 (0.19, 0.54)	0.21 (0.10, 0.35)**	0.25 (0.16, 0.40)	0.44 (0.29, 0.74)
Age, years	51.6±13.0	51.0±11.8	52.3±12.9	51.5±13.2
Male	813 (42.4)	170 (43.7)	256 (40.8)	387 (43.0)
Body mass index, kg/m^2				
23.0-27.4	821 (42.8)	178 (45.8)**	299 (47.6)	344 (38.2)
≥27.5	238 (12.4)	60 (15.4)	95 (15.1)	83 (9.2)
Smoking				
Current	445 (23.2)	109 (28.0)	130 (20.7)	206 (22.9)
Former	61 (3.2)	15 (3.9)	21 (3.3)	25 (2.8)
Drinkers[a]	489 (25.5)	98 (25.2)	174 (27.7)	217 (24.1)
Total energy intake, kcal/day	2167.3±647.0	2125.4±566.8	2195.8±702.6	2165.5±638.8
Rice intake, g/day	640.0±420.2	636.7±326.3**	395.4±432.8	811.9±358.3
Recent fish consumer	752 (39.2)	137 (35.2)**	220 (35.0)	395 (43.8)
Urbanization				
Low	642 (33.5)	189 (48.6)**	200 (31.9)	253 (28.1)
Medium	720 (37.5)	99 (25.5)	239 (38.1)	382 (42.4)
High	556 (29.0)	101 (26.0)	189 (30.1)	266 (29.52)
Total physical activity, METS/week	228.5±221.5	303.8±295.6**	208.7±199.2	209.8± 189.9
2009 Fasting glucose, mg/dL	92.11±10.99	89.27±11.93**	93.31±10.09	92.50±10.96
2009 Fasting insulin, µU/mL	12.16± 9.35	11.97±8.94**	13.20±11.22	11.52±7.93
2009 Hemoglobin A1c, %	5.44±0.46	5.45±0.35**	5.68±0.39	5.27±0.46
2009 HOMA2-IR[b]	1.51±0.89	1.48±0.93**	1.60±0.92	1.47±0.86
2009 HOMA2-β, %[c]	119.53±45.40	125.33±48.46**	121.98±47.44	115.33±42.11
Outcome measures at 6-years follow up (i.e.2015)				
2015 Fasting glucose, mg/dL	96.8±16.0	91.6±15.0**	97.1±14.5	98.9±16.9
2015 Fasting insulin, µU/mL	7.1±4.1	6.8±3.9*	6.8±3.5	7.5±4.5
2015 Hemoglobin A1c, %	5.6±0.6	5.6±0.5	5.6±0.5	5.6±0.6
2015 HOMA2-IRs[d]	1.1±0.6	1.0±0.6**	1.0±0.5	1.1±0.7
2015 HOMA2-βs, %[e]	86.5±32.8	93.6±35.3**	83.6±28.9	85.6±33.8
2015 Dysglycemia[f]				
Diabetes	128 (6.7)	21 (5.4)**	29 (4.6)	78 (8.7)

Prediabetes	893 (46.6)	146 (37.5)**	319 (50.8)	428 (47.5)

Data are n (%), mean±SD, or median (25th-75th percentile) among individuals involved in the complete case analysis (N=1918). [a] Any alcohol consumption ≥once per month. [b] HOMA2-IR: Regular insulin resistance calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin, regular HOMA2 is suggested for insulin measured using radioimmunoassay. [c] HOMA2-β: Regular β-cell function calculated by HOMA2. [d] HOMA2-IRs: Specific insulin resistance calculated by HOMA2, specific HOMA2 is suggested when insulin is measured using a chemiluminescent immunoassay. [e] HOMA2-βs: Specific β-cell function calculated by HOMA2. [f]Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. *p<0.10, **p<0.05 for differences across regions using one-way analysis of variance (ANOVA), Pearson's chi-square, or Kruskal-Wallis test.

Supplemental Figure 3.1. Adjusted Mean (SE) Differences in Indicators of Glucose Homeostasis at Follow-up (i.e.2015) as well as Odds Ratio of Incident Type 2 Diabetes (T2D) Associated with Toenail Arsenic at Baseline (i.e.2009) Stratified by Sex, Age, Weight Status, Smoking and Drinking Status, and Region.



Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models adjusted for all the confounders in our study identified by stepwise backward selection ($p<0.10$) (Figure 3.1b). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, and region. [a] HOMA2-βs: Specific β-cell function calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. [b] HOMA2-IRs: Specific insulin resistance calculated by HOMA2. *$p<0.05$, **$p<0.01$ for differences in indicators of glucose homeostasis (i.e. FG, FI, HOMA2-IRs, and HOMA2-βs at follow-up) or odds of incident prediabetes and diabetes. ǂ $p< .0.05$, ł $p<0.10$ for significance of interaction between ln-transformed toenail arsenic at baseline× sex, age, weight status, smoking and drinking status, and region.

Supplemental Table 3.2. Cross-sectional Associations between Baseline (i.e. 2009) Toenail Arsenic and Measures of Glucose Homeostasis as well as Odds of Prediabetes and Diabetes[a] at Baseline

	ln-toenail arsenic at baseline		
	Model 1: Age, sex, weight status[b], smoking, drinking status, daily total energy intake, dietary quality index, rice intake, fish intake, urbanization index, physical activity level, and region adjusted	Model 2: Age, sex, weight status[b], drinking status, rice intake, physical activity level, and region adjusted	Model 3: Age, sex, weight status[b], drinking status, rice intake, and physical activity level adjusted
Fasting glucose	-0.51±0.36	-0.53±0.36	-0.33±0.33
Fasting insulin	**-0.30±0.17***	**-0.33±0.19***	**-0.32±0.18***
hemoglobin A1c	**-0.03±0.01***	**-0.03±0.01***	**-0.05±0.01****
HOMA2-β[c]	-0.52±0.92	-0.56±0.92	-1.23±0.85
HOMA2-IR[c]	-0.03±0.02	-0.03±0.02	-0.02±0.02
Dysglycemia			
Diabetes	**0.82 (0.70 - 0.98)****	**0.83 (0.73 - 0.98)****	**0.85 (0.74 - 0.97)****
Prediabetes	1.00 (0.90 - 1.06)	1.00 (0.92 - 1.10)	1.00 (0.92 - 1.09)

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Model 1 adjusted for potential confounders based on literature (Figure 3.1a), including age and sex, weight status (body mass index<18, 23.0-27.4, or ≥27.5), smoking, drinking status, daily total energy intake, rice intake, fish intake, urbanization index, physical activity level and region. Model 2 adjusted for all the confounders in our study identified by stepwise backward selection (p<0.10) (Figure 3.1b). Model 3 adjusted for all identified confounders in our study except region. [a] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. [b] Weight status defined as Underweight: BMI<18kg/m^2; Normal weight: BMI≥23.0 to <27.5kg/m^2; Obese: BMI≥27.5kg/m^2. [c] Regular pancreatic β-cell function (HOMA2-β) and insulin resistance (HOMA2-IR) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. *p<0.10, **p<0.05 for coefficients of associations between baseline toenail arsenic and baseline measures of glucose homeostasis as well as odds of prediabetes and diabetes. **Coefficients were bolded when they reached significance** (P < 0.10).

Supplemental Table 3.3. Baseline (i.e. 2009) Characteristics among Participants Included in The Complete Case Analysis vs. Excluded Due to Missing Data.

Characteristics	Participants included in complete case analysis	Participants excluded due to missing data
Age, years	52 (41, 62)**	49 (38, 61)
Men	42.4%**	50.6%
Body mass index, kg/m^2		
23.0-27.4	42.8%**	35.6%
≥27.5	12.4%	9.4%
Smoking		
Current	23.2%**	30.0%
Former	3.2%	3.6%
Drinker	25.5%**	29.6%
Urbanization		
Low	33.5%	35.7%
Medium	37.5%	30.5%
High	29.0%	33.9%
Region		
North	20.3%	21.6%
Central	32.7%	33.2%
South	47.0%	45.2%
Toenail arsenic, µg/g	0.32 (0.19, 0.54)	0.30 (0.18, 0.52)

Data are percentage or median (25th-75th percentile) *p<0.10, **p<0.05 for differences among participants involved in complete case analysis vs. excluded due to missing data using one-way analysis of variance (ANOVA), or Kruskal-Wallis test.

Supplemental Table 3.4. Associations between Baseline (i.e. 2009) Toenail Arsenic and Measures of Glucose Homeostasis as well as Odds of Prediabetes and Diabetes[a] at Follow-up (i.e. 2015) based on Multiple Imputation

	ln-toenail arsenic at baseline		
	Model 1: Age, sex, weight status[b], smoking, drinking status, daily total energy intake, dietary quality index, rice intake, fish intake, urbanization index, physical activity level, and region adjusted	Model 2: Age, sex, weight status[b], drinking status, rice intake, physical activity level, and region adjusted	Model 3: Age, sex, weight status[b], drinking status, rice intake, and physical activity level adjusted
time	**0.73±0.23****	**0.77±0.27****	**0.97±0.27****
Fasting insulin	0.03±0.15	0.06±0.15	0.20±0.15
hemoglobin A1c	-0.00±0.01	-0.00±0.01	0.01±0.01
HOMA2-β[c]	**-2.01±0.82****	**-2.05±0.88****	**-1.59±0.90***
HOMA2-IR[c]	0.00±0.02	0.00±0.02	0.02±0.01
Dysglycemia			
Diabetes	1.03 (0.95 - 1.18)	1.06 (0.95 - 1.18)	**1.12 (1.00 - 1.26)***
Prediabetes	**1.05 (1.01 - 1.11)***	**1.05 (1.00 - 1.11)***	**1.07 (1.01 - 1.13)****

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Model 1 adjusted for potential confounders based on literature (Figure 3.1a), including age and sex, weight status (body mass index<18, 23.0-27.4, or ≥27.5), smoking, drinking status, daily total energy intake, rice intake, fish intake, urbanization index, physical activity level and region. Model 2 adjusted for all the confounders in our study identified by stepwise backward selection (p<0.10). Model 3 adjusted for all identified confounders in our study except region. [a] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. [b] Weight status defined as Underweight: BMI<18kg/m^2; Normal weight: BMI≥23.0 to <27.5kg/m^2; Obese: BMI≥27.5kg/m^2. [c] Regular pancreatic β-cell function (HOMA2-β) and insulin resistance (HOMA2-IR) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. **p*<0.10, ***p*<0.05 for coefficients of associations between baseline toenail arsenic and baseline measures of glucose homeostasis as well as odds of prediabetes and diabetes. **Coefficients were bolded when they reached significance** (P < 0.10).

Supplemental Table 3.5. Sensitivity Analysis of Associations between Baseline (i.e.2009) Toenail Arsenic and Measures of Diabetes[a] Development during Follow-up, (i.e.2015)

	Age, sex, weight status[b], drinking status, physical activity level, and region adjusted
Fasting glucose	**1.06±0.33****
Fasting insulin	-0.07±0.08
Hemoglobin A1c	0.01±0.01
HOMA2-βs[c]	**-1.62±0.67****
HOMA2-IRs[c]	-0.01±0.01
Dysglycemia	
Diabetes	**1.19 (0.99, 1.42)***
Prediabetes	**1.10 (0.92 - 1.31)****

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Adjusted for all identified confounders (p for stepwise backward regression <0.10) in our study except rice intake. [a] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. [b] Weight status defined as Underweight: BMI<18kg/m^2; Normal weight: BMI≥23.0 to <27.5kg/m^2; Obese: BMI≥27.5kg/m^2. [c] Specific pancreatic β-cell function (HOMA2-βs) and specific insulin resistance (HOMA2-IRs) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. *p<0.10, **p<0.05 for coefficients of associations between baseline ln-transformed toenail arsenic and measures of diabetes development at follow-up. **Coefficients were bolded when they reached significance** (P< 0.10).

CHAPTER 4. THE ASSOCIATIONS BETWEEN INTAKE OF MAGNESIUM AND ZINC—ALONE AND IN COMBINATION WITH INORGANIC ARSENIC EXPOSURE—AND DEVELOPMENT OF type 2 DIABETES: A LONGITUDINAL STUDY IN A POPULATION LOW TO MODERATELY EXPOSED TO INORGANIC ARSENIC

Overview

Laboratory data suggests that the adverse health effects of toxic metals, such as inorganic arsenic (iAs), could be reduced with optimal intake of dietary essential metals, such as magnesium (Mg) and zinc (Zn), which may decrease uptake or counter adverse metabolic effects of toxic metals. In addition, previous studies also found that adequate intake of Mg and Zn may independently reduce the risk of Type 2 diabetes (T2D) given their roles as cofactors in enzymes and proteins critical for insulin synthesis and secretion from pancreatic β-cells. However, human evidence that whether such dietary strategies could effectively counter iAs-associated T2D is limited.

Using unique data from China, a population with low-to-moderate level of iAs exposure and comparatively low prevalence of obesity, we examined the association between baseline (i.e.2009) Mg and Zn intake—alone and in combination with iAs exposure—and the development of T2D over six years. We took advantage of rich longitudinal data from the China Health and Nutrition Survey (CHNS). The dataset has detailed measurements of fasting glucose and insulin at follow-up (i.e.2015), toenail concentration of arsenic that reflects iAs exposure level in recent months at baseline, as well as dietary intake of Mg and Zn estimated from the

mean of the three consecutive days high-quality 24-hour recalls reported by each individual using the Chinese food composition table.

Our results further confirmed the association between deficiency of Zn intake and development of T2D, and the association was most apparent for measures of β-cell dysfunction through which Zn deficiency may influence the development of T2D. In contrast with previous literature, we found no statistically significant association between Mg intake and development of T2D in a population with relatively high prevalence of Mg deficiency.

Our findings support the idea that the susceptibility of iAs-associated diabetes varies depending on levels of Zn intake, and participants with adequate intake of Zn are more susceptible to iAs-associated β-cell dysfunction. Our results also indicate that the mixed results between low-to-moderate iAs exposure and development of T2D could be partially correlated with the varied susceptibility among participants due to different intake of essential metals, such as Zn.

Though the association between iAs exposure and development of T2D was stronger among participants with adequate intake of Zn, the estimated joint associations of Zn intake and iAs exposure in association with T2D stress the importance of having adequate Zn intake. Our findings support the idea that promoting intake of Zn may potentially be an effective strategy to counter the diabetogenic effects of iAs.

Introduction:

Type 2 diabetes (T2D), which is a well-recognized cause of premature death and disability, is a complex disease with multiple contributing factors, such as obesity, environmental pollution, diet, and genetic components[1,44-48]. Compelling evidence has linked high exposure of inorganic arsenic (iAs), a common contaminant in drinking water and foods such as rice, with

increased risk of T2D[55,57,90]. There is growing concern that low-to-moderate level of iAs exposure, which is much more commonly worldwide, may contribute substantially to the current epidemic of T2D, especially in the countries with low prevalence of obesity such as China[51,55-57].

Effective strategies to counter the adverse health effects of low-to-moderate iAs exposure are critical as it is challenging to eliminate iAs from daily life[18,19]. Recently, Petriello et al.[20] has advocated identifying nutritional strategies to mitigate cardio-metabolic effects of toxic metals, such as iAs. Laboratory data also suggests that the adverse health effects of toxic metals could be reduced with optimal intake of dietary essential metals, such as magnesium (Mg) and zinc (Zn), which may decrease uptake or counter adverse metabolic effects of toxic metals[21-27]. However, human evidence on the effectiveness of such dietary strategies to reduce the adverse health effects of iAs is limited, and epidemiological studies have yet to access whether susceptibility to the diabetogenic effects of iAs may vary depending on levels of essential metals intake[28-30]. Indeed, population differences in intake of essential metals may contribute to the heterogeneous findings on iAs-associated diabetes to date, especially among the population low-to-moderately exposed to iAs[21,26,30,58,59]. Thus, studies are urgently needed to examine the association between intake of essential metals and iAs-associated diabetes, especially in the population low-to-moderately exposed to iAs.

Essential metals, notably Mg and Zn, which are cofactors in enzymes and proteins critical for insulin synthesis and secretion from pancreatic β-cells, may also independently affect glucose homeostasis[165-173]. Growing data suggests Mg and Zn may reduce the risk of diabetes, but findings on Zn are inconsistent[169,174-183]. Recent reviews conclude that evidence is insufficient to promote the intake of essential metals to prevent or control diabetes[184,185]. Longitudinal studies

are urgently needed to examine the association between Mg and Zn intake with the development of T2D.

To address this gap, we prospectively assessed whether the susceptibility to the diabetogenic effects of low-to-moderate iAs exposure may vary depending on levels of Mg and Zn intake at baseline (i.e.2009). We also examined the association between baseline Mg and Zn intake with the development of T2D, characterized by fasting glucose, fasting insulin, glycated hemoglobin, and odds of incident prediabetes and diabetes six years later (i.e. 2015), in a population-based cohort with relatively high prevalence of Mg and Zn deficiency. We used the Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) to calculate steady state pancreatic β-cell function and insulin resistance based on measured fasting glucose and fasting insulin at follow-up (i.e.2015)[41-43]. The associations between baseline intake of Mg and Zn—alone and in combination with iAs exposure—and the measures of specific mechanism of T2D at follow-up (i.e. β-cell function and insulin resistance) can help us better understand the early stage change in glucose hemostasis associated with intake of Mg and Zn.

Method

China Health and Nutrition Survey. The China Health and Nutrition Survey (CHNS) is an ongoing longitudinal survey, which collected health, economic, sociological, and demographic data in nine diverse provinces throughout China from 1989-2015. Using a multistage random cluster design, a stratified probability sample was used to select counties and cities stratified by income using State Statistical Office definitions, and then communities and households were selected from these strata. Questionnaires were used to collect demographic, socioeconomic, anthropometric, behavioral and health information. Biomarker data, including

fasting glucose (FG), fasting insulin (FI), and hemoglobin A1c (HbA1c) were collected at both baseline (i.e.2009) and follow-up (i.e.2015). Toenail clippings were collected at baseline and analyzed for arsenic concentration. Survey procedures have been described elsewhere[97,98]. The CHNS cohort initially mirrored national age-gender-education profiles and the nine provinces in the CHNS constituted 44% of China's population in 2009 (according to 2009 census).

The study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill, the China-Japan Friendship Hospital, National Institute for Nutrition and Health in China, and the Chinese Center for Disease Control and Prevention.

Analysis sample. We restricted analyses to non-pregnant adults (≥18y) at time of measurement (i.e. 2009 and 2015) with toenail arsenic data available at the 2009 exam (N=6843). We excluded participants who missed baseline (i.e. 2009) data of body mass index (N=511), smoking or drinking status (N=5), diet (N=80), urbanization index (N=96), physical activity (N=34), province (N=7), any blood biomarkers (FG and HbA1c, N=478). We further excluded participants missed any blood biomarkers (i.e. FG, FI, and HbA1c) in 2015 (N=3479). Participants with extreme biomarkers, which were defined as 6 standard deviation above or below the means, were also excluded (N=15). Individuals with diabetes at baseline were excluded to reduce the possibility of reverse causality (N=220). In addition, participants age>70 at baseline (N=143) were excluded due to differences in metabolism of Mg and Zn in this age group[186,187], resulting in final analytic samples for the complete case analysis N=1775.

Ascertainment of diabetes. Blood samples of all participants were collected by venipuncture after an overnight fast. Whole blood was immediately centrifuged, serum was aliquoted and frozen at -70 degrees. Aliquots were collected in EDTA-coated vacutainers containing sodium fluoride to prevent glucose degradation. All blood samples were analyzed in a

national central lab in Beijing (medical laboratory accreditation certificate ISO15189:2007). Both 2009 and 2015 FG were measured by colorimetric assay methods (GOD-PAP; Randox Laboratories Ltd, UK) using a Hitachi 7600 analyzer (Hitachi Inc., Tokyo, Japan). Both 2009 and 2015 HbA1c was measured with high performance liquid chromatography (HPLC) system (model HLC-723 G7; Tosoh Corporation, Tokyo, Japan). FI in 2015 was measured using chemiluminescent immunoassay.

Diabetes was defined by FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Pre-diabetes was defined by FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. Measured FG and FI were used to calculate steady state pancreatic β-cell function and insulin resistance using the Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php). The HOMA2 calculator is an updated version (computer model) of the original HOMA model (HOMA1). HOMA2 is suggested as a more accurate method than the original HOMA1, since HOMA2 accounts for variations in hepatic and peripheral glucose resistance and the reduction of peripheral glucose-stimulated glucose uptake[41,42]. Specific HOMA2, including specific HOMA2-insulin resistance (HOMA2-IRs) and specific HOMA2-β cell function (HOMA2-βs), was calculated for 2015 since specific HOMA2 is suggested for insulin measured using chemiluminescent immunoassay[41,42].

Measurement of toenail arsenic. Toenail arsenic was used as a measurement of individual chronic iAs exposure since toenails have shown to preferentially sequester iAs species, which is a more toxic form than organic arsenic, and better reflects iAs exposure in recent months compared with routinely used spot urine samples, especially among participants low-to-moderately exposed[70-72,75]. Participants were asked to let toenails grow for at least two weeks,

and stainless-steel nail scissors were used to collect toenail clippings from all ten toes. Toenail clippings were washed and sonicated for 30 minutes and dried at 80℃. After microwave-assisted digestion, toenails were analyzed using inductively coupled plasma mass spectroscopy[99]. The limit of detection (LOD) for arsenic by this method is 0.01μg/g. Toenail arsenic concentrations lower than the LOD were imputed at half of the LOD as 0.005μg/g (N=71/1775). Toenail arsenic was either categorized in tertiles or natural log-transformed to normalize the distribution.

Dietary assessment. Dietary intake was assessed using three consecutive days 24-hour recalls (two weekdays and one weekend day) at the individual level and a food inventory at the household level during the same 3-day period. Trained investigators interviewed the participants to collect detailed types and amounts (in grams) of all foods and beverages consumed during the preceding 24 hours with the assistance of food pictures to aid quantification. Daily intake was calculated by the mean of the three days 24-hour recalls. Chinese food composition table was applied to estimate the mean daily energy intake (kcal), Mg intake (mg), and Zn intake (mg)[100,101] and tertiles of their distributions. In addition, we characterized dietary adequacy of Mg and Zn intake according to the US Recommended Dietary Allowance[188,189] and the Recommended Nutrient Intake[190]. We used the tailored Alternate Healthy Eating Index (tAHEI), which was generated by tailoring the Harvard AHEI 2010, to assess diet quality in our study[191]. Details of tAHEI have been described elsewhere[191].

Other variables. Standard height (in meters) and weight (in kilograms) were measured using standard protocol by trained nurses. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). Weight status was then generated based on BMI according to the World Health Organization cutoffs for Asian adults ($<18kg/m^2$ for underweight, 23.0-27.4kg/m^2 for overweight, and $\geq 27.5kg/m^2$ for obese)[102,103].

Information on age (in years), sex, smoking (current, former, never smokers), drinking status (< vs. ≥once per month) was collected using questionnaires.

A validated urbanization index was used to measure degrees of urbanization[104]. The index contains 10 validated components including communication, economic, housing, transportation infrastructure, the availability of schools, markets, and health care, environmental sanitation, and population size and density. The urbanization index, which was positively correlated with degree of urbanization, ranged from 10 to 100 and was categorized into tertiles. The nine provinces in CHNS were categorized into three geographic regions (North: Heilongjiang, Liaoning; Central: Shandong, Henan, Jiangsu; South: Hunan, Hubei, Guangxi, Guizhou).

Individual physical activity data was collected through an in-depth interview on time (hours) spent in domestic, occupational, transportation, and leisure-time activity. Separate questions were asked for each of the four domains. Energy expenditure from each activity domain was calculated by multiplying time (hours) spent on each type of physical activities by its specific metabolic equivalent (MET) intensity value, resulting in measures of total MET-hours/week for each domain. Overall energy expenditure for each individual was then calculated by summing up MET-hours spent in each domain and categorizing into tertiles.

Statistical analyses. Toenail arsenic was either categorized in tertiles or natural log-transformed to make the distribution more normal. Dietary intake of Mg and Zn were categorized into adequate vs. deficient based on US Recommended Dietary Allowances (RDA) to better compare with previous literature[189,192,193]. ANOVA (continuous variables) and chi-square tests (categorical variables) were used to determine the significance of differences in baseline characteristics across regions.

To determine whether development of T2D during follow-up (i.e.2015) is related to the intake of Mg and Zn at baseline, we first compared indicators of glucose homeostasis (i.e. FG, FI, HbA1c, HOMA2-βs, and HOMA2-IRs) at follow-up among participants with adequate vs. deficient baseline Mg and Zn intake. Medians were used rather than means given the highly non-normal distribution of the indicators of glucose homeostasis (Shapiro-Wilk $P<0.01$). We used a non-parametric Kruskall-Wallis test to identify statistically significant differences in each of the indicators of glucose homeostasis across adequate vs. deficient levels of Mg and Zn intake. We also compared baseline intake of Mg and Zn among participants with vs. without prediabetes and diabetes at follow-up.

We further evaluated associations between deficiency of baseline Mg and Zn intake (vs. adequacy) and T2D development using multivariable-adjusted linear regression models for indicators of glucose homeostasis, and multinomial logistic regression models for odds of incident prediabetes and diabetes. Coefficients from these models were used to estimate the mean differences in each measure of T2D development during follow-up among participants with deficient vs. adequate baseline Mg and Zn intake. Next, we examined whether the associations between baseline iAs exposure and development of T2D varied depending on baseline levels of Mg and Zn intake. We tested the significance of baseline dietary Mg and Zn status × baseline iAs exposure level (ln-transformed toenail arsenic).

Potential confounders of iAs-associated T2D identified based on literature include: age, sex, drinking status, smoking status, urbanization index, baseline weight status (weight status may influence the metabolism and storage of iAs[105-107] and it is a well-known risk factor for T2D), fish intake (an important source of non-toxic organic arsenic compounds (arsenobetaine and arsenosugars)[108,109] and is associated with T2D risk[110,111]), rice intake (an important source of

iAs exposure in the study population[112] and is also associated with risk of T2D[113]), and region (there is substantial regional variation in exposure sources, diet, cooking method, and lifestyle factors across China). We also included daily total energy intake and physical activity level since they are associated with risk of T2D. In addition, we included dietary quality index (i.e. tailored Alternate Healthy Eating Index) in our model to reduce the impact of other dietary factors in Mg and Zn rich foods, such as fibers, on the development of T2D development.

In our study, we adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index.

We also stratified models by levels of baseline Mg and Zn intake to compare the magnitude of the associations between iAs exposure and development of T2D among participants with adequate vs. deficient intake of Mg and Zn, regardless of the significance of the interactions. In addition, we stratified the participants based on both iAs exposure (i.e. tertiles) and intake of Mg and Zn (i.e. adequate vs. deficient) to generate the joint variable for iAs exposure and intake of Mg and Zn. We tested the association between the joint variable and the development of T2D. The coefficients from these models estimated the mean differences in each measure of T2D development after follow-up among participants with different tertiles of iAs exposure and intake of Mg and Zn, relative to participants with lowest tertile of iAs exposure and adequate intake of Mg and Zn.

We conducted several sensitivity analyses. First, we excluded participants with pre-diagnosed diabetes at follow-up (N=18), who might have medication or adjusted their diet pattern after diagnosis. Second, we also excluded participants with extreme self-reported daily

energy intake, which was defined as self-reported daily total energy intake <600kcals (N=2) or >4,500kcals (N=3)[194,195]. Third, we examined whether use of Chinese versus US definitions of Mg and Zn adequacy differed. We set statistical significance of main effects and interactions at $p<0.05$ with $p<0.10$ indicating marginal significance. We used complete case analysis for all the longitudinal analysis. In addition to complete case analysis, we conducted multiple imputation using predictive mean matching (five closest observations was drawn) to take into consideration of the impact from missing data.

Statistical significance of main effects was set at $p<0.05$ with $p<0.10$ indicating marginal significance. All the statistical analyses were performed in Stata, version 13.1 (StataCorp LP, College station, Texas).

Results:

Characteristics and deficiency of Mg and Zn in the target population

Baseline (i.e. 2009) sociodemographic and anthropometric characteristics of the participants involved in the complete case analysis (N=1775), as well as biomarkers at baseline and follow-up (i.e.2015), baseline arsenic exposure level characterized by toenail arsenic are provided in Table 4.1.

The toenail arsenic ranged from below the LOD to 115.6μg/g, with a median of 0.32μg/g, which intersected with ranges reported in the US population (toenail arsenic range=0-3.26μg/g)[77]. Though there is no established cut-point for iAs exposure level (high vs. low) based on toenail arsenic, the overlapping range of toenail arsenic between US studies and our study population suggests that the participants in CHNS are exposed to low-to-moderate levels of iAs[70,117].

Mean intake of Mg and Zn was 298.1mg/day and 10.9mg/day across all participants respectively. We categorized participants into adequate vs. deficient intake of Mg and Zn according to US Recommended Dietary Allowance (Supplemental Table 4.2)[189]. There were 78.9% participants (N=1400) with Mg deficiency and 33.5% participants (N=594) with Zn deficiency. Participants from South China had a significantly higher prevalence of Mg deficiency (84.1%, N=699) and a lower prevalence of Zn deficiency (28.7%, N=241) compared to participants from North or Central China. In addition, the tailored Alternate Healthy Eating Index was statistically significantly lower among participants from South. We also found statistically significantly lower tailored Alternate Healthy Eating Index among participants with higher tertile of toenail arsenic (Supplemental Table 4.1).

In the complete case analysis, baseline (i.e.2009) FG was significantly lower than follow-up (i.e.2015) FG (91.9±11.0 vs. 96.8±16.1mg/dL, $p<0.05$), whereas we found no statistically significant difference in HbA1c at baseline vs. follow-up (5.4±0.4 vs. 5.6±0.6%). The mean 2015 FI was 7.1μU/mL, mean HOMA2-IRs and HOMA2-βs at 2015 were 1.1 and 86.8%. Approximately 6.6% and 45.2% of study participants without diabetes at baseline developed diabetes and prediabetes during six years of follow-up.

Comparing indicators of glucose homeostasis across regions, we found participants from South and Central China had statistically significantly higher FG at baseline and follow-up compared with participants from North China. Individuals from Central China had significantly higher HbA1c at baseline, but we found no statistically significant difference in 2015 HbA1c among participants from different parts of China. We found FI at 2015 was significantly higher among participants from South versus North and Central China (South vs. North vs. Central: 7.5±4.6 vs. 6.8±3.5 vs. 6.9±3.9 μU/mL), while HOMA2-βs was significantly higher among

participants from North versus Central and South China (North vs. Central vs. South: 93.5±34.8 vs. 84.1±28.9 vs. 85.7±33.9%). In addition, we found no statistically significant difference in HOMA2-IRs at 2015 among participants from different parts of China.

Fasting glucose and pancreatic β-cell function at follow-up differed substantially among participants with different levels of baseline Mg and Zn intake.

We compared indicators of glucose homeostasis (i.e. FG, FI, HbA1c, HOMA2-βs, and HOMA2-IRs) at follow-up among participants with different levels of baseline Mg and Zn intake (Table 4.2**)**. Participants with adequate intake of Mg at baseline had significantly ($p<0.05$) lower FG (93.6 vs. 95.2mg/dL) but higher HOMA2-βs (82.4 vs. 79.9%) compared with participants with deficient Mg intake. Similarly, participants with adequate Zn intake at baseline had significantly ($P<0.05$) lower FG (94.3 vs.96.1mg/dL) and higher HOMA2-βs (80.9 vs. 78.6%) compared with participants had deficient intake of Zn at baseline. We found no statistically significant difference in Mg and Zn intake among participants with vs. without prediabetes and diabetes at follow-up (Supplemental Table 4.3).

Based on multivariable-adjusted models (Table 4.3), there was an adjusted mean difference of -5.18±2.09% in HOMA2-βs among participants with deficient Zn intake at baseline compared with participants had adequate Zn intake at baseline. We found positive (though not statistically significant) association between baseline Zn deficiency and follow-up FG (0.82±1.04 mg/dL). In contrast with the descriptive analysis suggesting participants with deficient Mg intake at baseline had significantly higher FG and lower HOMA2-βs at follow-up, we did not find statistically significant association between baseline Mg intake and development of T2D during follow-up in multivariable-adjusted models.

Associations between iAs exposure and development of T2D varied by levels of baseline Zn intake.

Among overall participants, we found negative association between iAs exposure (i.e. ln-transformed toenail arsenic at baseline) and HOMA2-βs at follow up (-1.37±0.67%, $p<0.05$). In addition, there was a positive association between iAs exposure and fasting glucose (0.98±0.32mg/dL, $p<0.05$) after adjusting for identified confounders in our study.

We found statistically significant interaction ($P<0.05$) between baseline iAs exposure (i.e. ln-transformed toenail arsenic) and deficiency of Zn intake at baseline for HOMA2-βs, suggesting that the association between baseline iAs exposure and HOMA2-βs varies depending on the level of Zn intake at baseline (Supplemental Table 4.4).

The adjusted mean differences FG and HOMA2-βs at follow-up associated with baseline iAs exposure stratified by levels of baseline Mg and Zn intake are presented in Figure 4.1. We found statistically significant positive association between baseline iAs exposure and FG among participants with deficient Mg intake at baseline (1.24±0.40mg/dL, $p<0.05$), and the association was significant but weaker among participants with adequate Mg intake at baseline (0.91±0.37mg/dL, $p<0.10$). The positive association between iAs exposure (i.e. ln-transformed toenail arsenic) and FG was only significant among participants with adequate Zn intake at baseline (1.32±0.42mg/dL, $p<0.05$), and the magnitude of the association between iAs exposure and FG among participants with adequate Zn intake was about twice the magnitude of the association among those with deficient Zn intake at baseline (adequate vs. deficient Zn: 1.32±0.42 vs. 0.75±0.69mg/dL). However, the interaction between iAs exposure and deficiency of Zn intake for FG did not reach statistical significance.

We found statistically significant negative association between baseline iAs exposure and HOMA2-βs among participants with deficient Mg intake (-2.34±0.82mg/dL, P<0.05), while the association was null among participants with adequate intake of Mg (-0.46±1.52mg/dL, P>0.10). The decline in HOMA2-βs associated with ln-transformed toenail arsenic was considerably as well as significantly larger among participants with adequate vs. deficient intake of Zn (-2.32% vs. -0.51%, p for interaction<0.05).

Estimated joint associations of iAs exposure (i.e. tertiles of baseline toenail arsenic) and adequacy of Mg and Zn intake on the development of T2D are presented in Table 4.4. Relative to participants with lowest tertile of iAs exposure and adequate intake of Mg at baseline, after multivariable adjustment, there was a statistically significant increment of 3.72mg/dL in FG among individuals exposed to highest tertile of iAs and deficient in Mg intake at baseline, vs. an increment of 2.92mg/dL in FG among participants exposed to highest tertile of iAs but with Mg intake in the adequate range.

Consistent with findings in Mg, we found participants with highest tertile of iAs exposure, including participants with both adequate and deficient intake of Zn, had statistically significantly higher FG compared with participants with lowest tertile of iAs exposure and adequate intake of Zn. After multivariable adjustment, there was a significant mean increment of 3.48mg/dL (p<0.05) in FG among individuals exposed to highest tertile of iAs and with deficient Zn intake at baseline, vs. a significant increment of 3.25mg/dL in FG among individuals with highest tertile of iAs exposure and adequate Zn intake at baseline.

In addition, we found statistically significant differences in HOMA2-βs among individuals with different levels of iAs exposure and Mg and Zn intake. Comparing with participants with adequate intake of Mg and lowest tertile of iAs exposure, there was no

significant difference in pancreatic β-cell function among participants with highest tertile of iAs exposure and adequate intake of Mg. however, we found a significant lower pancreatic β-cell function among those with highest tertile of iAs exposure and deficient intake of Mg (-5.18±2.43%, $p<0.05$). Consistent with what was found for Mg, we also found a statistically significant adjusted mean difference in pancreatic β-cell function among participants with highest tertile of iAs exposure and deficient Zn intake at baseline (-8.50±3.14%, $p<0.05$), but no statistically significant difference was found among those with adequate intake of Zn relative to participants with adequate Zn intake and lowest tertile of iAs exposure.

In models predicting variation in the FI, HbA1c, HOMA2-IRs, odds ratios of incident prediabetes and diabetes, we found no statistically significant effect modification by iAs exposure and intake of Mg and Zn (data not shown).

In sensitivity analyses exploring the effects of additionally excluding pre-diagnosed diabetes at follow-up, or participants with extreme self-reported daily energy intake (i.e. energy intake <600kcals/day or >4,500kcals/day), there was no meaningful difference in results. In addition, we also defined adequacy of Mg and Zn intake based on Chinese Recommended Nutrient Intake[190], the results did not differ meaningfully.

Discussion:

Using unique data from China, a population with low-to-moderate level of iAs exposure and comparatively low prevalence of obesity, we examined the association between baseline (i.e.2009) Mg and Zn intake—alone and in combination with iAs exposure—and the development of T2D over six years. Deficiency of Zn intake at baseline was associated with decreased pancreatic β-cell function, which was measured by HOMA2-βs, and the association remained marginally significant after adjusting for regions. Moreover, we found statistically

significant interaction between baseline Zn intake and iAs exposure. The negative association between iAs exposure and pancreatic β-cell function was stronger among participants with adequate vs. deficient intake of Zn at baseline. These findings suggest that Zn deficiency may be associated with development of diabetes through pancreatic β-cell dysfunction, and susceptibility of iAs-associated diabetes varies depending on the level of Zn intake at baseline in this population.

Based on literature, major sources of iAs exposure in our study population include iAs contaminated air due to coal or charcoal combustion, rice and drinking water contaminated by iAs from natural sources, such as mining[70-72 118-121]. Thus, instead of water iAs, toenail arsenic was used as a measurement of individual chronic iAs exposure in our study to better capture the iAs exposure from different sources[70-72 118,119]. A few studies estimate individual iAs exposure based on urinary arsenic, which is generally regarded as the most reliable indicator of recent exposure to iAs. Nonetheless, a previous rice-feeding trial conducted by Meharg et al.[75] found considerable variation (up to 13-fold) in an individual's total iAs urine content throughout the day, calling into question the robustness of the routinely used spot urine samples, especially among participants low-to-moderately exposed[75]. Compared to a spot urine sample, the long-term accumulation of iAs in nails makes the toenail arsenic more reliable as the biomarker of chronic iAs exposure[74,117].

A large proportion of our sample was deficient in Mg intake (78.9%), whereas Zn deficiency was lower (33.5%) in our sample. The prevalence of Mg and Zn deficiency in our study population is consistent with previous studies conducted in China[54,65,67]. The relatively high prevalence of Mg and Zn deficiency provided an outstanding context to examine such deficiencies in relation to T2D.

We found statistically significant interaction between iAs exposure and Zn intake for pancreatic β-cell dysfunction as measured by HOMA2-βs at follow-up (i.e. 2015). Our finding suggests that the association between iAs exposure and pancreatic β-cell dysfunction varies across population, depending on levels of Zn intake. Very limited studies have examined how intake of Mg and Zn is associated with the susceptibility of iAs-associated diabetes. Steinmaus et al.[26] have stressed the association between Zn intake and iAs metabolism among participants from western United States, who are known low-to-moderately exposed to iAs. They found participants in the higher quartile of Zn intake had lower urinary percent monomethyl arsenic (MMAs%) and higher percent dimethyl arsenic (DMAs%), and the associations remained significant in multivariable adjusted regression models. Based on literature, the relevance of a high vs. low DMAs% and MMAs% for health risks is uncertain, and may depend on the level of iAs exposure. Findings of studies conducted in the population highly exposed to iAs support the positive association between MMA% and risk of cancer and other health outcomes, including cardiovascular diseases and diabetes[39,87,196,197]. However, numerous studies in settings with more moderate exposure, such as in United States, have suggested a higher DMA% associated with increased risk of diabetes and other cardiometabolic outcomes [80-83]. Thus, the findings of Steinmaus et al.[26] support a higher risk of iAs-associated health outcomes among participants with higher intake of Zn, which is consistent with our findings. In contrast, Mitra et al.[28] found no statistically significant association between Zn intake and iAs associated adverse health outcomes in the cross-sectional study conducted in West Bengal, where people are known to be highly exposed to iAs. Inconsistent with our findings, previous laboratory data supports a lower risk of iAs-associated health outcomes among participants with higher intake of Zn, since Milton et al.[27] found Zn decreased iAs-induced apoptosis from in vitro study..

In contrast with the stronger association between iAs exposure and β-cell dysfunction among participants with adequate Zn intake, we found no statistically significant interaction between iAs exposure and Mg intake at baseline. However, the association between iAs exposure and β-cell dysfunction was only statistically significant among individuals with deficient vs. adequate intake of Mg, and the magnitude of the association between iAs exposure and HOMA2-βs was about twice among participants with deficient vs. adequate intake of Mg. Thus, our study suggests the associations between baseline iAs exposure and pancreatic β-cell dysfunction varied among participants with different levels of Mg and Zn intake, and the mixed results of the studies on iAs-associated T2D could be due in part to varied susceptibility among participants with substantial different Mg and Zn intake[21,51,55-60].

To the extent of our knowledge, others have not formally examined the joint associations of essential metals, such as Mg and Zn, and iAs exposure on development of diabetes. However, others have suggested that intake of essential metals could be a strategy to reduce the health effects of toxic metals, such as iAs[24,58]. Notably, though iAs had somewhat stronger association with β-cell dysfunction among individuals with adequate intake of Zn, the joint associations of iAs exposure and adequacy of Zn intake on development of T2D support the idea that adequate Zn intake is beneficial for β-cell function.

Several studies have addressed the associations between Zn level and risks of T2D, but findings are inconsistent[177-183]. The significant negative association between deficiency of baseline Zn intake and pancreatic β-cell function in our study is consistent with laboratory data, which indicates that adequate Zn intake is associated with lower risk of T2D by exerting insulin-like effects and reducing the production of cytokines, which leads to pancreatic β-cell death[167,168,183,198,199]. Similarly, in several epidemiological studies researchers found that Zn was

positively associated with pancreatic β-cell function[167,177-183,198]. However, Karamali et al.[36] and Islam et al.[93] have reported that higher Zn was associated with decreased β-cell function. In contrast with the null association we found between Zn intake and insulin resistance, Vashum et al.[42] and Islam et al.[93] reported higher Zn level was associated with decreased insulin resistance. However, both of these studies were cross-sectional. The statistically significant association found between Zn level and insulin resistance in cross-sectional studies could be explained by the fact that individuals with T2D may progressively develop insulin resistance over time, even if this is not the primary pathway involved in Zn-associated diabetes[13,14,144,200]. With our prospective study design and by excluding participants with diabetes at baseline, we are able to capture the early stage change of T2D development that is associated with Zn intake. In line with most studies, we did not find statistically significant association between Zn intake and FG, HbA1c, as well as the risk of prediabetes and diabetes[201-203]. The null associations could be partially due to the participants who were able to maintain glucose homeostasis at the early phase of T2D development[13,14,144,200]. Additional research is needed to assess the association between baseline Zn intake and the development of T2D for a longer follow-up.

In contrast with a considerable literature indicating lower Mg and Zn levels among diabetic individuals[174,178,181,182,198,203,204], we found no statistically significant difference in baseline Mg and Zn intake among participants with vs. without diabetes. In line with our findings, Fernandez-Cao et al.[198] also reported no statistically significant difference in Zn intake among participants with diabetes vs. non-diabetes based on their systematic literature review, while they found significantly lower blood Zn among participants with diabetes. Thus, the inconsistent results could be partially due to our use of dietary intake whereas most of others have used serum Mg and Zn[174,178,181,182,198,203,204]. Several studies have addressed the impact of

diabetes on the metabolism of metals, posing the risk that altered serum Mg and Zn in diabetic individuals may be a consequence rather than a cause of disease[17,166,204,205].

Several epidemiological studies have addressed the association between dietary Mg deficiency and increased risk of diabetes, and previous findings also indicate Mg deficiency may cause insulin resistance as shown by several studies both in human and in experimental animals[173,175,176,206]. However, we found no statistically significant association between baseline Mg intake and risk of T2D in our study population, which has a relatively high prevalence of Mg deficiency. Moreover, instead of the association between Mg intake and insulin resistance, we found participants with adequate Mg intake had significantly elevated β-cell function. Compared with studies conducted in different countries, the heterogenetic results may be related with substantial variation in dietary composition according to different geographic territory[166,173,175,176,206]. Moreover, as limited participants in our study with adequate intake of Mg, further study is needed to determine the impact of Mg intake in a population with a wide range of Mg intake.

We utilized the unique data from China, a population with relatively high prevalence of Mg and Zn deficiency and comparatively low prevalence of obesity, as well as low-to-moderate level of iAs exposure, which is more typical than high level of iAs exposure around the world[55,207]. To the best of our knowledge, ours is the first prospective longitudinal study designed to examine whether higher intake of Mg and Zn may associate with susceptibility to the diabetogenic effects of iAs. Our findings contribute substantially to the current literature as there is an urgent need to identify dietary strategies to counter the adverse effects of toxic metals, such as iAs, and it has yet been studied by epidemiological studies about how intake of Mg and Zn relates to the association between low-to-moderate iAs exposure and development of T2D[23,27].

Strengths of our study include the standard protocol used to collect data over the follow-up period, high-quality laboratory methods used for individual and direct measurement of chronic iAs exposure. Moreover, few studies on toxic metals, such as iAs, include the high-quality dietary data. The potential for essential metals to reduce the risk of toxic metals has been largely examined using biomarkers of essential metals (e.g. serum and urinary biomarkers). However, others have suggested that toxic metals and their related metabolic effects may diminish essential metals biomarkers, limiting their utility as markers of intake[208,209]. Our study is one of the few studies that directly examine how intake, rather than biomarkers, of Mg and Zn relate to iAs burden. We also excluded participants with diabetes at baseline to better capture the early stage changes of glucose homeostasis to reveal the underlying biological mechanism of Zn and Mg-associated diabetes.

Our study has several limitations. First, though the sample size was larger than in many previous studies, we had limited cell sizes for stratified analyses. The small cell sizes may have reduced our power to detect the interactions. Thus, statistical significance should be interpreted with caution, especially for the interactions and stratified analyses. Second, though three-day 24-hour recall is frequently used in studies to estimate the usual dietary intake, the result of it can be affected by several factors, such as over and under report[210,211]. In our study, we used sensitivity analysis to examine differences in model results when we excluded participants with extreme self-reported daily energy intake (<600 kcal/d and >4,500 kcals/d) to assess the effects from misreporting. We found no statistically significant difference in results before vs. after this exclusion. Third, limited information about supplement intake might have altered our estimates of daily intake of Mg and Zn. However, since mineral supplements, except calcium, are not widely used in this population and none of the participants included in the analysis reported

using supplements, it is unlikely that this study limitation influenced our findings[212]. In addition, our study used toenail arsenic as the measurement of iAs exposure. Though toenail arsenic has been suggested as a reliable biomarker for iAs exposure over an integrated period of months, toenail arsenic cannot separate different forms of iAs, such as trivalent vs. pentavalent, which may associate with health outcomes differently[90]. Therefore, we were unable to examine the associations between different forms of iAs exposure and measures of T2D development. Moreover, compared with urinary arsenic profiles, toenail arsenic was not able to capture the information about iAs metabolism, a more expanded knowledge of the correlations between toenail arsenic and existing biomarkers (i.e. urinary arsenic) is needed to better interpret our findings. In addition, missing data in our study could be a potential concern. We took into account of missing data by conducting multiple imputation, and our multiple imputation results are highly consistent with the complete case analysis, which ensures that data analysis samples in our study reflect the full population.

Tables and figures

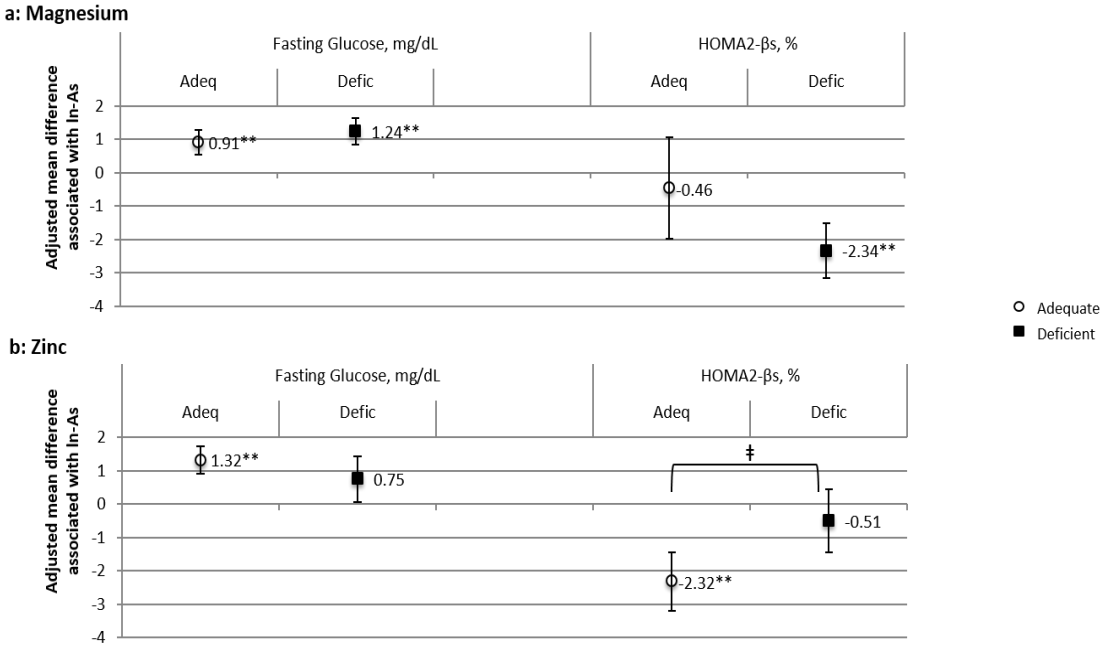
Table 4.1. Characteristics of Study Participants by Regions

Characteristics	All participants	Region		
		North	Central	South
Total N	1775	367	580	828
Baseline (i.e.2009) characteristics				
Age, years	49.7±11.3	49.5±10.5	50.4±11.4	49.4±11.6
Men	747 (42.1)	157 (42.8)	234 (40.3)	356 (43.0)
Body mass index, kg/m^2				
23.0-27.4	787 (44.3)	143 (39.0) **	214 (36.9)	430 (51.9)
≥27.5	756 (42.6)	165 (45.0)	272 (46.9)	319 (38.5)
Smoking				
Current	416 (23.4)	104 (28.3)**	119 (20.5)	193 (23.3)
Former	50 (2.8)	10 (2.7)	17 (2.9)	23 (2.8)
Drinking status[a]	468 (26.4)	95 (25.9)	165 (28.5)	208 (25.1)
Total energy intake, kcal/day	2186.6±639.2	2128.4±566.7	2201.0±684.5	2202.4±636.0
Magnesium intake, mg/day	298.1±106.6	313.7±101.8 **	311.8±112.3	281.7±102.3
Magnesium deficiency	1400 (78.9)	281 (76.8) **	420 (72.5)	699 (84.1)
Zinc intake, mg/day	10.9±3.5	10.8±3.1 **	10.5±3.7	11.2±3.4
Zinc deficiency	594 (33.5)	124 (33.9) **	229 (39.6)	241 (28.7)
Rice intake, g/day	642.8±423.2	646.0±327.6 **	390.1±428.8	818.4±363.9
Fish consumer	710 (40.0)	127 (34.6) **	206 (35.5)	377 (45.5)
Urbanization				
Low	599 (33.8)	187 (51.0) **	184 (31.7)	228 (27.5)
Medium	677 (38.1)	92 (25.1)	228 (39.3)	357 (43.1)
High	499 (28.1)	88 (24.0)	168 (29.0)	243 (29.4)
Total physical activity, METS/week	240.4±224.1	319.9±296.6 **	220.2±201.0	219.3±192.1
Toenail arsenic, µg/g	0.71±4.71	0.29±0.40 **	0.33±0.25	1.17±6.86
Tailored Alternate Healthy Eating Index	39.2±10.7	40.9±9.9**	40.7±10.9	37.3±10.7
Baseline Fasting glucose, mg/dL	91.9±11.0	88.9±11.7 **	93.2±10.2	92.3±10.9
Baseline Hemoglobin A1c, %	5.4±0.5	5.4±0.4 **	5.7±0.4	5.3±0.5
Follow-up (i.e. 2015) Fasting glucose, mg/dL	96.8±16.1	91.6±14.9 **	97.0±14.7	98.9±17.1
Follow-up Fasting insulin, µU/mL	7.1±4.1	6.8±3.9 *	6.8±3.5	7.5±4.6
Follow-up Hemoglobin A1c, %	5.6±0.6	5.6±0.5	5.6±0.5	5.6±0.6
Follow-up HOMA2-βsb, %	86.8±32.7	93.5±34.8 **	84.1±28.9	85.7±33.9
Follow-up HOMA2-IRsc	1.1±0.6	1.0±0.6	1.0±0.5	1.1±0.7
Follow-up Dysglycemiad				

Incident Diabetes	117 (6.6)	19 (5.2) **	28 (4.8)	70 (8.5)
Incident Prediabetes	802 (45.2)	136 (37.1)	284 (49.0)	382 (46.1)

Data are n (%), mean±SD, or median (25th-75th percentile) among individuals involved in the complete case analysis (N=1775). [a] Any alcohol consumption ≥once per month. [b] HOMA2-βs: Specific β-cell function calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin, specific HOMA2 is suggested when insulin is measured using a chemiluminescent immunoassay. [c] HOMA2-IRs: Specific insulin resistance calculated by HOMA2. [d] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. *p<0.10, **p<0.05 for differences across regions using one-way analysis of variance (ANOVA), Pearson's chi-square, or Kruskal-Wallis test.

Figure 4.1. Adjusted Mean (SE) Difference in Follow-up (i.e.2015) Indicators of Glucose Homeostasis Associated with Baseline (i.e.2009) iAs Exposure Stratified by Levels of Magnesium (Mg) and Zinc (Zn) Intake among Non-diabetic Individuals at Baseline



Adjusted mean (SE) difference in follow-up fasting glucose and HOMA2-βs associated with baseline iAs exposure (i.e. ln-transformed toenail arsenic) among participants with adequate vs. deficient baseline Mg and Zn intake. Estimated from linear regression adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index. *$p<0.10$, **$p<0.05$ for differences in indicators of glucose homeostasis associated with ln-transformed baseline toenail arsenic. Ł$p<0.10$, ǂ$p<0.05$ for interactions between baseline Mg or Zn intake (adequate vs. deficient) × baseline iAs exposure (ln-transformed toenail arsenic).

Table 4.2. Indicators of Glucose Homeostasis, Characterized by Fasting Glucose, Fasting Insulin, Glycated Hemoglobin, Pancreatic β-cell Function, and Insulin Resistance at Follow-up (i.e. 2015), by Intake of Magnesium (Mg) and Zinc (Zn)[a] at Baseline (i.e. 2009) (Median, 25th, and 75th Percentiles)

	N	%	Fasting Glucose, mg/dL		Fasting insulin, µU/mL		Hemoglobin A1c, %		HOMA2-βs[b], %		HOMA2-IRs[b]	
			p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)	p50	(p25, p75)
All Participants	1775	100	95.0	(87.5, 102.6)	6.0	(4.5, 8.4)	5.5	(5.3, 5.8)	80.0	(63.4, 103.1)	0.9	(0.7, 1.3)
By Mg intake:												
Adequate Mg	375	21.1	**93.6****	(86.4, 101.7)	5.9	(4.4, 8.3)	5.5	(5.3, 5.8)	**82.4****	(65.2, 106.5)	0.9	(0.6, 1.3)
Deficient Mg	1400	78.9	**95.2**	(87.7, 103.1)	6.0	(4.5, 8.4)	5.5	(5.3, 5.8)	**79.9**	(63.1, 102.1)	0.9	(0.7, 1.3)
By Zn intake:												
Adequate Zn	1181	66.5	**94.3****	(86.9, 102.2)	6.0	(4.4, 8.5)	5.5	(5.3, 5.8)	**80.9****	(64.0, 106.0)	0.9	(0.7, 1.3)
Deficient Zn	594	33.5	**96.1**	(88.4, 103.5)	5.8	(4.5, 8.1)	5.5	(5.3, 5.8)	**78.6**	(62.8, 97.5)	0.9	(0.7, 1.2)

[a] Intakes of Mg and Zn were categorized as Adequate vs. Deficient based on recommended dietary allowances (RDA), details are presented in Supplemental Table 4.2. [b] Specific pancreatic β-cell function (HOMA2-βs) and specific insulin resistance (HOMA2-IRs) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. Specific HOMA2 is suggested when insulin is measured using a chemiluminescent immunoassay. *P<0.10, **P<0.05 for Kruskall Wallis test for differences in indicators of glucose homeostasis among participants with adequate vs. deficient intake of Mg and Zn. **Medians were bolded when the Kruskall Wallis test reached significance** (P < 0.10).

Table 4.3. Associations between Deficiency of Magnesium and Zinc Intake[a] at baseline (i.e.2009) and T2D Incidence and Indicators of Glucose Homeostasis at Follow-up (i.e.2015)

	Adjusted for age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index
Associations with Baseline Magnesium Deficiency	
Follow-up Fasting glucose, mg/dL	-0.04±1.14
Follow-up Fasting insulin, μU/mL	-0.24±0.29
Follow-up Hemoglobin A1c, %	0.03±0.04
Follow-up HOMA2-βs[d], %	-1.91±2.31
Follow-up HOMA2-IRs[d]	-0.04±0.04
Dysglycemia	
Incident Diabetes	1.13 (0.69, 1.86)
Incident Prediabetes	0.97 (0.58, 1.39)
Associations with Baseline Zinc Deficiency	
Follow-up Fasting glucose, mg/dL	0.82±1.04
Follow-up Fasting insulin, μU/mL	-0.31±0.27
Follow-up Hemoglobin A1c, %	-0.06±0.04
Follow-up HOMA2-βs[d], %	**-5.18±2.09****
Follow-up HOMA2-IRs[d]	-0.04±0.04
Dysglycemia	
Incident Diabetes	0.83 (0.46, 1.51)
Incident Prediabetes	0.81 (0.44, 1.46)

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index. [a] Dietary intake of Mg and Zn were categorized into deficient vs. adequate intake based on recommended dietary allowances, details are presented in Supplemental Table 4.2. [b] Diabetes: fasting glucose ≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: fasting glucose≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. [c] Obesity status defined as Underweight: BMI<18kg/m^2; Normal weight: BMI≥23.0 to <27.5kg/m^2; Obese: BMI≥27.5kg/m^2. [d] Specific pancreatic β-cell function (HOMA2-βs) and specific insulin resistance (HOMA2-IRs) calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin. **p*<0.10, ***p*<0.05 for coefficients of associations between dietary intake of Mg and Zn and development of diabetes. **Coefficients were bolded when they reached significance** ($P < 0.10$).

Table 4.4. Estimated Joint Associations of Inorganic Arsenic (iAs) Exposure[a] at Baseline (i.e.2009) and Dietary Intake of Magnesium (Mg) and Zinc (Zn)[b] at Baseline on Measures of Diabetes[c] Development at Follow-up (i.e.2015)

		Follow-up Measures	
	N	**Fasting glucose, mg/dL**	**HOMA2-βs, %**
Estimated Joint associations of Baseline Mg intake and iAs exposure			
Adeq Mg+Tertile1 As[c]	*148*	*ref*	*ref*
Adeq Mg+Tertile2 As	*123*	0.51±2.29	-2.18±3.72
Adeq Mg+Tertile3 As	*108*	**2.92±1.42****	-2.52±3.52
Def Mg+Tertile1 As	*444*	0.47±1.67	-1.16±3.37
Def Mg+Tertile2 As	*468*	1.32±1.58	-2.14±3.42
Def Mg+Tertile3 As	*488*	**3.72±1.88****	**-5.18±2.43****
Estimated Joint associations of Baseline Zn intake and iAs exposure			
Adeq Zn+Tertile1 As	*392*	*ref*	*ref*
Adeq Zn+Tertile2 As	*394*	0.72±1.33	-2.02±2.69
Adeq Zn+Tertile3 As	*399*	**3.25±1.62****	-3.88±3.37
Def Zn+Tertile1 As	*200*	1.81±1.56	-5.64±3.48
Def Zn+Tertile2 As	*197*	2.39±1.55	**-6.27±3.12****
Def Zn+Tertile3 As	*197*	**3.48±1.52****	**-8.50±3.14****

Results (Mean±SE) are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index. [a] Baseline iAs exposure was captured by toenail arsenic and categorized into tertiles. [b] Dietary intake of Magnesium (Mg) and zinc (Zn) were categorized into deficient (Def) vs. adequate (Adeq) based on Recommended Dietary Allowances (RDA), details are presented in Supplemental Table 4.2. [c] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. *$p<0.10$, **$p<0.05$ for adjusted means difference in development of diabetes relative to participants with lowest tertile of iAs exposure and adequate intake of Mg and Zn at baseline. **Coefficients were bolded when they reached significance** ($P < 0.10$).

Supplemental Table 4.1. Characteristics of Study Participants by Concentrations of Toenail Arsenic, China Health and Nutrition Survey

Characteristic	All participants	Toenail arsenic tertiles (μg/g) at 2009		
		<0.23	≥0.23 and <0.44	>0.44
Total *n*	1775	589	594	592
Baseline (i.e.2009) characteristics				
Age, years	49.7±11.3	49.4±11.4	49.7±11.0	50.1±11.5
Men	747 (42.1)	231 (39.2)	253 (42.6)	263 (44.4)
Body mass index, kg/m^2				
23.0-27.4	787 (44.3)	227 (38.5) **	257 (43.3)	303 (51.2)
≥27.5	756 (42.6)	257 (43.6)	271 (45.6)	228 (38.5)
Smoking				
Current	416 (23.4)	129 (21.9)	156 (26.3)	131 (22.1)
Former	50 (2.8)	15 (2.5)	17 (2.9)	18 (3.0)
Drinking status[a]	468 (26.4)	157 (26.7)	163 (27.4)	148 (25.0)
Total energy intake, kcal/day	2186.6±639.2	2170.5±641.8	2199.8±629.8	2189.4±646.8
Magnesium intake, mg/day	298.1±106.6	309.2±114.8 **	295.8±99.4	289.5±104.3
Magnesium deficiency	1400 (78.9)	444 (75.5) **	468 (79.2)	488 (81.8)
Zinc intake, mg/day	10.9±3.5	10.8±3.6	11.0±3.4	11.0±3.4
Zinc deficiency	594 (33.5)	200 (34.0)	197 (33.3)	197 (32.6)
Rice intake, g/day	642.8±423.2	517.1±381.1 **	678.4±428.1	732.1±429.3
Recent fish consumer	710 (40.0)	225 (38.2) *	260 (43.8)	225 (38.0)
Urbanization				
Low	599 (33.8)	147 (25.0) **	233 (39.2)	219 (37.0)
Medium	677 (38.1)	227 (38.5)	193 (32.5)	257 (43.4)
High	499 (28.1)	215 (36.5)	168 (28.3)	116 (19.6)
Total physical activity, METS/week	240.4±224.1	215.9±215.2 **	241.9±222.8	263.2±231.7
Region				
North	367 (20.7)	204 (34.6) **	106 (17.9)	57 (9.6)
Central	580 (32.7)	263 (44.7)	200 (33.7)	117 (19.8)
South	828 (46.7)	122 (20.7)	288 (48.5)	418 (70.6)
Toenail arsenic, μg/g	0.71±4.71	0.14±0.07 **	0.33±0.06	1.68±8.07
Tailored Alternate Healthy Eating Index	39.2±10.7	40.3±10.8**	39.4±10.6	38.0±10.7
Baseline Fasting glucose, mg/dL	91.9±11.0	91.4±11.0	91.7±10.9	92.5±10.9
Baseline Hemoglobin A1c, %	5.4±0.5	5.5±0.4 **	5.4±0.5	5.3±0.5
Follow-up (i.e.2015) Fasting glucose, mg/dL	96.8±16.1	95.1±16.0 **	96.5±14.5	98.6±17.5
Follow-up Fasting insulin, μU/mL	7.1±4.1	7.1±3.8	7.1±4.1	7.2±4.4
Follow-up Hemoglobin A1c, %	5.6±0.6	5.6±0.5	5.6±0.6	5.6±0.6
Follow-up HOMA2-βs, %b	86.8±32.7	89.8±32.5 **	86.7±32.8	83.8±32.7

Follow-up HOMA2-IRsc	1.1±0.6	1.1±0.6	1.1±0.6	1.1±0.7
Follow-up Dysglycemiad				
Incident Diabetes	117 (6.6)	31 (5.3)	46 (7.7)	40 (6.8)
Incident Prediabetes	802 (45.2)	253 (43.0)	260 (43.8)	289 (48.8)

Data are n (%), mean±SD, or median (25th-75th percentile) among individuals involved in the complete case analysis (N=1775). [a] Any alcohol consumption ≥once per month. [b] HOMA2-βs: Specific β-cell function calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) based on fasting glucose and fasting insulin, specific HOMA2 is suggested when insulin is measured using a chemiluminescent immunoassay. [c] HOMA2-IRs: Specific insulin resistance calculated by HOMA2. [d] Diabetes: FG≥126mg/dL, or HbA1c≥6.5%, or self-reported diabetes diagnosis or medication use. Prediabetes: FG≥110mg/dL to <126mg/dL, or HbA1c≥5.7% to <6.5%. *p<0.10, **p<0.05 for differences across tertiles of toenail arsenic using one-way analysis of variance (ANOVA), Pearson's chi-square, or Kruskal-Wallis test.

Supplemental Table 4.2. Recommended Daily Allowances (RDA) for Magnesium (Mg) and Zinc (Zn)

Age	**Men**		**Women**	
	Mg	Zn	Mg	Zn
19–30 years	400mg	11mg	310mg	8mg
≥31 years	420mg	11mg	320mg	8mg

Supplemental Table 4.3. Comparison of Baseline (i.e.2009) Magnesium (Mg) and Zinc (Zn) Intake among Participants with vs. without Prediabetes and Diabetes at Follow-up (i.e. 2015)

	Follow-up Measures					
	Non-diabetic individuals		**Pre-diabetes**		**Diabetes**	
	Median	**p25, p75**	**Median**	**p25, p75**	**Median**	**p25, p75**
2009 Mg intake	283.1	234.2, 353.4	279.7	225.4, 348.5	289.7	226.1, 345.5
2009 Zn intake	10.4	8.6, 12.9	10.4	8.4, 12.9	11	8.9, 13.3

*P<0.10, **P<0.05 for differences in baseline Mg and Zn intake among participants with vs. without prediabetes and diabetes at follow-up based on Kruskal-Wallis test.

Supplemental Table 4.4. Interactions between ln-transformed Toenail Arsenic at Baseline (i.e.2009) and Deficiency of Magnesium (Mg) and Zinc (Zn) Intake[a] at Baseline for Measures of Diabetes Development at Follow-up (i.e.2015) Based on Multivariable-adjusted Linear Regression or Multinomial Logistic Regression Models

	Follow-up Measures						
	Fasting Glucose	**Fasting Insulin**	**HbA1c**	**HOMA2-βs**	**HOMA2-IRs**	**Prediabetes**	**Diabetes**
Baseline Mg	-0.24±0.83	-0.30±0.21	0.02±0.03	-1.87±1.68	-0.05±0.03	0.06±0.12	0.03±0.24
Baseline Zn	-0.37±0.71	0.20±0.18	0.03±0.02	**2.86±1.44****	0.03±0.03	0.08±0.10	0.05±0.20

Results are derived from multivariable-adjusted linear regression (continuous outcomes) or multinomial logistic regression (diabetes, prediabetes, vs. neither) models. Adjusted for the confounders that were identified based on stepwise backward selection ($p<0.10$). The confounders identified in our study include age, sex, weight status, drinking status, physical activity level, rice intake, region, daily energy intake, and dietary quality index. [a] Deficiency of baseline Mg and Zn intake was defined based on US Recommended Dietary Allowances (Supplemental Table 4.2). *$p<0.10$, **$p<0.05$ for significance of interaction between ln-transformed toenail arsenic× deficiency of baseline Mg and Zn intake.

CHAPTER 5. ASSOCIATION BETWEEN VARIANTS IN ARSENIC (+3 OXIDATION STATE) METHYLTRANSFERASE (*AS3MT*) AND URINARY METABOLITES OF INORGANIC ARSENIC: ROLE OF EXPOSURE LEVEL[60]

Overview

Variants in *AS3MT*, the gene encoding arsenic (+3 oxidation state) methyltranserase, have been shown to influence patterns of inorganic arsenic (iAs) metabolism. Several studies have suggested that capacity to metabolize iAs may vary depending on levels of iAs exposure, with shifts in the proportions of different iAs metabolites in urine reported when levels of inorganic As in drinking water exceed 50 ppb. However, it is not known whether the influence of variants in *AS3MT* on iAs metabolism may also vary by level of exposure. We investigated, in a population of Mexican adults exposed to drinking water As, whether associations between seven candidate variants in *AS3MT* and urinary iAs metabolites were consistent with prior studies, and whether these associations varied depending on the level of exposure. Overall, associations between urinary measures of iAs metabolism and *AS3MT* variants were consistent with the literature. Referent genotypes, defined as the genotype previously associated with a higher percentage of urinary dimethylated As (DMAs%), were associated with significant increases in the DMAs% and in the ratio of DMAs to monomethylated As (MAs), and significant reductions in MAs% and iAs%. For three variants, associations with between genotypes and measures of iAs metabolism were significantly stronger among subjects exposed to water As >50 vs. ≤50 ppb (water As X genotype interaction $P<0.05$). For example, for rs10748835, the multivariable-adjusted mean (SE) reduction in DMAs/MAs associated with having polymorphisms other than

the referent genotype was -2.2 (0.8) vs. -0.4 (0.4) among highly vs. less exposed participants. In contrast, for one variant (rs17881215), associations were significantly stronger at exposures ≤50 ppb. Results suggest that iAs exposure may influence the extent to which several *AS3MT* variants affect iAs metabolism. The variants most strongly associated with iAs metabolism—and perhaps with susceptibility to iAs-associated disease—may vary in settings with lower vs. higher exposure.

Introduction:

Chronic exposure to inorganic arsenic (iAs) has been associated with increased risk of several types of cancer, with a substantial literature suggesting iAs exposure may also be associated with other health outcomes, including cardiovascular diseases and diabetes[16,57,90,207]. There are multiple sources of exposure to iAs, including contaminated drinking water, food, soil, and air, as well as occupational settings[16]. Contaminated drinking water is a common source of high exposure, and is a widespread public health problem, estimated to affect around 140 million people worldwide[16,213]. There is growing evidence that, along with levels of exposure to iAs, inter-individual variation in the capacity to metabolize iAs is an important determinant of toxicity, and thus of health risks related to this exposure[89,214]. Using measures of proportions of iAs metabolites in urine, which have been postulated to reflect capacity to metabolize iAs, numerous studies suggest that individual variation in patterns of iAs metabolism may influence susceptibility to adverse health outcomes among subjects exposed to iAs in drinking water[81,82], or may be directly associated with health risks[51,80,87,214,215]. Although the indicators of iAs metabolism most strongly related to risk have varied, associations between measures of iAs metabolism and health risks have been reported in settings with widely varying levels of iAs exposure[80-82,87,196,216].

In humans, the primary pathway for metabolism of iAs involves sequential methylation to form monomethylated As (MAs) and dimethylated As (DMAs) metabolites, which are excreted in the urine[31]. Higher percentages of total urinary As represented by DMAs (DMAs%), and lower percentages of MAs or the unmethylated iAs (MAs% and iAs%, respectively) in urine have been hypothesized to be indicators of higher capacity to metabolize iAs[31,78,79]. The ratios of MAs to iAs (MAs/iAs) and of DMAs to MAs (DMAs/MAs) in urine have also been widely used as indicators of capacity for the first and second methylation steps. However, the measures of iAs metabolism most predictive of increased health risks remain to be established, given the conflicting associations reported in recent studies [31,80-83].

Arsenic (+3 oxidation state) methyltrasferase (AS3MT) is a key enzyme in the pathway for the methylation of iAs, and variants in the *AS3MT* have been shown to be associated with inter-individual differences in iAs metabolism [32-35,84,85]. Previous studies have linked polymorphic sites in this gene to significant differences in urinary measures of iAs metabolism in various populations [32-35,84,86]. It has been suggested that iAs exposure level may modify iAs metabolism, as reflected by changes in urinary As methylation profiles, with a shift in the proportions of urinary metabolites among persons exposed to levels approximately >50ppb vs. ≤50ppb [85,87-89]. We have previously reported based on laboratory experiments that levels and proportions of the methylated products, including DMAs/MAs ratio, differ between recombinant variants of human *AS3MT* and depend on the substrate concentration [36]. However, to our knowledge, no population study has formally explored to what extent associations between *AS3MT* variants and measures of iAs metabolism may vary depending on levels of iAs exposure. Such heterogeneity, if present, could lead to inconsistencies across populations with varying iAs

exposure in the extent to which genetic variants either relate to measures of iAs metabolism, or modify health risks associated with environmental iAs exposure.

The aims of this study were to examine the consistency of previously established associations between multiple *AS3MT* variants and the profiles of urinary iAs metabolites in a population with substantial variability in exposure, and to assess evidence of heterogeneity in the magnitude of these associations depending on the extent to which subjects are exposed to iAs in drinking water.

Method

Study population. Participants were originally recruited for a cross-sectional study on the association of iAs exposure with prevalence of diabetes mellitus in Chihuahua, Mexico, which has been described previously [81]. Briefly, in the parent study, a total of 1160 adults were recruited with a minimum of five year uninterrupted residency in the area. Pregnant women, subjects with urinary tract infection, and individuals with potential occupational exposure to iAs (e.g. those working with pesticides or in mines or smelters) were excluded since these conditions affect the urinary profiles of iAs metabolites. Participants provided samples of household drinking water and spot urine samples in which As metabolites were measured, and interviewer-administered questionnaires were used to collect a wide array of information on factors including health status (including diagnosed diabetes), use of medications, smoking, use of alcohol. Physical exams included an oral glucose tolerance test (OGTT) for detecting undiagnosed diabetes, as well as measures of weight, height, body mass index (BMI), waist and hip circumferences, blood pressure, and skin lesions associated with iAs exposure. For use in sensitivity analyses exploring effects of diabetes, subjects were classified as having diabetes based on fasting plasma glucose (FPG≥126mg/dL) or two-hour post OGTT glucose

(2HPG≥200mg/dL), or on self-reported diagnosis or use of diabetes medication [217,218]. All subjects provided signed informed consent, and the study was approved by the Institutional Review Boards of UNC-Chapel Hill and the Centro de Investigación y de Estudios Avanzados of the Instituto Politécnico Nacional in Mexico City.

Measurements of water and urinary As. The analyses of As in water and urines were described in detail in the parent study [81]. Drinking water was collected in subjects' homes, and the concentration of iAs in these water samples was measured by hydride generation (HG)-cryotrapping (CT)-atomic absorption spectrometry (AAS) [219]. Spot urine samples were collected during morning medical exams as described previously [220]. Concentrations of iAs, MAs and DMAs in spot urine were measured by HG-CT-AAS. Certified standard reference materials (SRMs) from the inter-laboratory comparison program in Quebec and the SRM 2669 (Arsenic Species in Frozen Human Urine) from National Institute of Standards & Technology were used as quality controls.

The limit of detection (LOD) for iAs in water as well As species in urine was 0.01 μg As/L. Concentrations of water iAs and urinary As species which were below LOD (1.9% for water iAs, 1.6% for urinary iAs) were imputed at LOD/2. Total speciated As in urine (tAs) was calculated as sum of the inorganic arsenic species iAs, MAs and DMAs. iAs metabolism was characterized using measures recommended in the literature [221,222], namely percentages of each iAs metabolite (DMAs%, MAs%, iAs%), as well as the ratios of MAs/iAs (also known as the primary methylation index) and DMAs/MAs (the secondary methylation index) in urine.

Genotyping. DNA was isolated from venous blood collected in the OGTT using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. We reviewed the available literature to look for candidate *AS3MT* variants associated with urinary As profiles.

Nine *AS3MT* variants linked to differences in iAs metabolism or susceptibility to iAs toxicity were identified for analysis in this study based on literature published through April 2011 [37-39,84,197,219,222-227]. These *AS3MT* variants included eight single nucleotide polymorphisms (SNPs), rs35232887 (Arg173Trp), rs34556438 (Thr306lle), rs11191439 (Met287Thr), rs17881215 (G4965C), rs3740393 (G12390C), rs3740390 (C14215T), rs11191453 (T35587C), rs10748835 (G35991A), and three variable number of tandem repeats (VNTR) variants, AB, A2B, and A3B. The *AS3MT* variants were analyzed either in the Mammalian Genotyping Core (UNC, Chapel Hill, NC, USA) or in our laboratory, using predesigned or custom TaqMan assays (Applied Biosystems, Carlsbad, CA, USA). The ABI Dual 384-Well GeneAmp PCR System 9700 and ABI PRISM 7900HT Sequence Detection System from Applied Biosystems was used for genotyping and the ABI SDS software for data analysis. VNTR variants and rs17881215 were identified by sequencing a PCR-amplified promoter region.

Due to funding constraints, samples for a random subset of approximately half the subjects in the full sample were sent to the Mammalian Genotyping Core (N=543). Subsequently, an additional random sample was analyzed for rs17881215 and VNTR in our laboratory. Due to genotyping failure, the analysis sample available for each variant measured in the core facility varied in size from N=500 (for rs10748835) to N=506 (for rs3740393), with N=715 available for rs17881215 and VNTR. 772 subjects had data on at least one candidate genetic marker along with measures of water As, iAs metabolism, age and gender. In addition to our primary analyses in the maximum sample with available data for each variant, we analyzed associations in a sample limited to subjects with data available for all candidate variants (N=483) to confirm that effects were not affected by varied sample size; results were not meaningfully different (Supplemental Tables 5.1).

Statistical analysis. We estimated associations between candidate variants in *AS3MT* and each measure of iAs metabolism described above. In all analyses, we defined the variant previously reported to be associated with a higher DMAs% as the referent genotype in order to facilitate comparisons with existing literature. Genotype frequencies were estimated and tested for departure from Hardy-Weinberg equilibrium (HWE) by calculating pairwise r^2 coefficients between variants.

To determine whether candidate *AS3MT* variants were related to biomarker estimates of overall iAs exposure vs. to measures of iAs metabolism, we first compared median (25^{th}, 75^{th} percentile) tAs, as well as each indicator of iAs metabolism, across allelic variants. Medians were used rather than means given the highly non-normal distribution of tAs (Shapiro-Wilk $P<0.01$). The non-parametric Kruskall-Wallis test was used to identify statistically significant differences in each As measure across genotypes for each polymorphism. For all analyses, $P<0.10$ was used *a priori* to define marginal significance and $P<0.05$ to define significance, given the moderate sample size. We further evaluated associations between *AS3MT* genotypes and indicators of metabolism using multiple linear regression models adjusting for age and gender, which are known to influence iAs metabolism [223,228-230]. Coefficients from these models estimated the mean differences in each measure of iAs metabolism among different genotypes of each variant, adjusting for age and sex.

Next, we explored whether associations between *AS3MT* variants and iAs metabolism appeared to vary depending on levels of exposure. To identify *AS3MT* variants for which associations with measures of iAs metabolism differed significantly with changes in the concentration of drinking water As, we tested the significance of polymorphism x water As interactions in age- and sex-adjusted linear regression models using global F tests. Based on

previous literature, interactions were tested defining high vs. low water As exposure with a cutoff of 50ppb, close to the sample median of 48.64 ppb. Age- and sex-adjusted models were also run stratified by higher vs. lower water As to compare the magnitude of associations between *AS3MT* variants and measures of iAs metabolism among individuals more vs. less exposed, regardless of the significance of interactions.

In addition to sensitivity analyses to evaluate the influence of varying sample size described above, we compared the associations between urinary As profiles and *AS3MT* variants before and after adjusting for or excluding persons with diabetes; results did not differ meaningfully (Supplemental Tables 5.2). Additionally, because numerous epidemiological studies have shown that BMI may influence the metabolism of iAs [105-107,153], we evaluated the impact of adjusting for BMI; again, results did not differ meaningfully (data not shown). Similarly, additionally adjusting for either total water As or urinary tAs had no meaningful effect (data not shown). Statistical analysis was performed using STATA version 13 (Stata Corporation, College Station, TX) except Global F test for the polymorphism x water As interactions, which was performed with PROC GLM in SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

Results:

Characteristics of the population and *AS3MT* genotype frequencies.

After excluding subjects with missing data on urinary As species, age, or gender (N=42), there were 772 individuals (520 females, 252 males) with available information on at least one candidate variant. Exposure to water As varied substantially, with a median (25^{th} – 75^{th} percentile) of 48.6 (37.1 – 74.1) ppb and range of 0.01ppb to 419.77ppb. Of the 9 initial candidate *AS3MT* variants, two SNPs (rs35232887 and rs34556438) had a very low frequency

(N<5) for the non-wildtype genotypes and were not used for further analysis. Genotype frequencies varied substantially for all other variants (Table 5.1). Three pairs of SNPs—rs11191439 and rs17881215, rs3740393 and rs3740390, rs3740390 and rs11191453—were in linkage disequilibrium with r^2>0.8. The referent genotype—which we defined as the genotype associated with a higher DMAs% in previously published studies—corresponded to the wildtype (based on global genotype frequency reports from National Center for Biotechnology Information) for only two SNPs (rs11191439 and rs17881215, highly correlated with each other) (Table 5.1). For rs11191453, since there was no observation in our sample with the homozygous variant (CC) previously associated with the highest DMAs%, we used the heterozygous variant (TC) as the referent. For VNTR, as only N=1 subject had the true referent genotype A3B, we defined A2B, which was also associated with a higher DMAs% [33,84], as the referent. A3B was excluded from further analyses due to low frequency (N=1).

Urinary measures of arsenic metabolism varied substantially with variants.

Table 5.1 also shows the median (25^{th}, 75th percentile) of each measure of iAs metabolism—DMAs%, MAs%, iAs%, DMAs/MAs, and MAs/iAs—overall and stratified by genotypes of each *AS3MT* variant. Values for the percentages and ratios of urinary metabolites were consistent with the full study population[81]. The median (25^{th}, 75th percentiles) for tAs, DMAs%, MAs%, iAs%, DMAs/MAs, and MAs/iAs were 62.9µg/L (34.2µg/L, 108.4µg/L), 76.9% (71.3%, 81.4%), 13.9% (10.9%, 17.4%), 8.8% (6.3%, 12.2%), 5.5 (4.2, 7.4), and 1.6 (1.2, 2.1), respectively. Water As was significantly positively correlated with urinary tAs (Spearman's R =0.23, P<0.01).

In descriptive analysis, no significant difference in tAs was observed across genotypes for any of the seven candidate variants (P>0.10; Table 5.1). Descriptive analyses also showed

that consistent with prior literature, the referent genotype was associated with increases in the DMAs% for all seven candidate markers. These differences were significant ($P<0.05$) for five variants, and marginally significant for two (VNTR and rs10748835, $P<0.10$). The five variants associated with significant increases in the DMAs% were also associated with significant reductions in the MAs% ($P<0.05$); VNTR and rs10748835 were associated with small but non-significant reductions in this measure. Similarly, the DMAs/MAs ratio was significantly higher among participants with the referent genotype for six variants, the exception being VNTR. There was a reduction in iAs% observed for the referent genotypes for six of the candidate markers examined, although differences were at least of marginally significance for only three variants. The exception to this pattern, rs3740393, showed lower iAs% for the heterozygous (GC) instead of referent homozygous (CC) genotype. In contrast to the patterns observed for all other measures of iAs metabolism, there were no clear pattern and no significant differences in the MAs/iAs ratio across genotypes in any of the candidate variants.

Adjusted mean differences in urinary measures of iAs metabolism associated with having polymorphisms other than the referent genotypes are shown in Table 5.2, based on multivariable linear models adjusted for age and gender. Results were similar to descriptive analyses. Having non-referent genotypes was associated with significant ($P<0.05$) decreases in the DMAs% for at least one genotype in all seven variants, significant increases in the MAs% for five variants, and marginally significant increases ($P<0.10$) in the MAs% for rs10748835 and VNTR. Excluding some genotypes with very small cell sizes ($N<5$), the five SNPs significantly associated with these measures of iAs metabolism (rs3740390 and two correlated SNPs rs3740393 and rs11191453; rs17881215 and the correlated SNP rs11191439) were associated with the largest adjusted mean differences in both DMAs% (from 3.3±0.7% to 4.6±2.2%) and MAs% (from

1.8±0.5% to 3.9±1.4%), with smaller differences associated with polymorphisms in rs10748835 and VNTR.

Significant differences in measures of iAs metabolism associated with genetic polymorphisms were most notable for the DMAs/MAs ratio. Compared to the referent genotype, all other genotypes in each variant were associated with at least marginally significant (P<0.10) differences in DMAs/MAs with only two exceptions: the homozygous variant of rs11191439, for which the cell size was small (N=3), and polymorphisms in VNTR, for which the association was null. The magnitude of association with DMAs/MAs was strongest—a >2-unit change—for variants in rs3740393 and the correlated SNP rs3740390, with the weakest associations—of about half this magnitude—for rs10748835. Like the descriptive analyses, few variants (n=3) were significantly associated with the iAs%, and none with the MAs/iAs ratio, after multivariable adjustment.

Associations between *AS3MT* variants and urinary measures of iAs metabolism among subjects with higher versus lower exposure to drinking water As.

There were interactions between elevated (>50ppb) exposure to water As and several genetic variants, suggesting that the degree to which genotype in these variants influence patterns of iAs metabolism may vary with increasing exposure. Interactions were significant primarily for the DMAs/MAs ratio (P<0.05 for three variants: the correlated SNPS rs3740390 and rs3740393, as well as rs10748835; Figures 5.1c, Supplemental Table 5.3). There were also marginal significant (P<0.10) interactions between exposure level and one variant (rs17881215) for the DMAs% and MAs%.

As shown in Figure 5.1c, the decline in the DMAs/MAs ratio associated with having genotypes other than the referent was considerably as well as significantly larger among more vs.

less highly exposed individuals for three SNPs: rs3740390, the correlated SNP rs3740393, and rs10748835 (see also Supplemental Table 5.4). In the sample as a whole, the magnitude of associations with the DMA/MAs ratio were strongest for the first two SNPs. However, except for the null relationship with VNTR, this last SNP—rs10748835—was the variant most weakly associated with the DMAs/MAs in the overall sample. Though interactions did not reach significance, the magnitude of associations with polymorphisms in rs10748835 and both the DMAs% and MAs% were more than two times larger among highly vs. more moderately exposed individuals (Figures 5.1a and 1b, Supplemental Table 5.4).

In contrast to the stronger associations seen at higher levels of exposure for these three variants, for polymorphisms in rs17881215 the magnitude of association with both the DMAs% and MAs% was marginally significantly weaker at higher exposure ($P<0.10$; Figures 5.1a and 1b, Supplemental Tables 5.3 and 5.4). There was, however, no difference in the magnitude of association between polymorphisms in this SNP and the DMAs/MAs ratio at high vs. low levels of exposure.

In models predicting variation in the iAs% and the MAs/iAs ratio, no significant effect modification by water As was observed. However, for both outcomes, the magnitude of associations appeared to vary depending on the level of water As exposure for polymorphisms that included both rs10748835 and rs17881215, for which interactions reached significance for other measures (Supplemental Table 5.4, Figure 5.1d and 1e).

In sensitivity analyses exploring the effects of additionally adjusting for diabetes (Supplemental Table 5.2), or for BMI, urinary tAs and water As (not shown), there were no meaningful differences in results.

Discussion

Inter-individual variation in urinary measures of iAs metabolism has been associated with the risk of adverse health outcomes associated with iAs exposure [51,81,89,214], indicating that factors influencing metabolism may affect susceptibility to disease. There is growing evidence—including a recent review—that a number of candidate variants in *AS3MT* affect iAs metabolism [32-35,84,85]. At present, evidence on the consistency with which these variants relate to markers of metabolism across populations is limited and mixed, perhaps in part due to the small sample size and modest power in most previous studies (N<300)[33,40]. Moreover, although it has been suggested that the extent to which some variants influence iAs metabolism might be stronger among subjects more highly exposed to iAs [89], to our knowledge, studies have yet to formally explore such heterogeneity. In this study, we aimed to confirm relationships between seven candidate *AS3MT* variants and urinary markers of iAs metabolism [32,37,40,84,216,219,223-226,231,232], and examine the extent to which these associations may vary depending on the level of exposure to drinking water As.

In this study, genotypes associated with a higher DMAs% in previous studies were consistently associated with significantly higher DMAs%, DMAs/MAs and lower MAs%; almost all associations were at least marginally significant at P<0.10. Reference genotypes also tended to be associated with a lower iAs%, though these relationships were largely not significant. However, associations with the ratio of MAs to iAs, which has been used as an indicator of the efficiency of the first methylation step, were consistently non-significant, and varied considerably in terms of direction and magnitude.

We found significant interactions between four of the seven candidate *AS3MT* variants and concentrations of As in drinking water. Three SNPs (rs3740393, rs3740390, and

rs10748835) had somewhat stronger associations with indicators of iAs metabolism among individuals exposed to higher vs. lower levels of As in drinking water, with significant differences in the magnitude of association for the DMAs/MAs ratio. In contrast, one SNP (rs17881215) was more strongly associated with two markers of iAs metabolism—the DMAs% and MAs%—among participants with lower rather than with higher exposure. This suggests that the genetic variants most influential for aspects of iAs metabolism may vary across populations, depending on prevailing levels of exposure. This finding also suggests the possibility that the variants most influential for modifying health risks may differ in more highly exposed populations than in settings with low exposure. Though earlier studies, to the best of our knowledge, have not formally examined whether the degree of exposure to iAs may modify the influence of *AS3MT* variants on iAs metabolism, prior literature has suggested that patterns of metabolism may vary by level of exposure [88,228,230,233,234]. Several studies have suggested that at exposures exceeding 50 ppb, there may be an increase in the MAs% in urine and decrease in DMAs%, perhaps due to saturated capacity for the secondary methylation step or the inhibition of AS3MT activity by high levels of iAs [88,228]. At these higher exposures, genetic variants may be either more, or less, influential on iAs metabolism. However, in our sample, we did not observe meaningful differences in urinary As profiles between groups exposed to higher (≥50 ppb) vs. lower concentrations of water As in our study. The median (25^{th}, 75^{th} percentile) for more vs. less exposed groups were 77.2% (71.4%, 81.8%) vs. 76.5% (70.9%, 81.2%), 14.0% (11.1%, 17.4%) vs. 13.6% (10.9%. 17.4%), and 5.4 (4.2, 7.3) vs. 5.6 (4.2, 7.4) for DMAs%, MAs% and DMAs/MAs accordingly (Kruskall-Wallis P>0.10 for all pairwise comparisons).

Interactions between *AS3MT* variants and levels of exposure (i.e. water iAs) did not depend on the magnitude of the association between those variants and urinary iAs profiles in the

overall population. For example, in the population as a whole, polymorphisms in rs10748835 were much more weakly associated with urinary iAs metabolites than those in rs3740393. However, there were significant differences in the magnitude of association with the DMAs/MAs ratio among participants with high vs. low exposure for both variants.

In our analyses, the referent genotype, defined as the one previously associated with a higher DMAs%--and postulated to be associated as well with a higher DMAs/MAs, lower MAs% and lower iAs%-- was not always the major (i.e. most frequent) genotypes in our sample. Indeed, the postulated beneficial referent genotype was the major genotype for only rs11191439 and rs17881215. However, defining the referent genotype based on the hypothesized direction of association facilitated comparisons with previous literature.

It is unclear what the implications of the *AS3MT* variant-iAs metabolism associations are for health of iAs-exposed individuals. Based on previous literature, the relevance of a high vs. low DMAs%, MAs% or DMAs/MAs ratio for health risks is uncertain, and may depend on the level of exposure. Several studies in high exposure settings have found a high MAs% to be associated with increased risk of cancer and other health outcomes, including cardiovascular diseases and diabetes [39,87,196,197]. However, numerous studies in settings with more moderate exposure have reported a higher DMAs% to be associated with increased risk of diabetes and other cardiometabolic outcomes [80-83]. The conflicting results for the associations between urinary iAs methylation profiles and health outcomes may be due to differences in the distribution of trivalent and pentavalent methylated metabolites (MAsIII vs. MAsV; DMAsIII vs. DMAsV) which exhibit different toxicities in laboratory models [39,79,80,83,235-238]. However, differentiating between the trivalent and pentavalent As species in urine is technically challenging and is rarely implemented in population studies [239]. Therefore, analyses performed in most population studies,

including the present study, are typically limited to measurements of total iAs, MAs and DMAs, and the percentages and ratios of these metabolites in urine.

This study was conducted in the Chihuahua area of Mexico, with moderately elevated levels of iAs in drinking water (median concentration of 48.6 ppb). Many previous studies have been conducted in areas with substantially higher exposure [90] (e.g. water iAs exposure level for studies done in Bangladesh is likely to be between 100 and 200 ppb [82,87,233,240]). Thus there is uncertainty regarding the health effects of more moderate levels of iAs exposure, and the influence of genetic polymorphisms on iAs metabolism and toxicity, in areas with moderate exposure, which are more typical population exposures around the world. The range of exposure in this Chihuahua cohort—with nearly 50% of subjects at levels below 50 ppb—enabled us to analyze interactions between *AS3MT* variants in the moderate range of water As exposure at which some studies have suggested iAs metabolism may shift [88,228].

A limitation of this study is that, although the sample size was larger than in many previous studies[33,37,40,223,224], we had small cell sizes for genotypes of several candidate *AS3MT* variants, particularly in analyses stratified by water As. The small cell sizes may have reduced our power to detect interactions between level of exposure and methylation profiles for those variants.

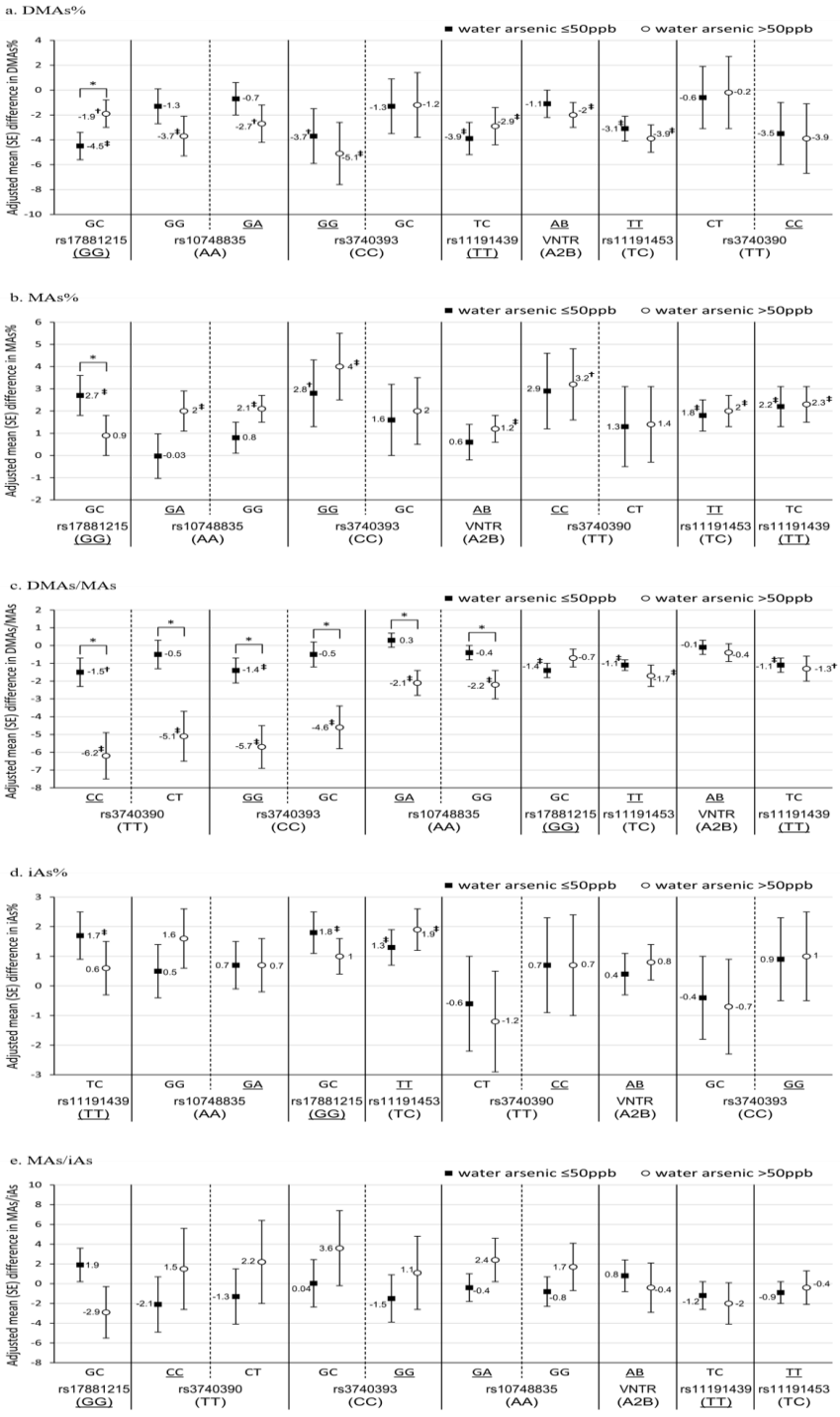
Tables and figures

Table 5.1. Urinary As profiles of arsenic metabolites overall and by *AS3MT* variants (median and 25th, 75th percentiles)

		N	%	Total Speciated Urine Arsenic (tAs)		Dimethylated Arsenic % (DMAs%)		Monomethylated Arsenic % (MAs%)		Unmethylated Inorganic Arsenic % (iAs%)		DMAs/MAs Ratio		MAs/iAs Ratio	
				p50	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***	**p5**	***(p25, p75)***
All subjects		772		62.9	*(34.2, 108.4)*	76.9	*(71.3. 81.4)*	13.9	*(10.9,17.4)*	8.8	*(6.3, 12.2)*	5.5	*(4.2, 7.4)*	1.6	*(1.2, 2.1)*
By AS3MT variant:															
rs11191439[1]	*TT	412	*82*	59.5	*(31.3, 105.5)*	**77.3‡**	*(71.7, 81.8)*	**13.5‡**	*(10.8, 16.7)*	8.9	*(6.1, 12.4)*	**5.7‡**	*(4.4, 7.6)*	1.5	*(1.1, 2.1)*
	TC	86	*17*	52.5	*(30.4, 99.7)*	**74.9**	*(66.5, 79.0)*	**15.8**	*(13.0, 20.0)*	9.8	*(6.3, 14.5)*	**4.7**	*(3.3, 6.0)*	1.8	*(1.2, 2.5)*
	CC	3	*1*	64.8	*(26.6, 151.0)*	**65.6**	*(59.2, 73.8)*	**19.7**	*(13.9, 27.8)*	13.0	*(12.2, 14.7)*	**3.3**	*(2.1, 5.3)*	1.3	*(1.1, 2.1)*
rs17881215[1]	*GG	579	*81*	61.4	*(33.1, 107.4)*	**77.6‡**	*(72.8, 82.5)*	**13.4‡**	*(10.7, 16.6)*	8.6	*(6.1, 11.6)*	**5.8‡**	*(4.4, 7.6)*	1.6	*(1.2, 2.1)*
	GC	124	*17*	67.5	*(38.0, 112.8)*	**74.9**	*(69.0, 80.5)*	**15.1**	*(12.8, 19.2)*	8.8	*(6.7, 13.9)*	**4.9**	*(3.6, 6.3)*	1.6	*(1.2, 2.3)*
	CC	12	*2*	45.2	*(34.3, 82.4)*	**73.9**	*(65.3, 78.7)*	**19.8**	*(12.6, 21.6)*	11.2	*(5.0, 13.1)*	**3.7**	*(3.0, 6.2)*	1.8	*(1.3, 3.0)*
rs3740393[2]	GG	280	*55*	59.0	*(30.9, 97.4)*	**75.3‡**	*(69.3, 79.6)*	**14.7‡**	*(11.7, 18.9)*	**9.9‡**	*(7.1, 13.1)*	**5.1‡**	*(3.8, 6.8)*	1.6	*(1.2, 2.1)*
	GC	200	*40*	57.2	*(30.6, 107.6)*	**78.6**	*(72.9, 83.3)*	**13.0**	*(10.8, 16.0)*	**8.3**	*(5.4, 12.0)*	**5.8**	*(4.6, 7.6)*	1.6	*(1.1, 2.3)*
	*CC	26	*5*	72.6	*(46.5, 125.0)*	**80.7**	*(74.0, 84.4)*	**12.0**	*(9.4, 15.0)*	**9.2**	*(4.3, 12.0)*	**6.7**	*(5.3, 9.0)*	1.2	*(1.0, 1.8)*
rs3740390[2, 3]	CC	293	*58*	59.9	*(32.4, 99.5)*	**75.5‡**	*(69.4, 79.7)*	**14.5‡**	*(11.8, 18.8)*	**10.0‡**	*(7.1, 13.3)*	**5.3‡**	*(3.8, 6.8)*	1.6	*(1.1, 2.1)*
	CT	192	*38*	56.6	*(30.1, 105.6)*	**78.7**	*(73.5, 83.4)*	**12.8**	*(10.5, 15.9)*	**8.1**	*(5.4, 11.9)*	**6.2**	*(4.7, 7.8)*	1.6	*(1.1, 2.3)*
	*TT	20	*4*	71.3	*(36.9, 119.2)*	**80.3**	*(70.2, 86.0)*	**12.6**	*(9.0, 15.3)*	**7.9**	*(4.4, 14.8)*	**6.3**	*(4.7, 9.5)*	1.2	*(0.9, 1.7)*
rs11191453[3]	TT	290	*58*	59.7	*(32.4, 99.5)*	**75.3‡**	*(69.2, 79.7)*	**14.6‡**	*(11.8, 18.9)*	**10.0‡**	*(7.1, 13.4)*	**5.2‡**	*(3.7, 6.8)*	1.6	*(1.2, 2.1)*
	*TC	212	*42*	57.9	*(30.1, 107.6)*	**78.8**	*(73.4, 83.5)*	**12.8**	*(10.3, 15.9)*	**8.1**	*(5.3, 12.0)*	**6.2**	*(4.7, 8.0)*	1.6	*(1.1, 2.3)*
rs10748835	GG	151	*30*	60.5	*(33.1, 99.5)*	**76.0†**	*(70.1, 80.2)*	14.5	*(12.0,18.1)*	9.5	*(7.1, 12.9)*	**5.4†**	*(3.9, 6.6)*	1.5	*(1.1, 2.1)*
	GA	251	*50*	57.2	*(29.4, 104.3)*	**76.7**	*(70.3, 81.3)*	13.7	*(10.8, 17.4)*	9.0	*(6.1, 12.9)*	**5.5**	*(4.1, 7.6)*	1.6	*(1.1, 2.1)*
	*AA	98	*20*	64.8	*(32.0, 112.6)*	**78.5**	*(73.8, 83.5)*	13.7	*(10.3, 15.9)*	8.3	*(5.1, 11.7)*	**5.7**	*(4.6, 7.7)*	1.6	*(1.1, 2.2)*
VNTR	AB	587	*82*	61.5	*(33.2, 112.0)*	**76.9†**	*(70.9, 81.5)*	13.9	*(11.0, 17.4)*	8.7	*(6.2, 12.3)*	5.5	*(4.1, 7.3)*	1.6	*(1.2, 2.1)*
	*A2B	127	*18*	60.5	*(34.2, 92.6)*	**77.9**	*(73.8, 82.1)*	13.6	*(10.6, 16.1)*	8.3	*(6.3, 10.5)*	5.6	*(4.6, 7.9)*	1.6	*(1.2, 2.2)*

***** Identifies the referent genotype, defined as the genotype associated in previous literature with a higher DMAs%. † P<0.10 ‡P<0.05 for Kruskall wallis test for differences in urinary As metabolites among different genotypes of the *AS3MT* variants. **Medians bolded when the Kruskall Wallis test reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Figure 5.1. Associations between AS3MT variants and urinary arsenic profiles among subjects with higher vs. lower water arsenic (As)



† P<0.10 ‡ P<0.05 for differences in urinary As profiles associated with having non-referent *AS3MT* variants genotypes compared with referent genotypes, where referent genotypes were defined as those associated with a higher DMAs% in previous studies. * Indicates interaction (P<0. 10) for variant X categorical water iAs (>50 vs. ≤50ppb). Results come from multiple linear regression models adjusted for age and gender.

Table 5.2. Adjusted associations between *AS3MT* variants and urinary profiles of arsenic metabolites[a]

Polymorphism		N	Dimethylated Arsenic % (DMAs%)		Monomethylated Arsenic % (MAs%)		Unmethylated Inorganic Arsenic % (iAs%)		DMAs/MAs Ratio		MAs/iAs Ratio	
			Coeff (SE)	*P*	Coeff (SE)	*P*	Coeff (SE)	*P*	Coeff (SE)	*P*	Coeff (SE)	*P*
rs11191439[1]	TC	86	**-3.5 (1.0)**	*0.00‡*	**2.3 (0.6)**	*0.00‡*	**1.2 (0.6)**	*0.04‡*	**-1.2 (0.4)**	*0.00‡*	-1.4 (1.2)	*0.23*
[ref TT, N=412]	CC	3	**-9.1 (4.7)**	*0.05†*	**6.5 (3.1)**	*0.04‡*	2.7 (2.9)	*0.36*	-2.6 (1.8)	*0.15*	-1.6 (5.8)	*0.79*
rs17881215[1]	GC	124	**-3.3 (0.8)**	*0.00‡*	**1.9 (0.5)**	*0.00‡*	**1.4 (0.5)**	*0.00‡*	**-1.1 (0.3)**	*0.00‡*	-0.2 (1.5)	*0.92*
[ref GG, N=579]	CC	12	**-4.6 (2.2)**	*0.04‡*	**3.9 (1.4)**	*0.01‡*	0.7 (1.4)	*0.63*	**-1.6 (0.9)**	*0.06†*	-1.5 (4.3)	*0.72*
rs3740393[2]	GC	200	-1.4 (1.7)	*0.41*	1.8 (1.1)	*0.10*	-0.4 (1.0)	*0.68*	**-2.1 (0.6)**	*0.00‡*	1.4 (2.1)	*0.50*
[ref CC, N =26]	GG	280	**-4.2 (1.7)**	*0.01‡*	**3.3 (1.1)**	*0.00‡*	0.9 (1.0)	*0.36*	**-3.1 (0.6)**	*0.00‡*	-0.5 (2.0)	*0.80*
rs3740390[2, 3]	CT	192	-0.6 (1.9)	*0.73*	1.4 (1.3)	*0.27*	-0.7 (1.2)	*0.53*	**-2.3 (0.7)**	*0.00‡*	0.2 (2.3)	*0.93*
[ref TT, N=20]	CC	293	**-3.8 (1.9)**	*0.05‡*	**3.0 (1.2)**	*0.02‡*	0.8 (1.2)	*0.50*	**-3.4 (0.7)**	*0.00‡*	-0.5 (2.3)	*0.82*
rs11191453[3] *[ref TC, N=212]*	TT	290	**-3.3 (0.7)**	*0.00‡*	**1.8 (0.5)**	*0.00‡*	**1.5 (0.5)**	*0.00‡*	**-1.3 (0.3)**	*0.00‡*	-0.7 (0.9)	*0.43*
rs10748835	GA	251	-1.5 (1.0)	*0.12*	0.7 (0.6)	*0.27*	0.8 (0.6)	*0.19*	**-0.6 (0.4)**	*0.10†*	0.7 (1.2)	*0.59*
[ref AA, N=98]	GG	151	**-2.2 (1.1)**	*0.04‡*	**1.3 (.7)**	*0.07†*	0.9 (0.7)	*0.16*	**-1.1 (0.4)**	*0.01‡*	0.1 (1.3)	*0.95*
VNTR *[ref A2B, N =127]*	AB	587	**-1.6 (0.8)**	*0.04‡*	**0.9 (0.5)**	*0.09†*	0.7 (0.5)	*0.14*	-0.2 (0.3)	*0.45*	0.2 (1.4)	*0.88*

[a] Linear regression model adjusted for age and gender; referent genotype defined as the genotype associated in previous literature with a higher DMAs%.
† P<0.10 ‡ P<0.05 for coefficients of associations between *AS3MT* variants and urinary As profiles, results come from multiple linear regression model adjusted for age and gender. **Coefficients (SE) were bolded when they reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.1a. Population characteristics and genotype frequencies among subjects with complete data available for all *AS3MT* variants and covariates

	N	**%**	**Water As (ppb) p50 *(p25, p75)***
All subjects	483	100%	46.0 *(33.1, 58.8)*
Gender			
Male	157	33%	45.4 *(28.9, 60.7)*
Female	326	67%	46.6 *(35.9, 58.0)*
Age (years)			
18-24	60	12%	43.9 *(31.9, 50.0)*
25-34	107	22%	43.9 *(28.7, 51.3)*
35-39	64	13%	48.1 *(36.8, 64.3)*
40-44	44	9%	48.0 *(32.1, 69.9)*
≥45	208	43%	47.5 *(35.4, 68.1)*
rs11191439			
TT	398	82%	45.8 *(32.4, 62.5)*
TC	82	17%	48.1 *(39.1, 55.5)*
CC	3	1%	42.1 *(0.7, 50.4)*
rs17881215			
GG	392	81%	45.7 *(32.5, 61.2)*
GC	82	17%	48.1 *(36.9, 54.2)*
CC	9	2%	50.4 *(41.5, 61.0)*
rs3740393			
GG	262	54%	46.7 *(30.3, 58.8)*
GC	195	40%	45.5 *(35.7, 53.8)*
CC	26	5%	46.5 *(35.9, 78.6)*
rs3740390			
CC	275	57%	46.6 *(30.3, 62.3)*
CT	188	39%	45.6 *(35.8, 52.5)*
TT	20	4%	46.5 *(34.4, 88.8)*
rs11191453			
TT	275	57%	46.6 *(30.3, 62.3)*
TC	208	43%	45.8 *(35.8, 52.8)*
CC	0	0%	--
rs10748835			
GG	143	30%	45.2 *(28.2, 58.3)*
GA	245	51%	46.0 *(32.5, 55.0)*
AA	95	19%	47.0 *(36.4, 67.2)*
VNTR			
AB	396	82%	46.3 *(32.5, 55.5)*
A2B	86	18%	45.4 *(37.6, 69.7)*
A3B	1	0%	1.24 *(--)*

Supplemental Table 5.1b. Urinary profiles of As metabolites overall and by *AS3MT* variants among subjects with complete data available for all *AS3MT* variants and covariates: median and 25th, 75th percentiles

		N	Dimethylated As % (DMAs%)		Monomethylated As % (MAs%)		Unmethylated Inorganic As % (iAs%)		DMAs/MAs Ratio		MAs/iAs Ratio	
			p50	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***	**p50**	***(p25, p75)***
All subjects		483	76.8	*(70.1, 81.3)*	13.9	*(10.9, 17.4)*	9.0	*(6.1, 12.7)*	5.5	*(4.1, 7.4)*	1.6	*(1.1, 2.2)*
By AS3MT variant:												
rs11191439[1]	*TT	398	**77.4‡**	*(71.7, 81.8)*	**13.5‡**	*(10.8, 16.5)*	8.9	*(6.1, 12.3)*	**5.7‡**	*(4.4, 7.6)*	1.5	*(1.1, 2.1)*
	TC	82	**74.7**	*(66.2, 78.5)*	**15.8**	*(13.0, 20.0)*	10.1	*(6.5, 14.6)*	**4.7**	*(3.3, 6.0)*	1.7	*(1.2, 2.5)*
	CC	3	**65.6**	*(59.2, 73.8)*	**19.7**	*(13.9, 27.8)*	13.0	*(12.2, 14.7)*	**3.3**	*(2.1, 5.3)*	1.3	*(1.1, 2.1)*
rs17881215[1]	*GG	392	**77.5‡**	*(71.8, 81.8)*	**13.4‡**	*(10.8, 16.5)*	**8.9‡**	*(6.1, 12.3)*	**5.7‡**	*(4.4, 7.6)*	1.5	*(1.1, 2.1)*
	GC	82	**74.4**	*(66.0, 78.5)*	**15.6**	*(13.0, 19.6)*	**10.2**	*(6.7, 15.0)*	**4.8**	*(3.3, 6.0)*	1.6	*(1.0, 2.3)*
	CC	9	**66.2**	*(65.0, 75.6)*	**19.8**	*(19.6, 22.7)*	**12.2**	*(4.6, 13.2)*	**3.3**	*(2.8, 4.0)*	1.9	*(1.4, 2.2)*
rs3740393[2]	GG	262	**75.5‡**	*(69.6, 79.6)*	**14.7‡**	*(11.7, 18.9)*	**9.8‡**	*(7.0, 13.4)*	**5.2‡**	*(3.8, 6.9)*	1.6	*(1.1, 2.1)*
	GC	195	**78.6**	*(72.8, 83.4)*	**13.0**	*(10.8, 15.9)*	**8.3**	*(5.3, 12.0)*	**5.9**	*(4.6, 7.6)*	1.6	*(1.1, 2.3)*
	*CC	26	**80.7**	*(74.0, 84.4)*	**12.0**	*(9.4, 15.0)*	**9.2**	*(4.3, 12.0)*	**6.7**	*(5.3, 9.0)*	1.2	*(1.0, 1.8)*
rs3740390[2, 3]	CC	275	**75.5‡**	*(69.4, 79.6)*	**14.5‡**	*(11.6, 18.8)*	**10.0‡**	*(7.0, 13.4)*	**5.3‡**	*(3.8, 6.9)*	1.6	*(1.1, 2.1)*
	CT	188	**78.7**	*(73.4, 83.4)*	**12.8**	*(10.5, 15.9)*	**8.1**	*(5.4, 11.9)*	**6.2**	*(4.7, 7.8)*	1.6	*(1.1, 2.3)*
	*TT	20	**80.3**	*(70.2, 86.0)*	**12.6**	*(9.0, 15.3)*	**7.9**	*(4.4, 14.8)*	**6.3**	*(4.7, 9.5)*	1.2	*(0.9, 1.7)*
rs11191453[3]	TT	275	**75.5‡**	*(69.4, 79.6)*	**14.5‡**	*(11.6, 18.8)*	**10.0‡**	*(7.0, 13.4)*	**5.3‡**	*(3.8, 6.9)*	1.6	*(1.1, 2.1)*
	*TC	208	**78.8**	*(73.3, 83.5)*	**12.8**	*(10.3, 15.9)*	**8.1**	*(5.3, 12.0)*	**6.2**	*(4.7, 7.9)*	1.6	*(1.1, 2.3)*
rs10748835	GG	143	**76.2†**	*(70.1, 80.2)*	14.4	*(12.0,18.1)*	9.5	*(7.1, 13.0)*	**5.4†**	*(3.9, 6.8)*	1.5	*(1.1, 2.1)*
	GA	245	**76.7**	*(70.3, 81.2)*	13.7	*(10.9, 17.4)*	9.0	*(6.1, 12.8)*	**5.5**	*(4.1, 7.4)*	1.6	*(1.1, 2.1)*
	*AA	95	**78.6**	*(73.8, 83.5)*	13.7	*(10.3, 15.9)*	8.2	*(5.0, 12.0)*	**5.7**	*(4.6, 7.8)*	1.6	*(1.1, 2.3)*
VNTR	AB	396	**76.6†**	*(69.9, 81.3)*	14.0	*(10.9, 17.7)*	**9.4†**	*(6.1, 13.2)*	5.4	*(4.0, 7.3)*	1.6	*(1.1, 2.1)*
	*A2	86	**77.9**	*(73.9, 81.4)*	13.6	*(10.8, 15.9)*	**8.3**	*(6.4, 10.5)*	5.6	*(4.6, 7.1)*	1.6	*(1.2, 2.4)*

* Identifies the referent genotype, defined as the genotype associated in previous literature with a higher DMAs%. † P<0.10 ‡P<0.05 for significant differences in urinary As measures across genotypes for each *AS3MT* variant based on global test adjusted for age and gender. **Medians bolded when the global test reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.1c. Adjusted associations between *AS3MT* variants and urinary profiles of As metabolites among subjects with complete data available for all *AS3MT* variants and covariates[a]

Polymorphism		N	Dimethylated As % (DMAs%)		Monomethylated As % (MAs%)		Unmethylated Inorganic As % (iAs%)		DMAs/MAs ratio		MAs/iAs ratio	
			β (SE)	*P*	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
rs11191439[1]	TC	82	**-3.8 (1.0)**	*0.00‡*	**2.3 (0.7)**	*0.00‡*	**1.5 (0.6)**	*0.02‡*	**-1.2 (0.4)**	*0.00‡*	-1.5 (1.2)	*0.21*
[ref TT, N=398]	CC	3	**-9.1 (4.7)**	*0.06†*	**6.5 (3.1)**	*0.04‡*	2.7 (3.0)	*0.37*	-2.6 (1.8)	*0.15*	-1.6 (5.9)	*0.79*
rs17881215[1]	GC	82	**-3.9 (1.0)**	*0.00‡*	**2.1 (0.7)**	*0.00‡*	**1.8 (0.6)**	*0.00‡*	**-1.1 (0.4)**	*0.00‡*	-1.7 (1.2)	*0.17*
[ref GG, N=392]	CC	9	**-6.9 (2.2)**	*0.01‡*	**5.4 (1.8)**	*0.00‡*	1.5 (1.7)	*0.39*	**-2.4 (1.1)**	*0.03‡*	-0.9 (3.4)	*0.80*
rs3740393[2]	GC	195	-1.4 (1.7)	*0.42*	1.8 (1.1)	*0.10*	-0.5 (1.0)	*0.67*	**-2.1 (0.6)**	*0.00‡*	1.4 (2.1)	*0.50*
[ref CC, N=26]	GG	262	**-4.2 (1.7)**	*0.01‡*	**3.3 (1.1)**	*0.00‡*	1.0 (1.0)	*0.35*	**-3.1 (0.6)**	*0.00‡*	-0.5 (2.1)	*0.82*
rs3740390[2, 3]	CT	188	-0.7 (1.9)	*0.72*	1.4 (1.3)	*0.26*	-0.7 (1.2)	*0.54*	**-2.4 (0.7)**	*0.00‡*	0.2 (2.4)	*0.92*
[ref TT, N=20]	CC	275	**-3.8 (1.9)**	*0.04‡*	**3.0 (1.3)**	*0.02‡*	0.8 (1.2)	*0.47*	**-3.4 (0.7)**	*0.00‡*	-0.5 (2.4)	*0.85*
rs11191453[3] *[ref TC, N=208]*	TT	275	**-3.2 (0.8)**	*0.00‡*	**1.7 (0.5)**	*0.00‡*	**1.5 (0.5)**	*0.00‡*	**-1.2 (0.3)**	*0.00‡*	-0.7 (0.9)	*0.47*
rs10748835	GA	245	-1.6 (1.0)	*0.12*	0.8 (0.7)	*0.26*	0.8 (0.6)	*0.20*	**-0.6 (0.4)**	*0.10†*	0.6 (1.2)	*0.60*
[ref AA, N=95]	GG	143	**-2.3 (1.1)**	*0.04‡*	**1.3 (0.7)**	*0.07†*	0.9 (0.7)	*0.17*	**-1.1 (0.4)**	*0.01‡*	0.1 (1.4)	*0.92*
VNTR *[ref A2B, N =86]*	AB	396	**-2.0 (1.0)**	*0.04‡*	0.7 (0.6)	*0.26§*	**1.3 (0.6)**	*0.04†*	-0.1 (0.4)	*0.77*	-0.3 (1.2)	*0.77*

[a] Linear regression model adjusted for age and gender; referent genotype defined as the genotype associated in previous literature with a higher DMAs%. †P<0.10 ‡ P<0.05 for coefficients of associations between *AS3MT* variants and urinary As profiles, results come from multiple linear regression model adjusted for age and gender. **Coefficients (SE) were bolded when they reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.2a. Urinary profiles of As metabolites overall and by *AS3MT* variants: median and 25th, 75th percentiles excluding subjects miss information for diabetes

		N	Dimethylated As % (DMAs%)		Monomethylated As % (MAs%)		Unmethylated Inorganic As % (iAs%)		DMAs/MAs Ratio		MAs/iAs Ratio	
			p50	***(p25,p75)***	**p50**	***(p25,p75)***	**p50**	***(p25,p75)***	**p50**	***(p25,p75)***	**p50**	***(p25,p75)***
All subjects		771	76.9	*(71.3. 81.4)*	13.9	*(10.9,17.4)*	8.8	*(6.3, 12.2)*	5.5	*(4.2, 7.4)*	1.6	*(1.2, 2.1)*
By AS3MT variant:												
rs11191439[1]	*TT	412	**77.3‡**	*(71.7, 81.8)*	**13.5‡**	*(10.8, 16.7)*	8.9	*(6.1, 12.4)*	**5.7‡**	*(4.4, 7.6)*	1.5	*(1.1, 2.1)*
	TC	86	**74.9**	*(66.5, 79.0)*	**15.8**	*(13.0, 20.0)*	9.8	*(6.3, 14.5)*	**4.7**	*(3.3, 6.0)*	1.8	*(1.2, 2.5)*
	CC	3	**65.6**	*(59.2, 73.8)*	**19.7**	*(13.9, 27.8)*	13.0	*(12.2, 14.7)*	**3.3**	*(2.1, 5.3)*	1.3	*(1.1, 2.1)*
rs17881215[1]	*GG	578	**77.6‡**	*(72.9, 82.5)*	**13.4‡**	*(10.7, 16.6)*	**8.6‡**	*(6.1, 11.6)*	**5.8‡**	*(4.4, 7.6)*	1.6	*(1.2, 2.1)*
	GC	124	**74.9**	*(69.0, 80.5)*	**15.1**	*(12.8, 19.2)*	**8.8**	*(6.7, 13.9)*	**4.9**	*(3.6, 6.3)*	1.6	*(1.2, 2.3)*
	CC	12	**73.9**	*(65.3, 78.7)*	**19.8**	*(12.6, 21.6)*	**11.2**	*(5.0, 13.1)*	**3.7**	*(3.0, 6.2)*	1.8	*(1.3, 3.0)*
rs3740393[2]	GG	280	**75.3‡**	*(69.3, 75.3)*	**14.7‡**	*(11.7, 18.9)*	**9.9‡**	*(7.1, 13.1)*	**5.1‡**	(3.8, 6.8)	**1.6†**	*(1.2, 2.1)*
	GC	200	**78.6**	*(72.9, 83.3)*	**13.0**	*(10.8, 16.0)*	**8.3**	*(5.4, 12.0)*	**5.8**	(4.6, 7.6)	**1.6**	*(1.1, 2.3)*
	*CC	26	**80.7**	*(74.0, 84.4)*	**12.0**	*(9.4, 15.0)*	**9.2**	*(4.3, 13.0)*	**6.7**	(5.3, 9.0)	**1.2**	*(1.0, 1.8)*
rs3740390[2, 3]	CC	293	**75.5‡**	*(69.4, 79.7)*	**14.5‡**	*(11.8, 18.8)*	**10.0‡**	*(7.1, 13.3)*	**5.3‡**	*(3.8, 6.8)*	1.6	*(1.1, 2.1)*
	CT	192	**78.7**	*(73.5, 83.4)*	**12.8**	*(10.5, 15.9)*	**8.1**	*(5.4, 11.9)*	**6.2**	*(4.7, 7.8)*	1.6	*(1.1, 2.3)*
	*TT	20	**80.3**	*(70.2, 86.0)*	**12.6**	*(9.0, 15.3)*	**7.9**	*(4.4, 14.8)*	**6.3**	*(4.7, 9.5)*	1.2	*(0.9, 1.7)*
rs11191453[3]	TT	290	**75.3‡**	*(69.2, 79.7)*	**14.6‡**	*(11.9, 18.9)*	**10.0‡**	*(7.1, 13.4)*	**5.2‡**	*(3.7, 6.8)*	1.6	*(1.2, 2.1)*
	*TC	212	**78.8**	*(73.4, 83.5)*	**12.8**	*(10.3, 15.9)*	**8.1**	*(5.3, 12.0)*	**6.2**	*(4.7, 8.0)*	1.6	*(1.1, 2.3)*
rs10748835	GG	151	**76.0†**	*(70.1, 80.2)*	14.5	*(12.0,18.1)*	9.5	*(7.1, 12.9)*	**5.4‡**	*(3.9, 6.6)*	1.5	*(1.1, 2.1)*
	GA	251	**76.7**	*(70.3, 81.3)*	13.7	*(10.8, 17.4)*	9.0	*(6.1, 12.9)*	**5.5**	*(4.1, 7.6)*	1.6	*(1.1, 2.1)*
	*AA	98	**78.5**	*(73.8, 83.5)*	13.7	*(10.3, 15.9)*	8.3	*(5.1, 11.7)*	**5.7**	*(4.6, 7.7)*	1.6	*(1.1, 2.2)*
VNTR	AB	587	**76.9†**	*(70.9, 81.5)*	13.9	*(11.0, 17.4)*	8.7	*(6.2, 12.3)*	5.5	*(4.1, 7.3)*	1.6	*(1.2, 2.1)*
	*A2B	126	**78.0**	*(73.9, 82.1)*	13.6	*(10.6, 16.1)*	8.2	*(6.3, 10.4)*	5.7	*(4.6, 7.9)*	1.6	*(1.2, 2.2)*

* Identifies the referent genotype, defined as the genotype associated in previous literature with a higher DMAs%. † P<0.10 ‡P<0.05 for significant differences in urinary As measures across genotypes for each *AS3MT* variant based on global test adjusted for age and gender. **Medians bolded when the global test reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.2b. Adjusted associations between *AS3MT* variants and urinary profiles of As metabolites [a]

Polymorphism		N	Dimethylated As % (DMAs%)		Mono-methylated As % (MAs%)		Unmethylated Inorganic As % (iAs%)		DMAs/MAs ratio		MAs/iAs ratio	
			Coeff (SE)	***P***	**Coeff (SE)**	***P***	**Coeff (SE)**	***P***	**Coeff (SE)**	***P***	**Coeff (SE)**	***P***
rs11191439[1]	TC	86	**-3.5 (1.0)**	*0.00‡*	**2.3 (0.6)**	*0.00‡*	**1.2 (0.6)**	*0.04‡*	**-1.2 (0.4)**	*0.00‡*	-1.4 (1.2)	*0.23*
[ref TT, N=412]	CC	3	**-9.2 (4.7)**	*0.05†*	**6.5 (3.1)**	*0.04‡*	2.7 (2.9)	*0.36*	-2.6 (1.8)	*0.14*	-1.6 (5.8)	*0.79*
rs17881215[1]	GC	124	**-3.4 (0.8)**	*0.00‡*	**1.9 (0.5)**	*0.00‡*	**1.4 (0.5)**	*0.00‡*	**-1.2 (0.3)**	*0.00‡*	-0.2 (1.5)	*0.91*
[ref GG, N=579]	CC	12	**-4.5 (2.2)**	*0.04‡*	**3.8 (1.4)**	*0.01‡*	0.7 (1.4)	*0.62*	**-1.5 (0.9)**	*0.08†*	-1.5 (4.3)	*0.73*
rs3740393[2]	GC	200	-1.1 (1.7)	*0.50*	1.6 (1.1)	*0.14*	-0.5 (1.0)	*0.64*	**-1.9 (0.6)**	*0.00‡*	1.3 (2.1)	*0.52*
[ref CC, N=26]	GG	280	**-4.1 (1.7)**	*0.02‡*	**3.2 (1.1)**	*0.00‡*	0.9 (1.0)	*0.39*	**-2.9 (0.6)**	*0.00‡*	-0.6 (2.0)	*0.78*
rs3740390[2, 3]	CT	192	-0.3 (1.9)	*0.89*	1.1 (1.3)	*0.39*	-0.8 (1.2)	*0.49*	**-2.0 (0.7)**	*0.01‡*	0.1 (2.4)	*0.97*
[ref TT, N=20]	CC	293	**-3.5 (1.9)**	*0.06†*	**2.8 (1.2)**	*0.03‡*	0.7 (1.2)	*0.54*	**-3.1 (0.7)**	*0.00‡*	-0.6 (2.3)	*0.79*
rs11191453[3] *[ref TC, N=212]*	TT	290	**-3.4 (0.7)**	*0.00‡*	**1.8 (0.5)**	*0.00‡*	**1.5 (0.5)**	*0.00‡*	**-1.3 (0.3)**	*0.00‡*	-0.7 (0.9)	*0.44*
rs10748835	GA	251	-1.4 (1.0)	*0.16*	0.6 (0.6)	*0.34*	0.8 (0.6)	*0.20*	-0.5 (0.4)	*0.16*	0.6 (1.2)	*0.61*
[ref AA, N=98]	GG	151	**-2.2 (1.1)**	*0.04‡*	**1.3 (0.7)**	*0.07†*	0.9 (0.7)	*0.16*	**-1.1 (0.4)**	*0.01‡*	0.1 (1.3)	*0.95*
VNTR *[ref A2B, N =127]*	AB	587	**-1.6 (0.8)**	*0.04‡*	0.8 (0.5)	*0.13*	0.8 (0.5)	*0.10*	-0.2 (0.3)	*0.61*	0.3 (1.4)	*0.85*

[a] Linear regression model adjusted for age, gender, and diabetes; referent genotype defined as the genotype associated in previous literature with a higher DMAs%. †P<0.10 ‡ P<0.05 for coefficients of associations between *AS3MT* variants and urinary As profiles, results come from multiple linear regression model adjusted for age, gender, and diabetes. **Coefficients (SE) were bolded when they reached significance** (P<0.10). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.3. Global test statistics for interactions between variants with categorical water iAs concentration[a]

Polymorphism	Dimethylated Arsenic % (DMAs%)		Monomethylated Arsenic % (MAs%)		Unmethylated Inorganic Arsenic % (iAs%)		DMAs/MAs Ratio		MAs/iAs Ratio	
	F	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
rs11191439[1]	0.07	*0.79*	0.09	*0.76*	0.57	*0.45*	0.10	*0.75*	0.04	*0.84*
rs17881215[1]	3.18	***0.08***	3.39	***0.07***	0.86	*0.35*	1.50	*0.22*	2.58	*0.11*
rs3740393[2]	0.40	*0.67*	0.38	*0.68*	0.14	*0.87*	5.79	***0.01***	0.44	*0.65*
rs3740390[2, 3]	0.14	*0.87*	0.03	*0.97*	0.20	*0.82*	5.31	***0.01***	0.35	*0.70*
rs11191453[3]	0.23	*0.63*	0.02	*0.88*	0.37	*0.54*	0.64	*0.43*	0.10	*0.75*
rs10748835	0.66	*0.52*	1.24	*0.29*	0.68	*0.51*	4.64	***0.01***	0.66	*0.52*
VNTR	1.02	*0.31*	1.17	*0.28*	0.24	*0.62*	0.51	*0.47*	0.21	*0.64*

[a] Categorical water iAs concentration was defined as higher (>50ppb) vs. lower (≤50ppb). [1, 2, 3] The pairs of variants shown were in linkage disequilibrium with r^2>0.80.

Supplemental Table 5.4. Associations between *AS3MT* variants and urinary profiles of As metabolites among subjects with higher (>50ppb) vs. lower (≤50ppb) drinking water As[a]

Variant: Water iAs level		**N**	**Dimethylated As % (DMAs%)**		**Monomethylated As % (MAs%)**		**Unmethylated Inorganic As% (iAs%)**		**DMAs/MAs Ratio**		**MAs/iAs Ratio**	
			Coeff (SE)	**P**	**Coeff (SE)**	**P**	**Coeff (SE)**	**P**	**Coeff (SE)**	**P**	**Coeff (SE)**	**P**
rs11191439[1] *[ref TT]:*												
Low *[ref N=263]*	TC	51	**-3.9 (1.3)**	0.00‡	**2.2 (0.9)**	0.02‡	**1.7 (0.8)**	0.03‡	**-1.1 (0.4)**	0.01‡	-1.2 (1.4)	0.39
	CC	2	**-12.5 (5.9)**	0.04‡	**9.3 (4.1)**	0.03‡	3.2 (3.7)	0.40	**-3.2 (1.9)**	0.09†	-1.3 (6.6)	0.85
High *[ref N=149]*	TC	35	**-2.9 (1.5)**	0.05‡	**2.3 (0.8)**	0.01‡	0.6 (0.9)	0.50	**-1.3 (0.7)**	0.08†	-2.0 (2.1)	0.34
	CC	1	-3.3 (7.8)	0.67	0.7 (4.5)	0.87	2.6 (4.7)	0.59	-1.3 (3.9)	0.74	-1.1 (11.4)	0.93
rs17881215[1] *[ref GG]:*												
Low *[ref N=322]*	GC	69	**-4.5 (1.1)**	0.00‡*	**2.7 (0.7)**	0.00‡*	**1.8 (0.7)**	0.01‡	**-1.4 (0.4)**	0.00‡	1.9 (1.7)	0.2
	CC	5	-5.0 (3.6)	0.17	**5.3 (2.5)**	0.04‡	-0.3 (2.3)	0.89	-1.9 (1.2)	0.12	-1.2 (5.7)	0.8
High *[ref N=257]*	GC	55	**-1.9 (1.1)**	0.08†	0.9 (0.6)	0.14	1.0 (0.6)	0.13	-0.7 (0.5)	0.15	-2.9 (2.6)	0.2
	CC	7	-4.4 (2.7)	0.11	**2.9 (1.6)**	0.07†	1.5 (1.6)	0.36	-1.4 (1.3)	0.27	-2.2 (6.6)	0.7
rs3740393[2] *[ref CC]:*												
Low *[ref N=16]*	GC	133	-1.3 (2.2)	0.57	1.6 (1.6)	0.29	-0.4 (1.4)	0.79	-0.5 (0.7)	0.50*	0.0 (2.4)	0.99
	GG	170	**-3.7 (2.2)**	0.09†	**2.8 (1.5)**	0.07†	0.9 (1.4)	0.52	**-1.4 (0.7)**	0.05‡*	-1.5 (2.4)	0.53
High *[ref N=10]*	GC	67	-1.2 (2.6)	0.63	2.0 (1.5)	0.19	-0.7 (1.6)	0.65	**-4.6 (1.2)**	0.00‡	3.6 (3.8)	0.34
	GG	110	**-5.1 (2.5)**	0.04‡	**4.0 (1.5)**	0.01‡	1.0 (1.5)	0.50	**-5.7 (1.2)**	0.00‡	1.1 (3.7)	0.77
rs3740390[2] *[ref TT]:*												
Low *[ref N=12]*	CT	128	-0.6 (2.5)	0.81	1.3 (1.8)	0.48	-0.6 (1.6)	0.69	-0.5 (0.8)	0.54*	-1.3 (2.8)	0.64
	CC	178	-3.5 (2.5)	0.16	2.9 (1.7)	0.10	0.7 (1.6)	0.67	**-1.5 (0.8)**	0.06†*	-2.1 (2.8)	0.46
High *[ref N=8]*	CT	64	-0.2 (2.9)	0.95	1.4 (1.7)	0.40	-1.2 (1.7)	0.48	**-5.1 (1.4)**	0.00‡	2.2 (4.2)	0.60
	CC	115	-3.9 (2.8)	0.17	**3.2 (1.6)**	0.05†	0.7 (1.7)	0.67	**-6.2 (1.3)**	0.00‡	1.5 (4.1)	0.71
rs11191453 *[ref TC]:*												
Low *[ref N=140]*	TT	178	**-3.1 (1.0)**	0.00‡	**1.8 (0.7)**	0.01‡	**1.3 (0.6)**	0.03‡	**-1.1 (0.3)**	0.00‡	-0.9 (1.1)	0.41
High *[ref N=72]*	TT	112	**-3.9 (1.1)**	0.00‡	**2.0 (0.7)**	0.00‡	**1.9 (0.7)**	0.01‡	**-1.7 (0.6)**	0.00‡	-0.4 (1.7)	0.80
rs10748835 *[ref AA]:*												
Low *[ref N=59]*	GA	161	-0.7 (1.3)	0.58	-0.0 (0.9)	0.98	0.7 (0.8)	0.36	0.3 (0.4)	0.54*	-0.4 (1.4)	0.79

	GG	96	-1.3 (1.4)	0.35	0.8 (1.0)	0.39	0.5 (0.9)	0.59	-0.4 (0.4)	0.35*	-0.8 (1.5)	0.61
High *[ref N=39]*	GA	90	**-2.7 (1.5)**	0.07†	**2.0 (0.9)**	0.02‡	0.7 (0.9)	0.46	**-2.1 (0.7)**	0.01‡	2.4 (2.2)	0.27
	GG	55	**-3.7 (1.6)**	0.02‡	**2.1 (0.9)**	0.03‡	1.6 (1.0)	0.10	**-2.2 (0.8)**	0.01‡	1.7 (2.4)	0.47
VNTR *[ref A2B]*												
Low *[ref N=66]*	AB	329	-1.1 (1.1)	0.33	0.6 (0.8)	0.45	0.4 (0.7)	0.55	-0.1 (0.4)	0.80	0.8 (1.6)	0.65
High *[ref N=61]*	AB	258	**-2.0 (1.0)**	0.04‡	**1.2 (0.6)**	0.04‡	0.8 (0.6)	0.17	-0.4 (0.5)	0.40	-0.4 (2.5)	0.88

[a] Linear regression model adjusted for age and gender; referent genotype defined as the genotype associated in previous literature with a higher DMAs%. †P<0.10 ‡ P<0.05 for coefficients of associations between *AS3MT* variants and urinary As profiles stratified by water As: higher (>50ppb) vs. lower (≤50ppb) drinking water As, results come from multiple linear regression model adjusted for age and gender. **Coefficients (SE) were bolded when they reached significance** (P<0.10). *Significant (P<0.10) Interaction with categorical drinking water As (higher vs. lower) based on global F test.

ENDNOTES

[60]This chapter previously appeared as an article in Toxicological Sciences. The original citation is as follows: Xu, X., et al., "Association between Variants in Arsenic (+3 Oxidation State) Methyltransferase (*AS3MT*) and Urinary Metabolites of Inorganic Arsenic: Role of Exposure Level" Toxicological Sciences, 2016.

CHAPTER 6. SYNTHESIS

Overview of findings

Though literature indicates that low-to-moderate level of iAs exposure may contribute to the current epidemic of T2D, the results of the few studies are inconsistent and most of them are cross-sectional[51,55-57]. In this research, we sought to examine the association between iAs exposure at baseline (i.e.2009) and development of T2D over 6-years follow-up in a population low-to-moderately exposed to iAs. In addition, we also aim to determine effective strategy to counter the diabetogenic effects of iAs and identify genetic susceptible group of iAs-associated adverse health outcomes due to impairment of iAs metabolism.

We used data from CHNS, which is an ongoing household-based cohort study designed to examine a series of economic, sociological, demographic, and health questions in the Chinese population, who are known to be low-to-moderately exposed to iAs. We are able to answer our study questions capitalizing on the rich CHNS data, which includes: concentration of toenail arsenic at baseline that reflects iAs exposure level in recent months; baseline dietary Mg and Zn intake assessed using three consecutive days 24-hour; indicators of glucose homeostasis at follow-up (i.e. 2015) including fasting glucose and insulin. Moreover, we took the different measures of T2D (insulin resistance vs. pancreatic β-cell dysfunction) into account: associations with both iAs and intake of Mg and Zn may be most apparent for measures of specific mechanism through which the iAs exposure and intake of Mg and Zn influence the development of T2D. Rather than only testing the associations using the measure of fasting glucose, we

examined the associations using measures of pancreatic β-cell function and insulin resistance, which were calculated by Homeostasis Model Assessment (HOMA2) calculator updated in 2015 (University of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php) to better understand the study question[41,42].

Answering these questions is important as it improves the understanding of the role of low-to-moderate level of iAs exposure, which is the exposure level common worldwide, in the current epidemic of T2D. Findings can also inform efforts to combat the diabetogenic effects of iAs by maximizing the effectiveness of Mg and Zn intake. It is extremely important as it is challenging to eliminate iAs exposure from our daily life. Our findings can also identify genetically susceptible subgroups in which there are augmented impaired iAs metabolism correlated with iAs exposure. Below we present a brief review of our findings, limitations and strengths of our research, its significance and public health impact, as well as future directions.

Whether iAs exposure at low-to-moderate level contribute to the current epidemic of T2D?

To understand how low-to-moderate level of iAs exposure at baseline is related to the T2D development over time, we examined the association between baseline iAs exposure and T2D development over 6 years in a population with low-to-moderate level of iAs exposure. Using multinomial logistic regression models and multivariable-adjusted linear regression models, we determined how baseline iAs exposure, which was captured by toenail arsenic, associated with T2D incidence and indicators of glucose homeostasis, which was characterized by fasting glucose, fasting insulin, HbA1c, pancreatic β-cell function, and insulin resistance at follow-up.

Our findings further confirm the diabetogenic effects of low-to-moderate iAs exposure, which may contribute greatly to the current epidemic of T2D, especially in the populations with

low prevalence of obesity. Moreover, our findings also suggest that instead of insulin resistance, pancreatic β-cell dysfunction is primarily involved in iAs-associated diabetes**.**

How the diabetogenic effects of low-to-moderate iAs exposure varied by population characteristics?

To evaluate the consistency of the associations between iAs exposure and development of T2D by participant characteristics, we tested the interactions between toenail arsenic at baseline and the participants characteristics that were suggested to modify the iAs-associated adverse health outcomes[114]. We conducted additional analyses stratified by age (<55, 55-64, or ≥65 years), sex, body mass index (<18, 23.0-27.4, or ≥27.5kg/m^2), smoking status (current, former, or never), drinking status (<, or ≥once/month), and region (North, Central, or South) to better inform how the characteristics of the participants relate to iAs-associated diabetes.

We found that men and participants who consumed alcohol were more susceptible to the diabetogenic effects of iAs than women and those who did not consume alcohol. Our findings inform that intervention strategies to reduce the iAs exposure or counter the diabetogenic effects of iAs are extremely important for men and alcohol consumers. In addition, though Fatmi et al.[160] suggests reduced susceptibility of iAs-associated adverse health outcomes among obese individuals, we found that low-to-moderate iAs exposure at baseline promoted rapid progression to more severe diabetes for obese individuals. Thus, instead of thinking obesity as a preventive factor for iAs-associated adverse health outcomes, our study stresses the adverse effects of low-to-moderate level of iAs exposure among obese individuals.

What are the potential reasons for the inconsistent results of iAs-associated diabetes from previous studies?

The results of the researches exploring the associations between low-to-moderate level of iAs exposure and risk of T2D are highly inconsistent[54,58-60]. In our study, we determined the potential reasons for the inconsistent results by 1) comparing the analyses using the cross-sectional vs. longitudinal data; 2) using different measures of T2D development (i.e. HbA1c vs. fasting glucose); 3) comparing the associations between iAs exposure and development of T2D among participants with different characteristics that thought to modify iAs-associated health outcomes, such as age, sex, weight status, drinking and smoking status.

There have been growing concerns that cross-sectional study design cannot capture the temporal relationship between iAs exposure and development of diabetes, and diabetes might affect iAs excretion and metabolism[53,17]. In line with this concern, we found different results for the associations between baseline iAs exposure and risk of diabetes using longitudinal vs. cross-sectional analysis. The longitudinal analysis suggests positive association between baseline iAs exposure and fasting glucose, β-cell dysfunction, odds of incident diabetes and prediabetes, whereas the cross-sectional analysis suggests lower odds of diabetes associated with iAs exposure. Our findings further indicate that the inconsistent results for the associations between iAs exposure and risk of diabetes reported in previous studies could be partially explained by the different study designs.

We also found significant differences in the associations between baseline iAs exposure and development of T2D while using different measures of T2D (i.e. fasting glucose vs. HbA1c). Our findings further confirm the concern about using HbA1c to diagnose diabetes among subjects exposed to iAs as laboratory data suggests iAs exposure is correlated with hemoglobin concentration[61,62]. Thus, our findings further support the idea that FG is more feasible to capture the change in glucose hemostasis induced by iAs exposure, and the real association between iAs

exposure and development of diabetes could be ignored if only HbA1c is used to define diabetes[139].

Moreover, as mentioned above, the associations between iAs exposure and T2D development varied by characteristics of the participants, such as sex, drinking status, and weight status. Thus, the different characteristics of the study populations may also partially explain the inconsistent results across studies.

Whether the diabetogenic effects of iAs is modified by dietary Mg and Zn intake at baseline?

The inconsistent results in previous studies on the associations between low-to-moderate iAs exposure and development of T2D further stress the importance of exploring factors that may be associated with the diabetogenic effects of iAs. To determine whether the diabetogenic effects of low-to-moderate iAs exposure are associated with intake of Mg and Zn, which are the macronutrients involved in glucose metabolism, we first determined the associations between Mg and Zn intake at baseline and T2D development over 6 years follow-up. We found deficiency of Zn intake at baseline might contribute to development of T2D through inducing pancreatic β-cell dysfunction. And our findings indicate that improvement of Zn intake could be a potential strategy to reduce T2D risk.

Second, we examined whether the diabetogenic effects of low-to-moderate iAs exposure varied depending on levels of Mg and Zn intake at baseline by testing the significance of the interactions between dietary Mg and Zn status × iAs exposure level at baseline. We found the susceptibility of iAs-associated diabetes varied depending on levels of Zn intake at baseline, and participants with adequate intake of Zn are more susceptible to iAs-associated β-cell dysfunction. Our results further suggest that the mixed results between low-to-moderate level of iAs exposure

and development of T2D among studies could be partially correlated with different levels of essential metals intake, such as Zn, across studies.

Does promoting Zn intake an effective strategy to counter the diabetogenic effects of low-to-moderate iAs exposure?

The positive association between low-to-moderate iAs exposure and T2D development observed in our study further stresses the need of identifying strategies to reduce the diabetogenic effects of iAs. We stratified participants based on both iAs exposure (i.e. tertiles) and intake of Mg and Zn (i.e. adequate vs. deficient) to generate the joint variable for iAs exposure and intake of Mg and Zn. We tested the association between the joint variable and the development of T2D.

We found comparing with the participants with the same level of iAs exposure, those with adequate intake of Mg or Zn were always with lower fasting glucose and better pancreatic β-cell function comparing with those with deficient Mg or Zn intake. Overall, the findings support the idea that promoting intake of Mg and Zn may potentially be an effective strategy to counter the diabetogenic effects of iAs.

Whether the associations between genetic variants of AS3MT and iAs metabolism varied by exposure level?

In this study, seven *AS3MT* variants which may play a role in iAs metabolism were examined based on results of previous studies. The patterns of association between markers of iAs metabolism and these variants were highly consistent with those reported in previous studies, confirming that these variants are in part responsible for the inter-individual differences in urinary profiles of iAs metabolites. We found that specific genotypes in five SNPs, rs17881215, rs3740393, rs3740390, rs11191439, and rs11191453, were associated with significantly higher

DMAs% and lower MAs% in urine. Polymorphisms in these SNPs, along with rs10748835, were also associated with the DMAs/MAs ratio.

Our results also suggested that the role of several of these *AS3MT* variants in iAs metabolism may differ among populations with different levels of iAs exposure. Three SNPs, rs3740393, rs3740390, and rs10748835, appeared to have significantly more potent effects, based on associations with larger decreases in the DMAs/MAs ratio, among subjects highly exposed to As in drinking water (> 50ppb). In contrast, rs17881215 had significantly more potent effects among subjects with lower water As levels. Since measures of iAs metabolism have been associated with risk of adverse health outcomes, these findings suggest that variants in *AS3MT* may influence susceptibility to health effects of iAs exposure, and that the role of these variants may depend on the level of iAs exposure. However, given that toxicity of iAs metabolites varies by oxidation status [79,80,83,235-238], further research focusing on whether and how these variants relate to the distribution of trivalent and pentavalent metabolites is needed to better clarify the influence of *AS3MT* polymorphism on iAs methylation profiles, and on health outcomes.

Limitations

Our study has several limitations. First, our study used toenail arsenic as the measurement of iAs exposure, and it has been suggested as a more reliable biomarker of chronic iAs exposure comparing with urinary arsenic[74,117]. However, a more expanded knowledge of toxicokinetic data and information on the correlations with existing biomarkers (i.e. urinary arsenic) is needed to better interpret our findings.

Second, though our sample size was larger than many previous studies, we had limited cell sizes for stratified analyses. The small cell sizes may have reduced our power to detect the

interactions, and the statistical significance should be interpreted with caution, especially for the interactions and stratified analyses.

Third, though three-day 24-hour recall is frequently used in studies to estimate the usual dietary intake, the result of it can be affected by several factors, such as over and under report[210,211]. In our study, we used sensitivity analysis to examine differences in model results when we excluded participants with extreme self-reported daily energy intake (<600 kcal/d and >4,500 kcals/d) to assess the effects from misreporting. We found no statistically significant difference in results before vs. after this exclusion.

Fourth, limited information about supplement intake might have altered our estimates of daily intake of Mg and Zn. However, since mineral supplements, except calcium, are not widely used in this population and none of the participants included in the analysis reported using supplements, it is unlikely that this study limitation influenced our findings[212].

Strengths

Despite the limitations, our study has several key strengths. First, the prospective, longitudinal design and the size of the CHNS cohort provide an outstanding opportunity to understand how, in a population with low-to-moderate iAs exposure, baseline (i.e.2009) iAs exposure relates to the development of T2D during follow-up, and potential effect modification by Mg and Zn intake. The CHNS with rich longitudinal data, especially high-quality dietary data, toenail concentration of arsenic that reflects iAs exposure level in recent months at baseline, provides a special opportunity for us to conduct this study

Second, we took accounted for different measures of T2D (insulin resistance vs. pancreatic β-cell dysfunction) to better inform the understanding of underlying biological mechanism of iAs-associated T2D. We also excluded participants with diabetes at baseline to

better capture the early stage changes of glucose homeostasis to reveal the underlying biological mechanism of Zn and Mg-associated diabetes

Third, few studies on toxic metals, such as iAs, include the high-quality dietary data. The potential for essential metals to reduce the risk of toxic metals has been largely examined using biomarkers of essential metals (e.g. serum and urinary biomarkers). However, others have suggested that toxic metals and their related metabolic effects may diminish essential metals biomarkers, limiting their utility as markers of intake[208,209]. Our study is one of the few studies that directly examine how intake, rather than biomarkers, of Mg and Zn relate to iAs burden. In addition, we accounted for potential confounders with progressive degrees of adjustment in our models.

Significance and public health impact

Our research has significant public health implications. First, our findings show significant and positive association between baseline iAs exposure and development of T2D over 6 years of follow-up. Our findings further confirm the diabetogenic effects of low-to-moderate level of iAs exposure, which is the exposure level very common worldwide [51,55-57], and indicate that the low-to-moderate level of iAs exposure may contribute substantially to the current epidemic of T2D. Moreover, our study highlights the needs for interventions to decrease iAs exposure from our daily life or strategies to counter the diabetogenic effects of iAs even in the regions with low-to-moderate iAs exposure.

Second, our research takes into account the different measures of T2D, such as fasting glucose, HbA1c, pancreatic β-cell dysfunction, and insulin resistance. We found positive association between iAs exposure and fasting glucose, whereas found null association between iAs exposure and HbA1c. Our finding further confirms the concern of using HbA1c to diagnose

diabetes among population exposed to iAs, as previous laboratory studies have indicated the association between iAs exposure on hemoglobin concentration[61,62]. Moreover, our study is the first to describe the association between iAs exposure and insulin resistance vs. pancreatic β-cell dysfunction. Our research strengthens the limited understanding of iAs-associated T2D and suggests that instead of insulin resistance, pancreatic β-cell dysfunction is primarily involved in iAs-associated T2D.

Our research is the first to describe and document the associations between baseline Mg and Zn intake and iAs-associated diabetes. Our findings support the idea that the susceptibility of iAs-associated diabetes varies depending on levels of Zn intake at baseline, and participants with adequate intake of Zn are more susceptible to iAs-associated β-cell dysfunction. Our findings contribute to better understanding of the mixed results between low-to-moderate iAs exposure and development of T2D, which could be partially due to the varied susceptibility among participants with different intake of essential metals, such as Zn. The estimated joint associations of Mg and Zn intake with iAs exposure in association with T2D stress the importance of having adequate Mg and Zn intake to reduce the risk of T2D. Overall, our findings suggest potential benefits of promoting intake of Mg and Zn to counter the diabetogenic effects of iAs, and emphasize the importance of intervention efforts on increasing intake of Mg and Zn for participants with different levels of iAs exposure.

Future directions

This study provides a foundation for further study of several important questions, including future research examining: (i) if dietary factors besides Mg and Zn may mitigate adverse effects of iAs (e.g. antioxidant vitamins); (ii) how a wider array of toxic and essential metals affect multiple cardio-metabolic outcomes (e.g. dyslipidemias); and (iii) how toxic and

essential metals relate to cardiometabolic risk after accounting for genetic susceptibility. In addition, a more expanded knowledge of toxicokinetic data and information on the correlations with existing biomarkers (i.e. urinary arsenic) is needed to better interpret our findings

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