OXFORD

SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 144(2), 2015, 338–346

doi: 10.1093/toxsci/kfu318
Advance Access Publication Date: January 9, 2015

Metabolomic Characteristics of Arsenic-Associated Diabetes in a Prospective Cohort in Chihuahua, Mexico

Elizabeth Martin*, Carmen González-Horta†, Julia Rager*, Kathryn A. Bailey*, Blanca Sánchez-Ramírez†, Lourdes Ballinas-Casarrubias†, María C. Ishida†, Daniela S. Gutiérrez-Torres†, Roberto Hernández Cerón‡, Damián Viniegra Morales‡, Francisco A. Baeza Terrazas‡, R. Jesse Saunders§, Zuzana Drobná§, Michelle A. Mendez§, John B. Buse¶, Dana Loomis‖, Wei Jia‖‖, Gonzalo G. García-Vargas‖‖‖, Luz M. Del Razo#, Miroslav Stýblo§,1, and Rebecca Fry*

*Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina, †Programa de Maestría en Ciencias en Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Chihuahua, México, ‡Colegio de Médicos Cirujanos y Homeópatas del Estado de Chihuahua, A.C., §Department of Nutrition, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, ¶Department of Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, ‖International Agency for Research of Cancer, Monographs Section, IARC/WHO, Lyon Cedex, France, ‖‖University of Hawaii Cancer Center, University of Hawaii, Honolulu, Hawaii, ‖‖‖Facultad de Medicina, Universidad Juárez del Estado de Durango, Gómez Palacio, Durango, México and #Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México DF, México

[1]To whom correspondence should be addressed at 2302 MHRC, CB 7461, Chapel Hill, NC 27599. Fax: (919) 843-0776. E-mail: styblo@med.unc.edu.

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

ABSTRACT

Chronic exposure to inorganic arsenic (iAs) has been linked to an increased risk of diabetes, yet the specific disease phenotype and underlying mechanisms are poorly understood. In the present study we set out to identify iAs exposure-associated metabolites with altered abundance in nondiabetic and diabetic individuals in an effort to understand the relationship between exposure, metabolomic response, and disease status. A nested study design was used to profile metabolomic shifts in urine and plasma collected from 90 diabetic and 86 nondiabetic individuals matched for varying iAs concentrations in drinking water, body mass index, age, and sex. Diabetes diagnosis was based on measures of fasting plasma glucose and 2-h blood glucose. Multivariable models were used to identify metabolites with altered abundance associated with iAs exposure among diabetic and nondiabetic individuals. A total of 132 metabolites were identified to shift in urine or plasma in response to iAs exposure characterized by the sum of iAs metabolites in urine (U-tAs). Although many metabolites were altered in both diabetic and nondiabetic 35 subjects, diabetic individuals displayed a unique response to iAs exposure with 59 altered metabolites including those that play a role in tricarboxylic acid cycle and amino acid metabolism. Taken together, these data highlight the broad impact of iAs exposure on the human metabolome, and demonstrate some specificity of the metabolomic response between diabetic and nondiabetic

individuals. These data may provide novel insights into the mechanisms and phenotype of diabetes associated with iAs exposure.

Key words: arsenic; chronic exposure; diabetes; metabolomics; urine; plasma

Drinking water contaminated with inorganic arsenic (iAs) is a major threat to human health with more than 100 million people worldwide exposed to levels that exceed the World Health Organization's (WHO) recommended limit of 10 μg As/L (WHO, 1993). Chronic exposure to iAs has been linked to an increased risk of cancer and noncancerous diseases, including cardiovascular disease and diabetes mellitus (Kuo *et al.*, 2013; Maull *et al.*, 2012; Moon *et al.*, 2012; Navas-Acien *et al.*, 2006; States *et al.*, 2009). Although many of the original reports refer to iAs-associated diabetes simply as Type-2 diabetes, growing evidence suggests that the disease phenotype and mechanisms of iAs-associated diabetes are more complex. Type-2 diabetes is often associated with obesity and is characterized primarily by hyperinsulinemia, insulin resistance with a compensatory increase in insulin secretion, followed by β-cell dysfunction (Adams, 2011). However, a recent study from areas with low to moderate concentrations of iAs in drinking water found positive association between iAs and diabetes yet negative correlations between iAs exposure and indicators of insulin resistance (i.e. fasting plasma insulin and homeostatic model assessment of insulin resistance (HOMA-IR)) (Del Razo *et al.*, 2011; Navas-Acien, 2012). Similarly, mice that developed diabetes as a result of chronic exposure to iAs in drinking water exhibited a phenotype characterized by a lack of insulin resistance and an impaired response of β-cells to glucose challenge (Paul *et al.*, 2011). Taken together these studies highlight that iAs-associated diabetes is potentially mechanistically different from Type 2 diabetes.

Laboratory studies using tissue culture systems have shown that arsenite (iAsIII) and its trivalent methylated metabolites inhibit insulin signaling and insulin-dependent glucose uptake in a Type-2 diabetes manner (Paul *et al.*, 2007). However, these arsenicals are also potent inhibitors of glucose-stimulated insulin secretion by pancreatic β-cells or isolated pancreatic islets (Douillet *et al.*, 2013; Fu *et al.*, 2010). Thus, the results of both population and laboratory studies of iAs-associated diabetes are not consistent with the typical Type 2 diabetes phenotype. These studies point instead to pancreatic β-cells as the primary target for iAs exposure and to β-cell dysfunction as the primary mechanism as opposed to insulin resistance. Understanding the phenotype of iAs-associated diabetes and mechanisms underlying this disease is essential for the development of effective treatment strategies in areas where drinking water is contaminated with iAs.

The north-eastern region of Mexico, Chihuahua, is an area with high concentrations of iAs in surface and underground water reservoirs. The concentrations of iAs in Chihuahua drinking water supplies have historically reached up to 800 μg/l (CNA, 2002). During recent years, a limited number of low-capacity purification systems have been installed in rural towns and villages, providing local residents with sources of clean water as an alternative to the contaminated water still supplied through the most municipal water networks. However, because of the cost of maintaining these purification systems and a limited accessibility, tens of thousands of Chihuahua residents continue using water with unsafe levels of iAs.

To examine the association between iAs exposure and diabetes in this area and to better characterize the phenotype of iAs-associated diabetes, we recently established a cohort of 1165 Chihuahua residents exposed to a wide range of iAs concentrations. All subjects in the cohort underwent medical exams focusing on diabetes and were assessed for iAs exposure in both urine and drinking water. The present study used a nested subcohort of 176 individuals, including both diabetic and nondiabetic individuals, to characterize urine and plasma metabolomes associated with iAs exposure. In the context of disease, a primary goal of this research was to identify both "shared" metabolites, i.e. metabolites that are associated with iAs exposure in either diabetic or nondiabetic individuals, as well as diabetes-unique metabolites that can help to characterize iAs-associated diabetes.

MATERIALS AND METHODS

The Chihuahua cohort. All procedures involving human subjects were approved by the IRBs of UNC Chapel Hill and Cinvestav-IPN, and all participants signed a written consent. A total of 1165 adults (≥18 years old) with a minimum of 5-year uninterrupted residency in the study area were recruited between 2008 and 2012 (Currier *et al.*, 2014). Pregnant women and subjects reporting kidney or urinary tract infection were excluded because these conditions may affect profiles of iAs metabolites in urine. Individuals with potential occupational exposure to iAs (e.g. those working with pesticides or in mines or smelters) were also excluded. Samples of drinking water were obtained from each subjects' households. An interviewer-administered study questionnaire was used to record data on residency, occupation, drinking water sources and use, smoking, alcohol consumption, and medical history. Spot urine and fasting venous blood were collected during medical exams which included an oral glucose tolerance test with blood drawn 2 h after a 75 g glucose dose. Plasma from both fasting and 2-h blood samples and urines were immediately frozen and stored at −80°C. Measures of body weight and height were obtained during the exams and used to calculate body mass index (BMI).

The nested subcohort. The following criteria and methods were used to select individuals for this subcohort: For the purpose of this study, only individuals with either fasting plasma glucose (FPG) ≥ 126 mg/dl or two hour blood glucose (2HPG) ≥ 200 mg/dl were considered diabetic. Individuals who reported previous diabetes diagnosis or use of diabetic medication but with both FPG < 126 mg/dl and 2HPG < 200 mg/dl were not treated as diabetic individuals. Using these criteria, 90 diabetic individuals were identified. For comparison, 90 nondiabetic individuals were randomly selected from the cohort within strata of iAs concentration in drinking water (<10, 10–49, 50–149, ≥150 μg As/L), BMI (<25, 25–29, ≥30), sex and age (≤39, 40–49, 50–59, ≥60) to match the distribution of these factors among the cases. The matching criteria were relaxed in four instances when there were no nondiabetic individuals in the same stratum as the case. In addition, four nondiabetic subjects were subsequently excluded from the study because of incorrect or missing data on iAs in water, BMI, or use of diabetic medication. Thus,

the final number of subjects included in the nested subcohort were 90 diabetic and 86 nondiabetic individuals.

Measures of iAs exposure. Hydride generation-atomic absorption spectrometry coupled with a cryotrap (HG-CT-AAS) was used to determine the concentration of iAs in drinking water (DW-iAs) and concentrations of As species in urine, including total iAs, total monomethyl-As (MAs), and total dimethyl-As (DMAs) (Hernandez-Zavala *et al.*, 2008). For quality control, a certified standard reference material Arsenic Species in Frozen Human Urine (SRM 2669; National Institute of Standards and Technology) was analyzed with every single batch of urine samples shipped from the field to UNC. The concentrations of As species measured by HG-CT-AAS in SRM 2669 ranged from 86.7% to 106.4% of the certified values. The limit of detection (LOD) for As in water was 0.1 μg As/L; LODs for As species in urine were 0.05 ng As/ml for MAs or DMAs and 0.1 ng As/ml for iAs. The concentration of total speciated As in urine (U-tAs) was calculated as sum of iAs, MAs, and DMAs. Creatinine concentration in urine was determined by a colorimetric assay (Cayman Chemical Company, Ann Arbor, MI).

Glucose analysis. FPG and 2HPG levels were measured using a Prestige 24i Chemistry Analyzer (Tokyo Boeki, Tokyo, Japan). To ensure accuracy, the analyzer was calibrated prior to analysis and reference human sera with normal and elevated glucose levels (Serodos and Serodos PLUS, Human Diagnostics Worldwide) were used for quality control.

Metabolomic data acquisition. Metabolic profiling was carried out in plasma and urine using gas chromatography (GC) and liquid chromatography (LC) with time of flight-mass spectrometry (TOF-MS) detection (Qi *et al.*, 2007, 2009, 2012; Xie *et al.*, 2008). Here, the GC-TOF-MS spectra were obtained with electron impact ionization (70 eV) at full scan mode (m/z 40–600) with a Rxi-5 ms capillary column (30 m × 250 μm i.d., 0.25-μm film thickness, Restek, PA) on a Pegasus HT system (Leco Co, St Joseph, MI) coupled with an Agilent 6890 GC (Agilent Co, Santa Clara, CA), using helium as the carrier gas at a constant flow rate of 1.0 ml/min. The acquired data files were analyzed by ChromaTOF software (Leco Co). The LC-TOF-MS mass spectra were obtained with Thean Agilent HPLC 1200 system coupled with 6220 MSD TOF-mass spectrometer (MS) (Agilent Corporation Co, Santa Clara, CA). The acquired GC-TOF-MS and LC-TOF-MS data were processed using ChromaTOF software (Leco Co), Agilent MassHunter Qualitative Analysis Program (vB.05.00), and XCMS package (v1.24.1, http://metlin.scripps.edu) (Cheng *et al.*, 2012; Xie *et al.*, 2013). Spectral data conversion was performed using the Agilent MassHunter Qualitative Analysis Program (vB.05.00) and XCMS package. Metabolites were verified and annotated using an in-house library of>800 mammalian metabolites and the online databases, including the Human Metabolome Database (http://www.hmdb.ca/), the National Institute of Standards and Technology library, and the LECO/Fiehn Metabolomics Library. Relative amounts of the metabolites (individual metabolite peak area divided by the sum of all metabolite peak areas) were used in statistical analyses.

Statistical analyses. Data were analyzed using the statistical packages SAS 9.3 (SAS Institute Inc, Cary, NC) and Partek® Genomics SuiteTM Software (St Louis, MO). A Spearman rank test was used to quantify the relationship between DW-iAs and U-tAs (Supplementary Fig. 1). Descriptive statistics were used to summarize iAs in drinking water, U-tAs, and other demographic factors. Because specific gravity was found to be associated with diabetes status within this cohort (Currier *et al.*, 2014) and urinary creatinine levels have been previously linked with iAs exposure, unadjusted values of U-tAs were used in the primary model in the analyses (Basu *et al.*, 2011; Gamble and Liu, 2005; Yassine *et al.*, 2012). The abundance levels of the 221 plasma metabolites and 294 urine metabolites were used for analyses. Human Metabolome Database Identifiers (HMDB IDs) were also determined for metabolites to facilitate comparison between other studies.

The GC-TOF-MS and LC-TOF-MS analyses identified 294 urinary and 221 plasma metabolites, including 89 metabolites that were detected in both plasma and urine with a total of 426 unique metabolites that were profiled in this study (Supplementary Tables 1 and 2). Multivariable regression models were used to establish relationships between metabolite levels and iAs exposure in diabetic and nondiabetic individuals, where U-tAs was used as the main predictor variable and metabolite levels the dependent variable. BMI, age, and sex were selected as covariates based on *a priori* association with diabetes. Statistical significance was set at $p < .05$.

Pathway analysis of statistically significant metabolites. Associations of statistically significantly metabolites with canonical metabolic pathways were determined using the MetPA tool within Metabolomics Pathway Analysis (Xia and Wishart, 2010). This tool maps metabolites onto KEGG pathways and determines the statistical significance as well as the impact of metabolite groups. Human Metabolome Database (HMDB) identifiers were used for these analyses as they provided greater coverage of metabolites than did the metabolite names (Xia and Wishart, 2010). As the metabolites had already been statistically filtered, a more lax statistical significance of $p < .1$ was used in the pathways analysis.

RESULTS

Basic Characteristics of the Subcohort

The subcohort selected for the present study ($n = 176$) consisted of 133 (75.6%) women and 43 (24.4%) men (Table 1). Men and women were equally represented in the diabetic and nondiabetic groups (Table 1). There were no statistically significant differences on average between the two groups for BMI, age, drinking water iAs or U-tAs (Table 1). Among the 176 individuals, a total of 152 (86.9%) individuals were exposed to DW-iAs levels exceeding 25 μg As/l, the current maximum allowable contaminant value for iAs for drinking water supplies in Mexico. Only 20 water samples (11.3%) had DW-iAs below the WHO recommended value of 10 μg As/L. DW-iAs was significantly correlated with U-tAs: $r = 0.37$, $p < .01$ (Supplementary Fig. 1). The key characteristics of the entire Chihuahua cohort were described elsewhere (Currier *et al.*, 2014).

Basic Characteristics of the Metabolome

Of the 426 unique metabolites measured, a total of 132 metabolites were significantly increased or decreased with exposure to iAs (characterized by U-tAs) among either diabetic or nondiabetic individuals (Supplementary Table 1). Of these, 103 were urinary and 32 were plasma metabolites (Table 2), with three metabolites common to both urine and plasma (Supplementary Table 1). However, the response to iAs exposure differed between the matrices. Although in urine the

TABLE 1. Basic Characteristics of the Subcohort

	Entire Subcohort ($n = 176$)		Nondiabetic Individuals ($n = 86$)		Diabetic Individuals ($n = 90$)	
A. Demographics	*N* (%)	Mean (range)	*N* (%)	Mean (range)	*N* (%)	Mean (range)
Sex						
Female	133 (75.6)	—	65 (75.6)	—	68 (75.6)	—
Male	43 (24.4)	—	21 (24.4)	—	22 (24.4)	—
Age (years)	—	50 (18–79)	—	50 (18–78)	—	51 (23–79)
BMI	—	30.4 (18.3–45.2)	—	30.1 (19.2–43.6)	—	30.7 (18.3–45.2)
Fasting blood glucose	—	131.1 (49.5–379.0)	—	88.7 (49.0.5–117)*	—	169.7(63.0–379.0)*
2HPG	—	163.8(50.0–437.0)	—	112.5(50.0–191.5)*	—	211.1 (50.5–437.0)*
B. Arsenic exposure						
DW-iAs (μg/l)	—	68.4 (LOD–292.9)		65.8 (LOD–284.7)		70.8 (LOD–284.7)
≤ 10 μg/l	20 (11.3)	—	11 (12.8)	—	9 (10.0)	—
≥ 10 μg/l	156 (88.7)	—	75 (87.2)	—	81 (90.0)	—
≤ 25 μg/l	24 (13.1)	—	14 (16.3)	—	10 (11.1)	—
≥ 25 μg/l	152 (86.9)	—	72 (83.7)	—	80 (89.9)	—
U-tAs[a]	—	86.8 (3.85–348.6)	—	80.5 (6.11–348.61)	—	92.6 (3.85–346.3)

[a]Sum of iAs, MMAs, DMAs.
*Statistically significant difference between diabetic and nondiabetic groups.

TABLE 2. Number of U-tAs Associated Metabolites in Either Plasma or Urine

	Urine[a], *n* (%)	Plasma[b], *n* (%)	Overall, *n*
Overall	**103**	**32**	**132**
Increased metabolite level	68 (66)	9 (31)	77
Decreased metabolite level	35 (34)	20 (68)	55
Nondiabetic individuals	**61**	**15**	**73**
Increased metabolite level	35 (57)	3 (20)	36
Decreased metabolite level	26 (43)	12 (80)	37
Diabetic individuals	**73**	**17**	**87**
Increased metabolite level	52 (71)	9 (53)	59
Decreased metabolite level	21 (29)	8 (47)	28

[a]Total number of urinary metabolites tested $n = 294$.
[b]Total number of plasma metabolites tested $n = 221$.
Numbers in bold represent total numbers of U-tAs associated metabolites.

concentrations for most of the altered metabolites ($n = 68$, 66%) increased, the concentrations of most of the plasma metabolites ($n = 20$, 63%) decreased (Table 2, Supplementary Table 1).

Urinary and Plasma Metabolites Associated with U-tAs in Nondiabetic and Diabetic Subjects

In urine, a total of 61 metabolites were associated with U-tAs among nondiabetic subjects and 73 metabolites were associated with U-tAs among diabetic subjects (Fig. 1a, Table 2, Supplementary Table 1). Of those metabolites, 30 were unique to nondiabetic individuals, 42 were unique to diabetic individuals, and 31 metabolites were associated with U-tAs in both groups (Fig. 1a). Among the 31 common metabolites were those related to amino acid metabolism, the tricarboxylic acid (TCA) cycle, and pyruvate metabolism. The majority of the urinary metabolites showed a positive association with iAs exposure: 35 of the 61 metabolites in nondiabetics, and 52 of the 73 metabolites in diabetic individuals (Table 2, Supplementary Table 1).

In plasma, a total of 15 metabolites were associated with U-tAs among nondiabetic individuals and 17 metabolites were associated with U-tAs among diabetic individuals (Fig. 1b, Supplementary Table 1). No U-tAs-associated metabolites in plasma were common among the diabetic and nondiabetic subjects (Fig. 1b, Supplementary Table 1). Many of the plasma metabolites were negatively associated with iAs exposure: 8 of the 17 metabolites in diabetics and 12 of the 15 metabolites in nondiabetic individuals (Table 2, Supplementary Table 1). Thus, across the two matrices diabetic individuals displayed altered levels of 59 metabolites (42 urinary metabolites and 17 plasma metabolites) (Supplementary Table 1).

Urinary and Plasma Metabolite-Enriched Pathways in Nondiabetic and Diabetic Subjects

Using METPA to perform KEGG enrichment analysis, the 61 urinary metabolites associated with U-tAs in nondiabetic individuals were enriched for 9 metabolic pathways (Fig. 1c, Table 3). Three of these pathways were associated with amino acid metabolism (alanine/aspartate/glutamate, phenylalanine, arginine and proline), 5 with carbohydrate/energy metabolism (TCA cycle, glyoxylate/dicarboxylate, galactose, pentose/glucuronate, and pyruvate), and 1 was associated with vitamin (riboflavin) metabolism (Table 3).

The 73 urinary metabolites identified in diabetic individuals enriched for 8 pathways (Fig. 1c, Table 3). Of these pathways, 3 were associated with amino acid metabolism (alanine/aspartate/glutamate, phenylalanine, taurine) and 4 with carbohydrate/energy metabolism (TCA cycle, glyoxylate, pentose, and pyruvate). One pathway was associated with nucleic acid (purine) metabolism (Table 3).

When examining the overlap between the study groups at a pathway level, there were 6 pathways that were associated with U-tAs in both nondiabetic and diabetic subjects. These were pathways for metabolism of alanine/aspartate/glutamate, glyoxylate/dicarboxylate, pentose/glucuronate, phenylalanine, and pyruvate, and TCA cycle (Fig. 1c, Table 3). Three pathways were unique to the metabolome of nondiabetic subjects, including the pathways of arganine/proline, galactose, and riboflavin metabolism. Two pathways involved in purine and taurine metabolism were unique to the metabolome of diabetic subjects.

When KEGG enrichment analysis was conducted for the 15 plasma metabolites associated with U-tAs in nondiabetic

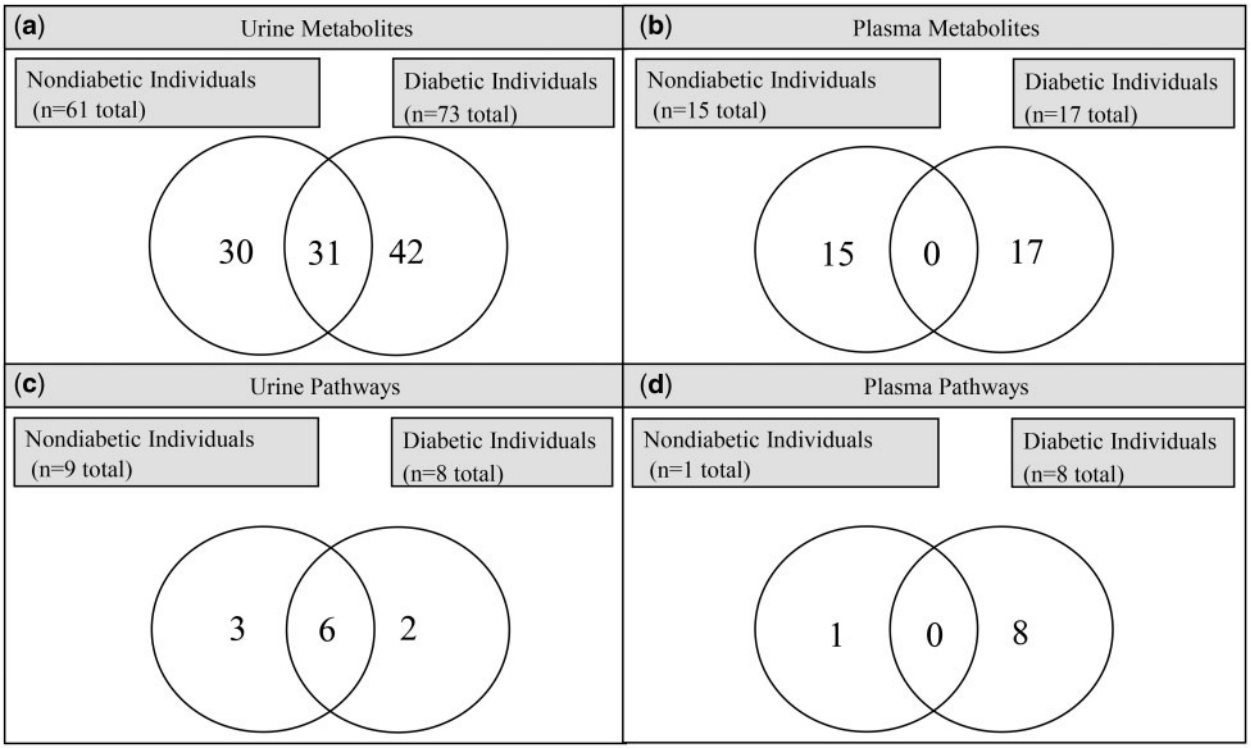

FIG. 1. U-tAs associated urinary and plasma metabolites and pathways identified for diabetic and nondiabetic individuals. Venn diagrams illustrate the overlap of metabolites in urine (a) or plasma (b) and of pathways in urine (c) or plasma (d) between nondiabetic individuals and diabetic individuals.

individuals, a single pathway was enriched, namely the pathway for biosynthesis of phenylalanine, tyrosine, and tryptophan (Fig. 1d, Table 3). The 17 plasma metabolites associated with U-tAs in diabetic individuals enriched for 8 pathways (Fig. 1d, Table 3). Of these 8, 5 are associated with amino acid metabolism (arginine/proline, lysine, phenylanine, and tyrosine and aminoacyl-tRNA biosynthesis), 2 are associated with energy metabolism (nitrogen metabolism and TCA cycle), and 1 pathway is associated with thiamine metabolism (Table 3). An examination of the overlap between the study groups at a pathway level revealed that there were no pathways associated with U-tAs in both nondiabetic and diabetic subjects (Fig. 1d, Supplementary Table 2). Specifically, there was 1 pathway unique to the response of nondiabetic subjects to arsenic exposure, and 8 pathways unique to the diabetic response to iAs exposure.

As a separate analysis, the 59 metabolites that were uniquely associated with U-tAs in diabetic individuals (i.e. 42 urinary metabolites and 17 plasma metabolites) were also assessed for their enriched metabolic pathways, a total of 10 metabolic pathways were identified (Table 3). Among these, were 5 pathways related to amino acid metabolism, 3 related to energy metabolism and 2 related to B vitamin (biotin, riboflavin, and thiamine) metabolism.

DISCUSSION

A consensus exists that moderate-to-high chronic exposures to iAs are associated with an increased risk of diabetes (Maull *et al.*, 2012). However, questions remain about pathophysiological processes that underlie this disease and about the phenotype of diabetes developed in the presence of iAs exposure. One way to address these questions is the identification of metabolic shifts associated with iAs exposure among diabetic and nondiabetic individuals. Here we examined plasma and urine metabolites that were altered in response to iAs exposure in both nondiabetic and diabetic individuals in our Chihuahua cohort where the association between iAs exposure and prevalent diabetes was recently established (Currier *et al.*, 2014). These metabolites and their corresponding pathways were then compared to identify features that were common or unique to the nondiabetic and diabetic individuals in their response to iAs exposure, to gain insight into iAs-associated disease.

We identified a total of 132 urinary and plasma metabolites that were associated with U-tAs in either nondiabetic or diabetic individuals, with 103 urinary metabolites and 32 plasma metabolites. Approximately, one-third of the urinary metabolites that were U-tAs associated (31/103, 30%) were identified as common to both nondiabetic and diabetic subjects. It was interesting to note that in contrast to urine, no overlap was observed in plasma, highlighting the nonconcordant results in the matrices measured. The 31 urinary metabolites that were common between the nondiabetic and diabetic individuals include but are not limited to metabolites that broadly play a role in amino acid metabolism (aspartic acid), the TCA cycle (malate, cAMP) and pyruvate metabolism (acetylphosphate). It is important to note that all of these metabolites were changed similarly in their levels between nondiabetic and diabetic subjects demonstrating a broad, disease-independent response to iAs exposure. As these pathways are critical for energy processing, including adenosine triphosphate (ATP) production (Dashty, 2013), alterations of these pathways could indicate impacts of iAs exposure on cellular energy production. In addition, the data suggest that effects of iAs exposure within the shared pathways may differ between diabetic and nondiabetic subjects. For example, the effects on TCA cycle in diabetics included shifts in succinate and fumarate, whereas isocitrate and oxaloacetate were shifted

TABLE 3. Pathways Enriched for U-tAs Associated Metabolites in Plasma and Urine of Diabetic and Nondiabetic Individuals

Class of Metabolism	Metabolic Pathway	Urine Nondiabetic Individuals ($n = 61$*)	Urine Diabetic Individuals ($n = 73$*)	Plasma Nondiabetic Individuals ($n = 15$*)	Plasma Diabetic Individuals ($n = 17$*)	Urine and Plasma Diabetes Unique Metabolites ($n = 59$*)
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	$p = .001$ Oxaloacetate (−), N-Acetyl-L-aspartate(+), Aspartate (−), Glutamine (−)	$p = .001$ N-Acetyl-L-aspartate (+), Aspartate (−), Succinate (+)			$p = .053$ Furmurate (+, P), succinate (+,U)
Amino acid metabolism	Aminoacyl-tRNA biosynthesis				$p < .001$ Glycine (+), Lysine (−), Tyrosine (−), Proline (+)	$p < .001$ Glycine (+, P), Serine (+, U), Methionine (+, U), Lysine (−, P), Proline (+, P)
Amino acid metabolism	Arginine and proline metabolism	$p = .061$ Glutamine (−), Guanidinoacetate (−), Creatinine (+), Pyrrole-2-carboxylate (−)			$p = .004$ Fumarate (+), Citrulline (−), Proline (+)	
Amino acid metabolism	Glycine, serine, threonine metabolism					$p = .039$ Serine (+, U), Glycine (+, P), 5-Aminolevulinic acid (−, U)
Amino acid metabolism	Lysine degradation				$p = .01835$ Glycine (+), Lysine (−)	$p = .006$ Glutaric acid (+, U), Lysine (−, P), Glycine (+, P), Carnitine (−, U)
Amino acid metabolism	Phenylalanine, tyrosine and tryptophan biosynthesis			$p = .086414$ Erythrose 4-phosphate (−)		
Amino acid metabolism	Phenylalanine metabolism	$p = .056$ Pheynlpurvate (−), Acetamide (−), 4-Hydrobenzoate (−)	$p = .010$ Phenylpyruvate (−), Acetamide (−), Succinate (+)		$p = .017$ Tyrosine (−), Fumarate (+)	$p = .005$ Tyrosine (−, P), Phenylglyoxylic acid (+, U), Fumarate (+, P), succinate (+, P)
Amino Acid Metabolism	Taurine and hypotaurine metabolism		$p = .055$ Acetylphosphate (−), Taurine (−)			
Amino acid metabolism	Tyrosine metabolism				$p = .045$ Tyrosine (−), Fumarate (+)	
Energy metabolism	Citrate cycle (TCA cycle)	$p < .001$ Malate (+), Oxaloacetate (−), Aconitate (+), Isocitrate (+)	$p = .006$ Malate (+), Succinate (+), Aconitate (+)		$p = .003$ Malate (+), Fumarate (+)	$p = .039$ Furmarate (+, P), Succinate (+, U)
Energy metabolism	Galactose metabolism	$p = .044$ Glyceraldehyde 3-phosphate (−), Galactitol (+), Sucrose (+)				
Energy metabolism	Glyoxylate and dicarboxylate metabolism	$p = .015$ Oxaloacetate (−), Aconitate (+), Malate (+), Isocitrate (+)	$p = .014$ Oxalate (−), Malate (+), Aconitate (+), Succinate (+)			

TABLE 3. Continued

Class of Metabolism	Metabolic Pathway	Urine	Urine	Plasma	Plasma	Urine and Plasma
		Nondiabetic Individuals ($n = 61$*)	Diabetic Individuals ($n = 73$*)	Nondiabetic Individuals ($n = 15$*)	Diabetic Individuals ($n = 17$*)	Diabetes Unique Metabolites ($n = 59$*)
Energy metabolism	Methane metabolism					**$p = .099$** Serine (+, U), Glycine (+, P)
Energy metabolism	Nitrogen metabolism				**$p = .012$** Tyrosine (−), Glycine (+)	**$p = .02$** Tyrosine (−, P), Taurine (−, U), Glycine (+, U)
Energy metabolism	Pentose and glucuronate interconversions	**$p = .019$** Glucaric Acid (+), Ribitol (+), Arabitol (+), Glyceraldehyde 3-phosphate (−)	**$p = .079$** Glutaric Acid (+), Ribitol (+), Arabitol (−)			
Energy metabolism	Pyruvate metabolism	**$p = .023$** Acetylphosphate (−), Oxaloacetate (−), Malate (+)	**$p = .022$** Homocitrate (+), Acetylphosphate (−), Malate (+)			
Nucleic Acid Metabolism	Purine metabolism		**$p = .096$** Adenosine (+), Aconitate (+), Oxalate (−), Cyclic AMP (+)			
Vitamin Metabolism	Biotin metabolism					**$p = .012$** Biotin (+, U), Lysine (−, P)
Vitamin metabolism	Riboflavin metabolism	**$p = .062$** Ribitol (+), Hydroquinone (+)				
Vitamin metabolism	Thiamine metabolism				**$p = .005$** Tyrosine (−), Glycine (+)	**$p = .054$** Tyrosine (−, P), Glycine (+, P)

Increases or decreases in metabolite levels are indicated with (+) or (−).
* n represents the total number of U-tAs associated metabolites.

only among nondiabetics; shifts in the concentrations of another two TCA cycle metabolites, aconitic acid and malate, were shared by both conditions. It is known that trivalent iAs, at high doses, can inhibit pyruvate dehydrogenase by binding directly (Hughes, 2002). This mode of action has also been suggested to decrease ATP production.

In the context of disease specificity, we identified a set of 59 U-tAs-associated metabolites that were unique to the diabetic individuals, specifically 42 U-tAs-associated urinary metabolites and 17 U-tAs-associated plasma metabolites. Among these diabetes-specific metabolites were those that play a role in two major areas of human metabolism: (1) the TCA cycle and energy-related pathways (succinate, furmarate, taurine) and (2) amino acid metabolism (glycine, serine, methionine, lysine, proline, 5-aminolevulinic acid, glutaric acid, carnitine, tyrosine, and phenylglyoxylic acid). Within this group of metabolites are also those associated with metabolism of three major B vitamins: biotin, riboflavin, and thiamine. Notably, these vitamins are known for their roles in both the energy and the amino acid metabolism. Additionally, both deficiencies for riboflavin and thiamine have been shown to be associated with increased incidence of skin lesions in human populations (Melkonian *et al.*, 2012). Also, methionine and thiamine, in conjunction with other antioxidants, have been shown to reverse the oxidative stress burden in target organs of mice exposed to arsenic (Nandi *et al.*, 2005).

Although diabetes developed in the presence of chronic exposure to iAs has been often referred to as Type-2 diabetes, an exact disease phenotype is only poorly characterized. When we compared the 59 U-tAs-associated metabolites unique to diabetic individuals in the Chihuahua cohort with the previously published Type 2 diabetes-associated metabolites (Kaur *et al.*, 2013; Wang *et al.*, 2011) or metabolites that predict the risk of Type 2 diabetes (Kaur *et al.*, 2013), we found only 4 shared metabolites: tyrosine, taurine, succinate, 3-hydroxy-3-methylglutaric acid even though 14 metabolites were common across all studies. Thus, most of the metabolites that shifted in the urine or plasma of diabetic individuals in response to iAs exposure were not among the Type 2 diabetes-associated metabolites reported in previous studies. These results point to a unique phenotype of iAs-associated diabetes apparent at the level of the metabolome. It is possible that the U-tAs-associated metabolites unique to diabetic individuals represent a metabolomic fingerprint of iAs-associated diabetes.

Although a metabolomics phenotype of iAs-associated diabetes begins to emerge, this study is not without limitations.

Most importantly, it was not possible to establish whether diabetic individuals' metabolomics differences were due to diabetes developing as a result of iAs exposure or if diabetic individuals respond differently to iAs. The response of an individual to environmental exposures is multifactorial. For this reason, the analysis performed controlled for factors known to influence disease such as age, BMI, and gender. As with similar study designs, it is not possible to assess the roles played by genetic susceptibility, diet, and other lifestyle factors. Another limitation of this study was that U-tAs was not strongly correlated to DW-iAs suggesting the potential for additional sources of As exposure, such as diet and occupational exposures. In addition, it is not possible to directly compare the metabolomes between diabetic and nondiabetic individuals as they were matched on levels of iAs exposure. Further investigations, including both population-based and laboratory models, are necessary to determine perturbation of these metabolites is a potential mechanism by which iAs influences diabetes status.

Taken together there are three major findings from this study: first, we have identified more than 100 metabolites that are altered in urine and plasma in response to iAs exposure. Second a set of these metabolites was identified that differentiates diabetic individuals from nondiabetic individuals. Third, only a minimal overlap was observed between previously identified Type 2 diabetes-associated metabolites and the diabetes-specific iAs exposure-associated metabolites, representing a putative fingerprint of iAs-associated diabetes. These data provide insight into the complex mechanism of action of iAs-associated disease in human populations with relevance to millions around the globe.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

NIH grants (R01ES015326 and 3R01ES015326-03S1 to M.S.) and in part by (P30ES010126, P42ES005948, R01ES019315 and T32ES007018). UNC Nutrition Obesity Research Center grant (DK056350).

ACKNOWLEDGMENTS

Disclosure: The authors declare that they do not have any competing financial interests to disclose.

REFERENCES

Adams, S. H. (2011). Emerging perspectives on essential amino acid metabolism in obesity and the insulin-resistant state. *Adv. Nutr.* **2**, 445–456.

Basu, A., Mitra, S., Chung, J., Guha Mazumder, D. N., Ghosh, N., Kalman, D., von Ehrenstein, O. S., Steinmaus, C., Liaw, J., and Smith, A. H. (2011). Creatinine, diet, micronutrients, and arsenic methylation in West Bengal, India. *Environ. Health Perspect.* **119**, 1308–1313.

Cheng, Y., Xie, G., Chen, T., Qiu, Y., Zou, X., Zheng, M., Tan, B., Feng, B., Dong, T., He, P., *et al.* (2012). Distinct urinary metabolic profile of human colorectal cancer. *J. Proteome Res.* **11**, 1354–1363.

CNA, 2002. Comisión Nacional del Agua, Vega-Gleason S. (2002). Riesgo sanitario ambiental por la Presencia de Arsénico en los Acuíferos de México.

Currier, J. M., Ishida, M. C., González-Horta, C., Sánchez-Ramírez, B., Ballinas-Casarrubias, L., Gutiérrez-Torres, D. S., Hernández Cerón, R., Viniegra Morales, D., Baeza Terrazas, F. A., Del Razo, L. M., *et al.* (2014). Associations between arsenic species in exfoliated urothelial cells and prevalence of diabetes among residents of Chihuahua, Mexico. *Environ. Health Perspect.* **122**, 1088–1094.

Dashty, M. (2013). A quick look at biochemistry: carbohydrate metabolism. *Clin. Biochem.* **46**, 1339–1352.

Del Razo, L. M., Garcia-Vargas, G. G., Valenzuela, O., Castellanos, E. H., Sanchez-Pena, L. C., Currier, J. M., Drobna, Z., Loomis, D., and Styblo, M. (2011). Exposure to arsenic in drinking water is associated with increased prevalence of diabetes: a cross-sectional study in the Zimapan and Lagunera regions in Mexico. *Environ. Health* **10**, 1–11.

Douillet, C., Currier, J., Saunders, J., Bodnar, W. M., Matousek, T., and Styblo, M. (2013). Methylated trivalent arsenicals are potent inhibitors of glucose stimulated insulin secretion by murine pancreatic islets. *Toxicol. Appl. Pharmacol.* **267**, 11–15.

Fu, Y. Y., Kang, K. J., Ahn, J. M., Kim, H. R., Na, K. Y., Chae, D. W., Kim, S., and Chin, H. J. (2010). Hyperbilirubinemia reduces the streptozotocin-induced pancreatic damage through attenuating the oxidative stress in the Gunn rat. *Tohoku J. Exp. Med.* **222**, 265–273.

Gamble, M. V. and Liu, X. (2005). Urinary creatinine and arsenic metabolism. *Environ. Health Perspect.* **113**, A442.

Hernandez-Zavala, A., Matousek, T., Drobna, Z., Paul, D. S., Walton, F., Adair, B. M., Jiri, D., Thomas, D. J., and Styblo, M. (2008). Speciation analysis of arsenic in biological matrices by automated hydride generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer (multiatomizer). *J. Anal. Atom. Spectrom.* **23**, 342–351.

Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicol. Lett.* **133**, 1–16.

Kaur, P., Rizk, N., Ibrahim, S., Luo, Y., Younes, N., Perry, B., Dennis, K., Zirie, M., Luta, G., and Cheema, A. K. (2013). Quantitative metabolomic and lipidomic profiling reveals aberrant amino acid metabolism in type 2 diabetes. *Mol. BioSys.* **9**, 307–317.

Kuo, C. C., Moon, K., Thayer, K. A., and Navas-Acien, A. (2013). Environmental chemicals and type 2 diabetes: An updated systematic review of the epidemiologic evidence. *Curr. Diabetes Rep.* **13**, 831–849.

Maull, E. A., Ahsan, H., Edwards, J., Longnecker, M. P., Navas-Acien, A., Pi, J., Silbergeld, E. K., Styblo, M., Tseng, C. H., Thayer, K. A., and Loomis, D. (2012). Evaluation of the association between arsenic and diabetes: A National Toxicology Program workshop review. *Environ. Health Perspect.* **120**, 1658–1670.

Melkonian, S., Argos, M., Chen, Y., Parvez, F., Pierce, B., Ahmed, A., Islam, T., and Ahsan, H. (2012). Intakes of several nutrients are associated with incidence of arsenic-related keratotic skin lesions in Bangladesh. *J. Nutr.* **142**, 2128–2134.

Moon, K., Guallar, E., and Navas-Acien, A. (2012). Arsenic exposure and cardiovascular disease: an updated systematic review. *Curr. Atherosclerosis Rep.* **14**, 542–555.

Nandi, D., Patra, R. C., and Swarup, D. (2005). Effect of cysteine, methionine, ascorbic acid and thiamine on arsenic-induced oxidative stress and biochemical alterations in rats. *Toxicology* **211**, 26–35.

Navas-Acien, A. (2012). Exploring links between arsenic and diabetes, with Ana Navas-Acien. Interview by Ashley Ahearn. *Environ. Health Perspect.* **120**, 10.1289/ehp.trp110112.

Navas-Acien, A., Silbergeld, E. K., Streeter, R. A., Clark, J. M., Burke, T. A., and Guallar, E. (2006). Arsenic exposure and type 2 diabetes: A systemic review of the experimental and epidemiological evidence. *Environ. Health Prespect.* **114**, 641–648.

Paul, D. S., Harmon, A. W., Devesa, V., Thomas, D. J., and Styblo, M. (2007). Molecular mechanisms of the diabetogenic effects of arsenic: inhibition of insulin signaling by arsenite and methylarsonous acid. *Environ. Health Perspect.* **115**, 734–742.

Paul, D. S., Walton, F. S., Saunders, R. J., and Styblo, M. (2011). Characterization of the impaired glucose homeostasis produced in C57BL/6 mice by chronic exposure to arsenic and high-fat diet. *Environ. Health Perspect.* **119**, 1104–1109.

Qi, Y., Li, P., Zhang, Y., Cui, L., Guo, Z., Xie, G., Su, M., Li, X., Zheng, X., Qiu, Y., *et al.* (2012). Urinary metabolite markers of precocious puberty. *Mol. Cell. Proteomics* **11**, M111 011072.

Qiu, Y., Cai, G., Su, M., Chen, T., Zheng, X., Xu, Y., Ni, Y., Zhao, A., Xu, L. X., Cai, S., *et al.* (2009). Serum metabolite profiling of human colorectal cancer using GC-TOFMS and UPLC-QTOFMS. *J. Proteome Res.* **8**, 4844–4850.

Qiu, Y., Su, M., Liu, Y., Chen, M., Gu, J., Zhang, J., and Jia, W. (2007). Application of ethyl chloroformate derivatization for gas chromatography-mass spectrometry based metabonomic profiling. *Anal. Chim. Acta* **583**, 277–283.

States, J. C., Srivastava, S., Chen, Y., and Barchowsky, A. (2009). Arsenic and cardiovascular disease. *Toxicol. Sci.* **107**, 312–323.

Wang, T. J., Larson, M. G., Vasan, R. S., Cheng, S., Rhee, E. P., McCabe, E., Lewis, G. D., Fox, C. S., Jacques, P. F., Fernadez, C., *et al.* (2011). Metabolite profiles and the risk of developing diabetes. *Nat. Med.* **17**, 448–453.

WHO (1993). Guidelines for drinking water quality. *World Health Organization*.

Xia, J., and Wishart, D. S. (2010). MetPA: A web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics* **26**, 2342–2344.

Xie, G., Plumb, R., Su, M., Xu, Z., Zhao, A., Qiu, M., Long, X., Liu, Z., and Jia, W. (2008). Ultra-performance LC/TOF MS analysis of medicinal Panax herbs for metabolomic research. *J. Separation Sci.* **31**, 1015–1026.

Xie, G., Zhong, W., Li, H., Li, Q., Qiu, Y., Zheng, X., Chen, H., Zhao, X., Zhang, S., Zhou, Z., *et al.* (2013). Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. *FASEB J.* **27**, 3583–3593.

Yassine, H., Kimzey, M. J., Galligan, M. A., Gandolfi, A. J., Stump, C. S., and Lau, S. S. (2012). Adjusting for urinary creatinine overestimates arsenic concentrations in diabetics. *Cardiorenal Med.* **2**, 26–32.