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Urinary Metabolomics Revealed Arsenic Internal Dose-Related Metabolic Alterations: A Proof-of-Concept Study in a Chinese Male Cohort

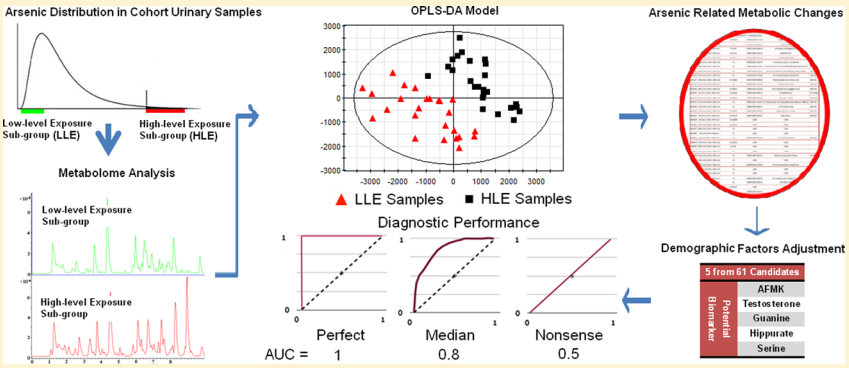
Jie Zhang,[†] Heqing Shen,*[,†] Weipan Xu,[†] Yankai Xia,[‡] Dana Boyd Barr,[§] Xiaoli Mu,[†] Xiaoxue Wang,[†] Liangpo Liu,[†] Qingyu Huang,[†] and Meiping Tian[†]

[†]Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen, Fujian 350002, China

[‡]Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing, Jiangsu 210029, China

[§]Rollins School of Public Health, Emory University, Atlanta, Georgia 30322, United States

Supporting Information



ABSTRACT: Urinary biomonitoring provides the most accurate arsenic exposure assessment; however, to improve the risk assessment, arsenic-related metabolic biomarkers are required to understand the internal processes that may be perturbed, which may, in turn, link the exposure to a specific health outcome. This study aimed to investigate arsenic-related urinary metabolome changes and identify dose-dependent metabolic biomarkers as a proof-of-concept of the information that could be obtained by combining metabolomics and targeted analyses. Urinary arsenic species such as inorganic arsenic, methylarsonic acid, dimethylarsinic acid and arsenobetaine were quantified using high performance liquid chromatography (HPLC)-inductively coupled plasma-mass spectrometry in a Chinese adult male cohort. Urinary metabolomics was conducted using HPLC-quadrupole time-of-flight mass spectrometry. Arsenic-related metabolic biomarkers were investigated by comparing the samples of the first and fifth quintiles of arsenic exposure classifications using a partial least-squares discriminant model. After the adjustments for age, body mass index, smoking, and alcohol consumption, five potential biomarkers related to arsenic exposure (i.e., testosterone, guanine, hippurate, acetyl-*N*-formyl-5-methoxykynurenamine, and serine) were identified from 61 candidate metabolites; these biomarkers suggested that endocrine disruption and oxidative stress were associated with urinary arsenic levels. Testosterone, guanine, and hippurate showed a high or moderate ability to discriminate the first and fifth quintiles of arsenic exposure with area-under-curve (AUC) values of 0.89, 0.87, and 0.83, respectively; their combination pattern showed an AUC value of 0.91 with a sensitivity of 88% and a specificity of 80%. Arsenic dose-dependent AUC value changes were also observed. This study demonstrated that metabolomics can be used to investigate arsenic-related biomarkers of metabolic changes; the dose-dependent trends of arsenic exposure to these biomarkers may translate into the potential use of metabolic biomarkers in arsenic risk assessment. Since this was a proof-of-concept study, more research is needed to confirm the relationships we observed between arsenic exposure and biochemical changes.

INTRODUCTION

Arsenic pollution is considered one of the top environmental health risks globally. Chronic exposure to inorganic arsenic (iAs) has been associated with an increased risk for developing various diseases.[1−5] Over the past 50 years, epidemiologic studies have uncovered arsenic-related adverse health effects primarily by investigating people living in highly contaminated geographical areas.[6,7] Dietary arsenic and inhaled particulates may be other important sources for general population exposures.[8,9] For a typical Chinese adult living in an urban area where safe drinking water is supplied, the daily dietary intake of iAs was estimated to

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be 42.81 μg/day.[9] This exposure source in combination with aggregate exposures from other pathways may result in exposure levels that may pose a health risk to the general population.[5,10]

Because of the multiple potential pathways of exposure including unknown sources, classic bottom-up environmental measurements to estimate risk may be incomplete.[9] A way to obtain additional information on the early biological effect related to these exposures is the top-down strategy.[11] The targeted urinary arsenic measurements combined with complementary omics data enable the evaluation of biological consequences associated with arsenic exposures.[12,13] A variety of protein and nucleic acid biomarkers were identified through genomics, transcriptomics, and proteomics in human populations;[14] however, metabolome-based biomarkers that result from arsenic exposures have not been investigated.

Metabolic biomarkers provide a logical mechanism to correlate potential environmental risk factors with adverse health outcomes. For example, the metabolic changes detected in blood and urine can reflect the molecular phenotypic response of an individual to an environmental insult. Such changes can be monitored readily in populations, especially those detectable in urine. Thus, a robust method of screening for metabolic biomarkers, especially in urine samples, could guide arsenic risk assessment processes by linking the exposure dose to the metabolite response. Metabolomics is a promising approach to comprehensively understand disrupted metabolic pathways. Arsenic-related metabolomic perturbations in mice, clam *Ruditapes philippinarum*, and *Daphnia magna* were reported.[15−18] In addition, epidemiologic studies have linked iAs exposure with an increased incidence of type 2 diabetes[4] and metabolic syndrome,[19] which suggests that arsenic could alter human metabolism. For the first time, we found that ambient arsenic exposure (i.e., general population exposure levels) was significantly associated with male infertility through oxidative stress and sexual hormone disrupting mechanisms, as indicated by infertility-related metabolic biomarkers.[5] Despite the growing body of evidence that arsenic is involved in metabolic perturbations and that similar observations were made with exposure to other environmental pollutants (e.g., laborers exposed to welding fumes,[20] rural pregnant women exposed to pesticides,[21] and people exposed to cadmium released from nearby smelters[22]), arsenic-related metabolome changes have not been addressed so far.

In this study, we hypothesized that ambient arsenic exposure could disrupt individual metabolism in the general Chinese population. We measured both the arsenic exposure and metabolomic alterations in the same urine samples, thus any uncertainties associated with sampling or sample differences were eliminated. Because the samples were collected from persons with no clear adverse health outcomes, the identified metabolomic biomarkers were expected to characterize early arsenic effects, which could potentially lead to a better understanding of the toxicities associated with arsenic exposure. In addition, the possibility of these metabolic biomarkers in refining risk assessment is also discussed.

MATERIALS AND METHODS

Subject Demographics and Sample Collection. Our study was approved by the institutional ethics committee and conducted in accordance with the Helsinki Declaration. One hundred twenty-seven adult men were enrolled from the affiliated hospitals of Nanjing Medical University (NJMU) from 2008−2009, who were the healthy controls of the NJMU Infertility Study.[5] All of the participants were ethnically Han Chinese. All of the participants provided their written informed consent. Each participant was asked to complete a questionnaire that provided information including age, weight, height, education level, annual family income, profession, and smoking and alcohol consumption (current, past, never).

Spot urine samples are commonly used to monitor individual exposure to arsenic;[23] thus, first morning void spot urine samples were collected to assess the arsenic exposure. The samples were centrifuged and filtered with 0.45 μm filters to remove cell sediment and then stored at −80 °C immediately after the sampling. The urine samples were transported on dry ice to the analytical laboratory in Xiamen and were kept at −80 °C until the analysis.

Arsenic Measurement. Urinary arsenic species (i.e., iAs^{III}, iAs^{V}, methylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AsB)) were measured according to our previous reported method.[10] The details are described in the Supporting Information. The limit of detection (LOD) was 0.2 μg/L for iAs^{III}, AsB, MMA, DMA, and 0.5 μg/L for iAs^{V}; the relative standard deviations (RSDs) were 5.53, 5.21, 3.62, 6.39, and 5.25% for iAs^{III}, iAs^{V}, AsB, MMA, and DMA, respectively. The total iAs was calculated as iAs^{III} + iAs^{V}, and the total As was calculated as iAs + MMA + DMA + AsB.

Urinary Metabolomics and Biomarker Identification. Details of the sample preparation, high performance liquid chromatography-quadrupole-time-of-flight-mass spectrometer (HPLC-qTOF-MS) acquisition, data processing, biomarker screening, biomarker identification, and quality control procedures are described in the Supporting Information. Briefly, the diluted urine samples were subjected to the metabolic profile acquisition using HPLC-qTOF-MS. The raw chromatograms were processed with Profile Analysis 2.0 (Bruker, USA) to obtain a table containing retention time, exact mass pairs, and normalized peak areas. The data set in this table were Pareto-scaled and then introduced to SIMCA-P v11.5 software (Umetrics, Sweden) for a multivariate statistical analysis. A tight quantum clustering (QC) was observed in the scores plot following the principal component analysis (Figure S1, Supporting Information), which indicates that the quality of the data was suitable for further analysis. To investigate arsenic-related metabolome alterations, the data set was grouped into quintiles from the lowest to the highest arsenic concentration. The first quintile was set as the lowest-level exposure group, while the fifth quintile was set as the highest-level exposure group. A partial least-squares discriminant analysis (PLS-DA), a supervised pattern recognition approach, was used for group differentiation between the first and the fifth quintile samples. The 999-time permutation test was performed to validate the developed PLS-DA models (Figure S2, Supporting Information). The biomarkers were screened from the most robust model, an iAs-based PLS-DA model, where the extracted variables that contributed the most in the grouping process were considered as potential biomarkers. The criteria used for identification of the biomarkers were: (1) variable importance plot (VIP) scores of the variables >3 (Figure S3, Supporting Information); (2) a jack-knifing confidence interval of the variables >0; (3) p-values of the variables between the first quintile and the fifth quintile samples <0.01; and (4) the variables significantly ($p < 0.05$) correlated with urinary iAs after adjustment by age, body mass index (BMI), and smoking and drinking status.

Statistical Analysis. The statistical analysis was performed using SPSS 18 (SPSS Inc.). The Mann−Whitney test was used to evaluate the significance of the biomarkers between the groups. The associations between the participant demographic factors, arsenic species concentrations, and biomarker levels were preliminarily

evaluated by a Spearman correlation analysis. Then, the partial correlation analysis was performed to investigate the association between the biomarkers and arsenic exposure after age, BMI, and smoking and drinking status were adjusted as covariates. A receiver operator characteristic (ROC) analysis is usually used to assess the performance of biomarkers in diagnostic models.[24] A biomarker with an area under the ROC curve (AUC) of 1.0 suggests a perfect classification, while 0.5 is equivalent to labels assigned randomly. In the present work, an ROC was conducted to evaluate the biomarkers' ability to classify the individuals into a high or low arsenic exposure category in the binary classifier system. The statistical significance was set at $p < 0.05$.

RESULTS

Demographic Factors. Because the concentration of total arsenic in the tap water of the recruitment area was much lower than that recommended by the World Health Organization (WHO) (10 μg/L), the participants were considered to be derived from the general Chinese adult male population with background sources of arsenic exposure. A total of 127 adult men provided their urine samples and questionnaires. The demographic information is described in Table 1. The subjects' ages ranged from 19−43 years, with an average age of 29. Current smokers and consumers of alcohol accounted for 37% and 51% of the participants, respectively.

Table 1. Demographic Data of the Participants

item	participants	missing data (*n*)
age (years)	28.47 (19.05−43.66)[a]	0
height (cm)	174 (160−187)[a]	0
weight (kg)	75 (51−110)[a]	0
BMI (kg/m^2)	24.77 (17.65−32.85)[a]	0
Education Level		2
<college	50 (39.4%)[b]	
≥college	75 (59.1%)[b]	
Annual Family Income		11
<RMB20,000	31 (24.4%)[b]	
≥RMB20,000	85 (66.9%)[b]	
Cigarette Smoking		0
never	47 (37.0%)[b]	
past	5 (3.9%)[b]	
current	75 (59.1)[b]	
Alcohol Drinking		0
never	65 (47.2)[b]	
past	1 (0.8%)[b]	
current	61 (52%)[b]	
Occupation		2
white collar	60 (47.2%)[b]	
blue collar	57 (44.9%)[b]	
others	8 (6.3%)[b]	

[a]Expressed as median (min−max). [b]Expressed as *n* (%).

Urinary Arsenic Concentrations. The urinary arsenic concentration is commonly recognized as the best indicator of arsenic exposure because it integrates all routes, pathways, and sources of exposure into one biologically relevant measurement. Although the collection of 24-h urine is preferred, it is usually difficult and expensive in a cohort study and involves an undue participant burden. Urinary arsenic does not appear to vary widely over time, so spot collection, especially the first morning void is usually recommended.[23] Urinary arsenic levels of the participants are listed in Table 2. The urinary arsenic species have concentrations with wide ranges, which were not normally distributed. The median creatinine-adjusted concentrations of total arsenic and iAs were 40.03 and 6.38 μg/g of creatinine. iAs was significantly correlated with the total arsenic ($r = 0.844$). iAs was also positively correlated with age, but negatively correlated with BMI (Table S1, Supporting Information). No significant differences in arsenic levels were observed for education level, annual family income, occupation, smoking, or alcohol consumption status (Table S2, Supporting Information).

Urinary Metabolome. To discover any arsenic-related metabolic alterations, PLS-DA models were established using total As and iAs, smoking, and drinking status as classifiers (Table S3, Supporting Information). Since smoking is suspected to be a source of arsenic exposure,[25] smoker-excluded models were also developed for the classifiers total As and iAs. Clear separations were obtained in the scores plots (Figure 1a−d), which suggests that the urinary metabolic disturbance was significantly different between the lowest-level arsenic exposure group (i.e., the first quintile samples) and the highest-level arsenic exposure group (i.e., the fifth quintile samples). To further validate the established models, the 999-time permutation tests were performed for the corresponding models. The goodness-of-fit (R^2) and predictive capability (Q^2) of the original model are indicated. Finally, strict criteria were used to validate the models: (1) R^2Y and Q^2 (cum) > 0.5 (Table S3, Supporting Information); (2) the R^2 and Q^2 values in the permutation tests were kept lower than in the original ones (Figure S2, Supporting Information); and (3) the *Y*-axis intercept for Q^2 is below zero (Figure S2, Supporting Information). For all of the arsenic species classifiers, the PLS-DA model on iAs data is the most robust, where the *Y*-axis intercept for Q^2 is (0, −0.134). This may result from the fact that the organic species (especially AsB) are less toxic than the inorganic species,[23] and their presence decreased the sensitivity of the other models to respond to arsenic-related dose-dependent metabolic effects. Therefore, arsenic exposure related biomarkers were screened based on iAs data derived from the PLS-DA model.

Sixty-one extracted variables (i.e., metabolites) that contributed the most to the intergroup differentiation were considered as

Table 2. Urine Arsenic Concentrations (Expressed as mg/g Creatinine) of the Participants

	mean (SD)	min	20% tile	40% tile	median	60% tile	80% tile	max
iAs^{III}	4.55(3.18)	0.12	2.27	3.52	4.03	4.54	5.80	19.11
iAs^{V}	39.94(98.37)	0.08	0.20	0.41	1.64	4.08	36.98	507.44
DMA	23.35(16.28)	1.69	11.79	16.68	19.05	20.66	36.64	86.2
MMA	3.65(2.62)	0.30	1.86	2.54	2.88	3.52	4.97	16.01
AsB	11.94 (18.33)	1.34	4.68	6.4	7.68	9.48	14.59	188.14
iAs[a]	44.50 (99.70)	0.89	3.50	4.93	6.38	9.58	41.87	513.16
total As[b]	83.44 (111.60)	4.79	25.01	33.17	40.03	46.18	99.42	590.99

[a]iAs = iAs^{III} + iAs^{V}. [b]Total As = iAs^{III} + iAs^{V} + MMA + DMA + AsB.

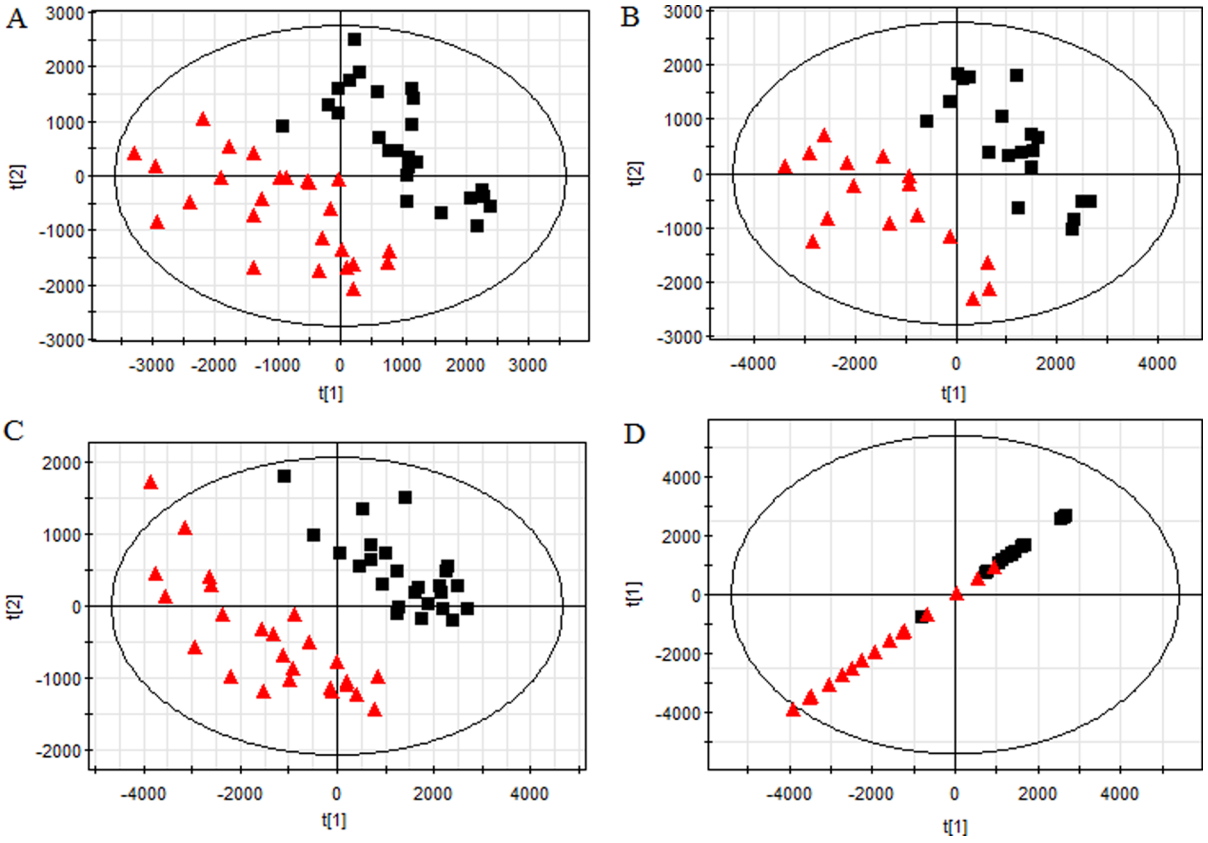

Figure 1. Scoring plots of the developed PLS-DA models. Classifiers for each PLS-DA model are as follows: A = total As; B = total As (current smokers excluded); C = iAs; D = iAs (current smokers excluded); the red triangles indicate the lowest-level arsenic exposure group (the 1st quintile samples); and the black squares indicate the highest-level arsenic exposure group (the 5th quintile samples).

Table 3. Identified As-Related Urinary Metabolic Biomarkers

			monitored adduct		parental molecule	mass uncertainty		
compound	VIP score[b]	p value[c]	structure	MW (Da)[a]	MW (Da)[a]	delta (Da)	fold change[d]	pathway
AFMK	11.94	7.20×10^{-03}	M+H	265.1183	264.1110	0.0098	1.46	tryptophan metabolism
testosterone	6.24	1.90×10^{-06}	M+Na	311.1981	288.2089	0.0051	1.91	androgen metabolism
guanine	5.45	8.47×10^{-06}	M+H	152.0567	151.0494	0.0085	2.59	purine metabolism
hippurate	3.88	6.16×10^{-05}	M+Na	202.0475	179.0582	0.0067	2.26	glycine metabolism
serine	3.14	6.61×10^{-04}	M+H	106.0399	105.0426	0.0067	1.80	glycine and serine metabolism

[a]MW = molecular weight; M = the parental molecule of the monitored adduct, usually one hydrogen or sodium atom is conjugated with the parental molecule to form the positive ion mass. [b]VIP score was obtained from the PLS-DA model. [c]*p*-values were calculated from nonparametric Mann–Whitney U test between the lowest-level arsenic exposure group (i.e., the 1st quintile samples) and the highest-level arsenic exposure group (i.e., the 5th quintile samples). [d]Mean of the 5th quintile samples/mean of the 1st quintile samples.

potential biomarkers of early arsenic-induced effects. After adjustment by age, BMI, and smoking and drinking status, a further partial correlation analysis showed that 5 metabolites were significantly related to arsenic levels. These biochemical metabolites were testosterone, guanine, hippurate, acetyl-*N*-formyl-5-methoxy kynurenamine (AFMK), and serine (Table 3). The biomarker changes ranged from 1.46- to 2.59-fold. The biomarkers also showed significant changes when total As classifiers were used in the PLS-DA models.

The alterations of the biomarker levels across all of the data sets were also investigated. As shown in Figure 2, when compared to the lowest-level exposure group (i.e., the first quintile samples), AFMK levels were significantly different in the highest-level exposure group (i.e., the fifth quintile samples) for iAs. Serine was only significantly increased in the fifth quintile samples for the iAs and total As doses. When compared to the first quintile samples, in the fourth and fifth quintile groups, guanine and hippurate showed significant increases for iAs doses; an increase in the fifth quintile was also observed for the total As dose. A dose-dependent increase of testosterone was observed for the iAs and total As doses. Because smoking may be associated with testosterone levels,[26] we further excluded the smoking participants from the models. The result showed that testosterone still increased when arsenic levels increased (Figure S4, Supporting Information), which indicates that the increase of testosterone was independent of smoking status. Hippurate and serine levels were significantly different between the drinkers and nondrinkers, but no significant differences were noted between these biomarker levels in the smokers and nonsmokers (Table S1, Supporting Information).

The association between the identified biomarkers and arsenic was also evaluated by the Spearman correlation (Table 4). These biomarkers were significantly positively correlated with iAs

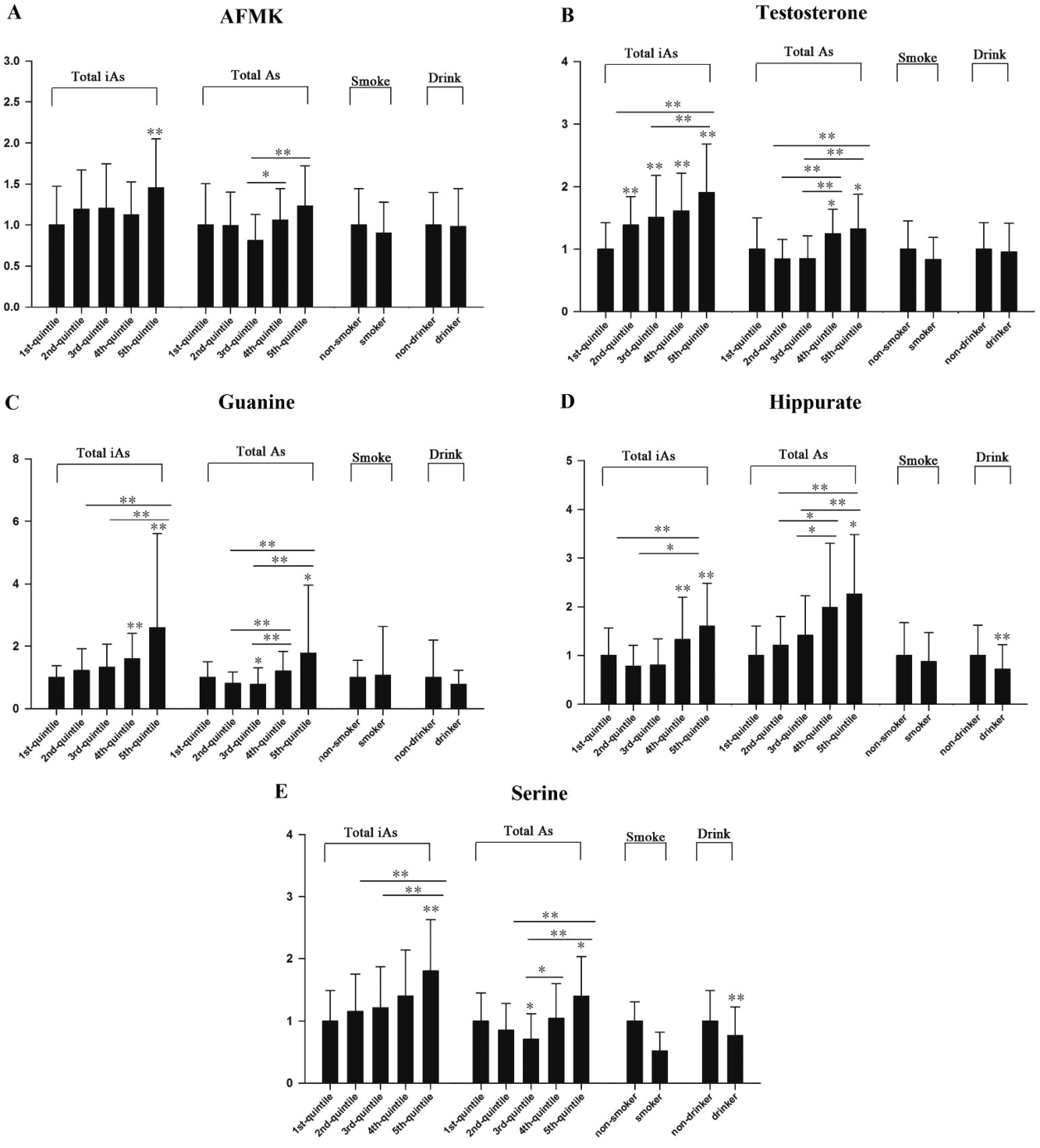

Figure 2. Changes of the identified biomarkers for all of the data set. A, AFMK; B, testosterone; C, guanine; D, hippurate; E, serine. *, $p < 0.05$; **, $p < 0.01$.

($p < 0.01$); they were also significantly correlated with the total As. Testosterone and guanine showed positive correlations with age, while AFMK and testosterone showed negative correlations with BMI. After the adjustment for age, BMI, and smoking and drinking status, the significant correlations remained between the five biomarkers and the total As and iAs (Table 4).

ROC Analysis. An ROC curve was extensively used in the evaluation of the biomarker diagnostic performance.[27] By using a binary classifier system, the sensitivity and specificity of the biomarkers can be evaluated by the ROC curve; the value of the AUC is commonly used to summarize the overall performance. The AUC values of the five biomarkers and one combinational pattern derived from the models of first quintile versus second quintile, first quintile versus third quintile, first quintile versus fourth quintile, and first quintile versus fifth quintile are plotted in Figure 3, and their statistical significances are listed in Table S4 of the Supporting Information. For the first quintile versus fifth quintile iAs model, the AUC values were 0.89, 0.87, 0.83, 0.78, and 0.72 for testosterone, guanine, hippurate, serine, and AFMK, respectively. The three biomarkers with AUC values > 0.80 were

Table 4. Spearman Correlation and the Adjusted Partial Correlation Analysis between the Biomarkers and Urinary Arsenic[a]

		AFMK	testosterone	guanine	hippurate	serine
	age	0.080	0.21*	0.23*	0.16	0.10
	bmi	−0.27**	−0.25**	−0.16	−0.16	−0.20*
total As	not adjusted	0.21*	0.35**	0.28**	0.31**	0.25**
	adjusted	0.29**	0.37**	0.26**	0.21*	0.22*
iAs	not adjusted	0.26**	0.47**	0.42**	0.40**	0.33**
	adjusted	0.29**	0.39**	0.28**	0.29**	0.29**

[a]The adjusted covariates are age, BMI, and smoking and drinking status in the partial correlation analysis; *, $p < 0.05$; **, $p < 0.01$.

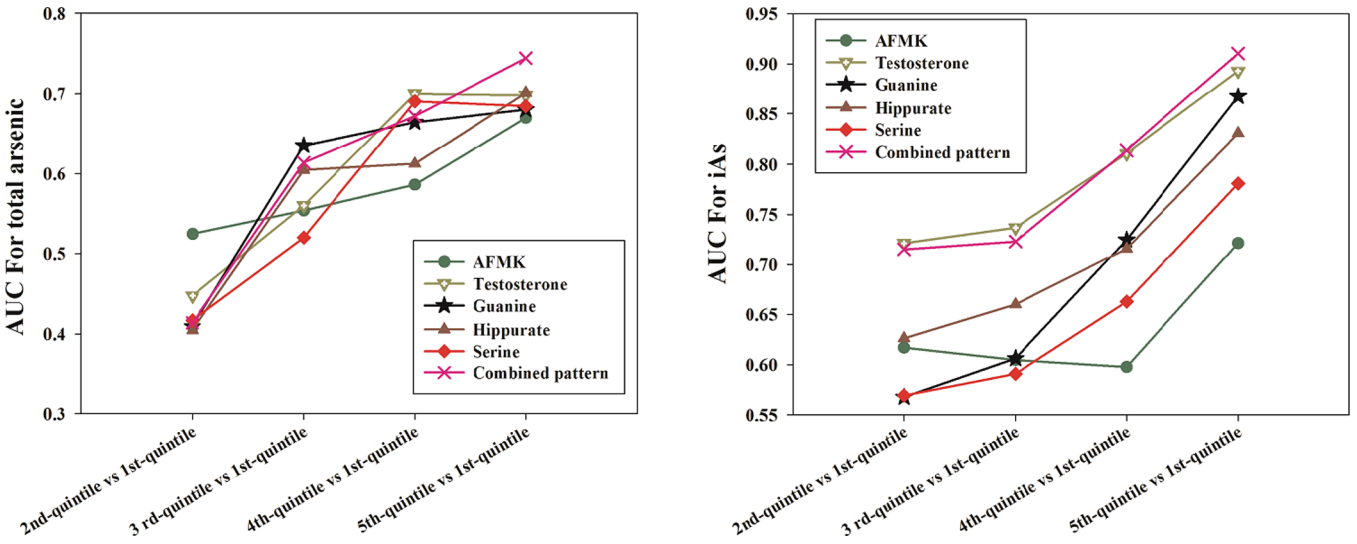

Figure 3. Dose-dependent AUC trends of the biomarkers and their combinational pattern. The AUC values derived from the iAs exposure groups of the 1st quintile versus 2nd quintile, 1st quintile versus 3rd quintile, 1st quintile versus 4th quintile, and 1st quintile versus 5th quintile; testosterone, guanine, and hippurate formed the combinational pattern; the statistical significances of the AUC values were listed in Table S4 of the Supporting Information.

used to form the combinational biomarker (i.e., composite index).[24] The AUC value increased up to 0.91 with 88% sensitivity and 80% specificity, respectively (Figure 3 and Table S4, Supporting Information), which may imply a synergetic response of testosterone, guanine, and hippurate to iAs stress. In other words, the three metabolites alone and together have well characterized the iAs exposure in the investigated cohort. Because the performance of the biomarkers in the classification of the total arsenic exposure was weaker than in the classification of iAs alone, some low-toxicity organic species may have diluted the biological responses to arsenic. The increased trends of the AUC values with arsenic concentrations implied a dose-dependent response of the biomarkers to arsenic exposure.

DISCUSSION

Although urinary arsenic levels are often monitored to evaluate individual or population exposures, an understanding of the biological response related to these exposures is required to understand the mode of action and characterize the exposure risk; thus, our complementary targeted and untargeted analyses to evaluate both arsenic exposure and metabolism responses related to the exposures are presented. We hypothesized that arsenic exposure could induce metabolic disruption based on both animal and human studies[3,19,28] and as such, have identified five urinary metabolites that are strongly linked to arsenic exposures. In addition, we observed some specific dose−response trends between arsenic and the identified biomarker levels, which further suggests that the changes were in some way related to arsenic exposure. To our knowledge, our study is the first metabolomics-based epidemiologic investigation of the metabolic effects from ambient arsenic exposure.

General Arsenic Exposure Characters. Humans are simultaneously exposed to iAs and organic arsenic species. The median concentrations of iAs and total As for the participants of this study were 6.38 and 40.03 μg/g of creatinine, respectively, which is lower than the levels published for populations exposed to arsenic via contaminated drinking water.[29] For our participants, consumption of rice and other As-containing foods was assumed to be the largest contributor to urinary iAs. In the Chinese urban residents, the estimated daily dietary intake of iAs was 42.81 μg/day.[9,30] Seafood may be one of the main sources of organic arsenic exposure, which also contributes to the generation of urine DMA and other metabolites. The significant correlation of AsB with iAs and DMA may suggest a coexposure to these arsenic species in the diet.[5] The weakly positive correlation between age and iAs may indicate a decrease in arsenic metabolism with age. Significant but weak inverse correlations were found between BMI and total As as well as iAs. The lowered BMI may be a toxic manifestation of the chronic exposure to arsenic.[31]

Urinary Arsenic Levels-Related Urinary Metabolomics Markers. People who are exposed to the highest levels of pollutants are expected to be at the highest risk of developing related adverse effects. Although the Agency for Toxic Substances and Disease Registry (ATSDR) has set guidelines of urinary total arsenic <100 μg/L (66.7−142 μg/g of creatinine for males 20−69 years old) to indicate "unexposed" individuals;[32] for iAs exposure, the suggested biological tolerance values are 50 and 35 μg/L of urinary arsenic for the Deutshe Forschungsgemeinschaft

(DFG) and American Conference of Governmental Industrial Hygienists (ACGIH),[33] respectively. In this study, 20% of the samples with the lowest iAs levels (0.89−3.50 μg/g of creatinine for iAs and 4.79−25.01 μg/g of creatinine for total As) and 20% of the samples with the highest iAs levels (41.87−513.16 μg/g of creatinine for iAs and 99.42−509.99 μg/g of creatinine for total As) were used to establish the multivariant statistical models. The samples in the two groups were clearly differentiated in the PLS-DA model, which suggests that the urinary metabolic profile could detect arsenic-related metabolic perturbations. Aside from the differentiation capacity of the model, the roles of the identified biomarkers in the metabolic process may suggest arsenic exposure related toxicities. At the trial-and-error stage, we found that many candidate biomarkers could correlate with urinary arsenic species; however, most of them also were correlated with age, BMI, and smoking and drinking status. To simplify and clarify the potential causal linkage between arsenic and its metabolism interruption and to specify the arsenic-related character of the potential biomarkers, the above-mentioned factors were adjusted in the further partial correlation analyses. Finally, five potential biomarkers were obtained from the 61 candidates: testosterone, guanine, hippurate, AFMK, and serine. These biomarkers provide clues that link arsenic exposure to androgen, amino acid, and purine metabolism.

Biological Significance of the Arsenic-Related Biomarkers. Endocrine disruption is one of the potential modes of action of arsenic exposure. At relatively high doses, arsenic affects the amount of hormones either by disrupting hormone synthesis and metabolism or by interfering with the binding of the hormones with their receptors. A recent in vivo study found that arsenic treatment (3−4 mg/kg of weight/day) resulted in lowered testosterone and increased luteinizing hormone levels in the serum, which in turn led to the inhibition of the testosterone synthesis pathway and resulted in infertility in male mice.[34] Similar results (5 mg/kg of weight/day) were observed in adult male rats.[35] Contrary to the trend of testosterone in rodent serum, we observed a slight increase in urinary testosterone in humans. Since smoking history is also associated with urinary testosterone, we excluded the smokers from the statistical model; the analysis showed that testosterone still increased with arsenic levels, which suggests that the result is robust. Therefore, arsenic from the ambient environment may be sufficient to induce testosterone alteration. The observed testosterone alteration may be different from the other in vivo models and human studies. Recently, researchers from Taiwan found that blood testosterone levels were inversely correlated with exposure to arsenic via well water in 129 elderly men (average age = 67 years, arsenic >50 μg/L in drinking water),[36] which may imply that there are important dosing and timing differences in the effects of arsenic as well as important gene−environment and coexposure interactions.[37,38] The three major differences between this study and ours were the ages of participants, magnitude of exposure, and biological matrix. The average age of the participants in our study was 29 years, much younger than the males in Hsieh's report; this could be an important difference since testosterone levels decrease significantly with age. The participants in our study were exposed to much lower doses of arsenic than those in Hsieh's study. Low-dose effects (homeostasis or adaptation) are remarkably common in natural hormones and endocrine disruptor exposures, but it cannot necessarily be predicted by effects observed at higher doses.[39] Previous studies have revealed that even exposure to very low concentrations of arsenic have altered steroid hormone receptor mediated genes and have disrupted the function of hormone nuclear receptors.[37,40] However, a larger scale epidemiologic study is needed to confirm this preliminary observation.

Oxidative stress is another major mode of action of arsenic exposure. The amino acid metabolism related biomarkers AFMK, serine, and hippurate are oxidation indicators. Arsenic could regulate some kinases of the mitogen-activated protein kinase (MAPK) family that are known to phosphorylate the hydroxyl groups of serine.[41] Reactive oxygen species (ROS)-mediated activations of MAPKs in response to arsenic treatment activate Bax and the phosphorylation of Bcl-2, which results in mitochondrial apoptotic cell death.[42] Increased hippurate levels were observed in the urine of mice exposed to arsenic.[16] In this study, for the first time we found that urinary hippurate was linked with human exposure to arsenic. Hippurate synthesis involves the binding of benzoate or dietary essential amino acid metabolites with glycine;[43] the latter tightly relates to serine metabolism and the γ-glutamyl cycle via glutathione. Additionally, arsenic metabolites might influence the binding of hippurate because of their high affinities to the -SH group,[44] which thereby results in the altered production of hippurate. The alteration of urinary guanine may also indirectly indicate arsenics-driven oxidative stress in humans. Arsenic exposure induced DNA damage via oxygen stress was widely reported.[2,45] The sensitive DNA oxidation biomarker 8-OH-G originates from the reaction of the hydroxyl radical with guanine. Guanine can also be catalyzed to form xanthine and then transformed to uric acid. Arsenic exposure was negatively correlated with uric acid.[46,47] AFMK is an antioxidant metabolite of melatonin, which reflects the depletion of melatonin by highly ROS.[48] As an important and powerful antioxidant, melatonin could reverse the As-driven changes in reduced glutathione level and lipid peroxidation.[49] Because melatonin of the pineal hormone marks the biologically circadian rhythm and may also regulate the testicular hormone testosterone,[50−53] the observed metabolic changes are biologically dependent (Figure S5, Supporting Information). Cellular enzymatic changes of these arsenic-induced biochemical changes must be tested further by using in vivo and in vitro animal models.

Assessing Metabolically Grounded Arsenic Exposure Risk? Usually an exposure assessment and a dose−response assessment are addressed before a risk assessment.[54] Although the bottom-up strategy on scenario models[55,56] for an exposure assessment brings more information on exposure factors and routes, the top-down strategy is believed to be the more accurate assessment.[57,58] The hazard characterizations are usually grounded on the different tiers of in vitro and in vivo toxicological studies,[54,59] which may introduce interspecies uncertainties into the human health risk. Few cohort studies[14,22,60] have tried to elucidate the effect of biomarkers from human exposure (with doses established through biomonitoring), in which the high-throughput omic techniques are employed because of their hypothesis-free advantage in biomarker screening.[61,62] An AUC value of 0.85 is considered to be an acceptable value for most clinical applications, so the present proof-of-concept observations suggest that testosterone, guanine, and hippurate, and especially their combinational pattern, are arsenic-related metabolic interruption signatures. The testosterone and biomarker combinational pattern that suggests moderate ROC performances can even be observed at comparably low exposure levels (i.e., 9.58−41.87 μg/g of creatinine), which may be a sensitive metabolic response to arsenic stimuli. In addition, the dose-dependent relationship of iAs and the biochemical marker suggests that this methodology and our results may offer a more

human-relevant approach to address hazard in a defined cohort. We anticipate further use of these biomarkers in the refinement of this human risk assessment.

Our study is not without limitations. Our cohort size is relatively small. Additional coexposure information (with the exception of phthalates) was not collected nor were the environmental pathway measurements. Long-term storage may have affected the urine metabolome, although the influence was not apparent in the developed PLS-DA models. Lastly, we recognize the role of creatinine in 2-carbon metabolism as is the case with arsenic, so the creatinine correction may not be the most appropriate way to correct for urine dilution. Despite these limitations, we feel that our data provide direct evidence that a metabolically based approach to risk assessment may be viable in the near future.

In summary, the proof-of-concept HPLC/MS-based metabolomics approach is powerful to screen general arsenic exposure-related potential biomarkers in humans. The metabolites related to endocrine disruption and oxidative stress effects were identified as potential biomarkers of arsenic exposure. This proof-of-concept work also suggests a general approach to evaluate the environmental chemicals' perturbation of normal metabolic regulation, which is the fundamental process in a biological response to environmental stimuli. We are encouraged that the potential metabolism disrupting effects could be useful in population-based risk assessments by linking biological monitoring data directly to metabolic effects. More importantly, if the high-throughput metabolomic and exposomic technologies[13] are integrated into a systematic approach, it may be possible to delineate the molecular imprint of the total chemical environmental exposure in an individual-based population study.

■ ASSOCIATED CONTENT

Supporting Information

The observed significant Spearman correlations among creatinine-adjusted arsenic concentrations and selected demographic factors (Table S1); a comparison of the urinary arsenic and biomarker levels between the factors of education, annual family income, occupation, smoking, and drinking (Table S2); established PLS-DA models by using total As, iAs, smoking, and drinking status as the classifiers (Table S3); areas under the receiver−operator characteristic curve and the statistic significances of iAs; and total arsenic-based models (Table S4). A principle component analysis (PCA) scores plot of individual urine samples and QC samples obtained by HPLC/QTOF-MS in positive ionization mode (Figure S1); validation of the developed PLS-DA models using the 999-time permutation tests (Figure S2); variable importance plot for the iAs-based PLS-DA model (Figure S3); changes of testosterone for the data set with the exclusion of smokers (Figure S4); and the suggested biological dependences of the observed metabolic biomarkers marked in red color (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (86)-592-6190997; fax: (86)-592-6190997; e-mail: hqshen@iue.ac.cn.

Notes

The authors declare no competing financial interest.

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