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Identifying Early Urinary Metabolic Changes with Long-Term Environmental Exposure to Cadmium by Mass-Spectrometry-Based Metabolomics

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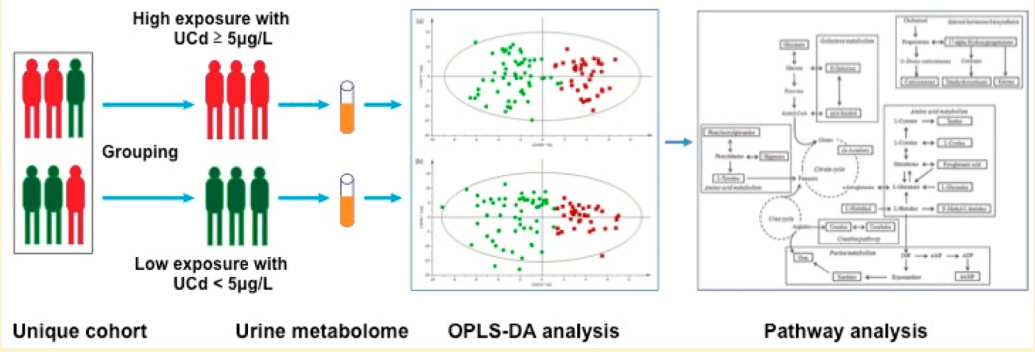
Supporting Information



ABSTRACT: Cadmium (Cd) is a common environmental pollutant, and urinary Cd (UCd) is generally used as a marker of exposure; however, our understanding on the related urinary metabolic changes caused by Cd exposure is still not clear. In this study, we applied a mass-spectrometry-based metabolomic approach to assess the urinary metabolic changes in human with long-term environmental Cd exposure, aimed to identify early biomarkers to assess Cd nephrotoxicity. Urine samples from 94 female never smokers aged 44−70 with UCd in the range of 0.20−68.67 μg/L were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-ToF-MS) and gas chromatography−mass spectrometry (GC−MS). It was found that metabolites related to amino acid metabolism (L-glutamine, L-cystine, L-tyrosine, *N*-methyl-L-histidine, L-histidinol, taurine, phenylacetylglutamine, hippurate, and pyroglutamic acid), galactose metabolism (D-galactose and *myo*-inositol), purine metabolism (xanthine, urea, and deoxyadenosine monophosphate), creatine pathway (creatine and creatinine), and steroid hormone biosynthesis (17-α-hydroxyprogesterone, tetrahydrocortisone, estrone, and corticosterone) were significantly higher among those with a UCd level higher than 5 μg/L. Moreover, we noticed that the level of *N*-methyl-L-histidine had already started to elevate among individuals with a UCd concentration of ≥2 μg/L. The overall findings illustrate that metabolomics offer a useful approach for revealing metabolic changes as a result of Cd exposure.

INTRODUCTION

Cadmium (Cd) pollution has become a major environmental hazard to human health, owing to human activities, such as mining, smelting, and refining.[1] Cd in the environment, especially soil and water, can be taken up by crops and aquatic organisms and finally accumulates in the human body through

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diet.[2,3] It has been reported that 70−80% of Cd intake was derived from cereals and vegetables for non-smokers.[4] Numerous studies indicate that kidney is the target organ of Cd exposure.[5,6] In addition, a high intake of Cd can lead to chronic obstructive lung diseases and osteoporosis.[7,8] With high exposure, it can be a cancer risk factor and has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC).[9]

Urinary Cd (UCd) is widely used as a marker to evaluate Cd exposure, because the level of Cd in urine is in proportion to the amount accumulated in the kidney.[10] In 1992, the World Health Organization (WHO) first proposed a health-based exposure limit of UCd at 5 μg/g of creatinine (or 5 μg/L).[11] Currently, the standard of the UCd level deemed safe to humans in most countries is also similar to the WHO reference value.[12] However, there is considerable controversy over the health-based biological limit (maximum contaminant level) of Cd in urine. Recent studies suggest that there is an underestimation of the risk of Cd exposure, with an exposure limit of UCd of 5 μg/L.[13,14]

Metabolomics is an emerging technique in the field of "omics" research. It focuses on the systematic study of low-molecular-weight metabolites within a cell, tissue, or biofluid of an organism in response to a stressor, such as drug toxicity, disease, environmental factor, and genetic variation.[15] Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the two most commonly used techniques in metabolomic studies.[16] Environmental metabolomics is viewed as the application of metabolomic techniques to analyze the metabolic responses of organisms to environmental stressors, ranging from abiotic stressors, such as temperature stress and pollution, to biotic−biotic interactions, such as infection and predation.[17,18] In 2012, Ellis et al. investigated the changes of urine metabolism in a population exposed to environmental Cd pollution using NMR, and six urinary metabolites associated with mitochondrial metabolism and one carbon metabolism were identified.[19] Most recently, Suvagandha et al. conducted a urinary metabolic profiling in Cd-exposed residents of Thailand using gas chromatography−mass spectrometry (GC−MS), and they reported that urinary citrate may be a useful biomarker for early detection and prevention of nephrolithiasis.[20] To the best of our knowledge, there is currently no systematic metabolomic study based on liquid chromatography−mass spectrometry (LC−MS) on human exposure to Cd. GC−MS is ideal for analyzing volatile and derivatized compounds, while LC−MS is better poised to detect a wider range of different metabolites. LC−MS is especially suitable for detecting low-concentration compounds that may not be detectable with GC−MS.[21,22] Thus, the integration of these two complementary techniques could offer a more comprehensive coverage of the metabolites in the biosystem.

In the present study, we applied an integrated MS-based approach to investigate the urinary metabolic profiles in humans with long-term Cd exposure. We also aimed to identify metabolites that could serve as early biomarkers to assist in assessment of Cd toxicity. In brief, urine samples from 94 female never smoke residents in two rural countries in China were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-ToF-MS) and GC−MS. The obtained spectral data were processed by multivariate analysis and Student's *t* test to identify metabolites that show significant changes in a population with different levels of environmental Cd exposure.

EXPERIMENTAL SECTION

Participants and Urine Samples. All of the 94 subjects for this study were recruited from two counties about 200 km apart in south China, but they belong to the same population with potential Cd exposure from foods grown on soil known to have different levels of Cd. The lifestyles and habits of the residents of these two counties are similar. The recruitment process is as follows. Adult female never smokers (age of ≥40 years and have been living in the same village for more than 20 years) without known kidney or other clinical diseases were included in the study. Residents under long-term medication were excluded. All participants completed a questionnaire that provided demographical information, including age, lifestyle factors, and medical family history. Data were anonymized for the present study. The morning urine sample was collected from all participating individuals and divided into two tubes. One stored in 4 °C and sent to the Guangdong Provincial Center for Disease Control and Prevention (CDC) lab for biomarkers assay and heavy metal (e.g., Cd, lead, and copper) detection within 4 h of collection. The other tube was frozen at −80 °C and used for subsequent metabonomic studies. The UCd concentration was determined by inductively coupled plasma−mass spectrometry (Agilent 7700x). Quality control was performed for 1 in 10 samples. Urinary *N*-acetyl-*β*-D-glucosaminidase (U-NAG) was determined by colorimetric assay using a Hitachi automatic biochemical analyzer, 7600-010. Urinary creatinine was assayed using the same Hitachi analyzer with modified Jaffe's kinetic method. On the basis of the WHO criteria, the subjects were divided into two groups: UCd concentration of ≥5 μg/L and UCd concentration of <5 μg/L. The mean values with standard deviation (SD) of age, local living, UCd, U-NAG, and body mass index (BMI) for the subjects in the two groups are shown in Table 1. No significant differences in age, local living, and BMI were found between the two groups, but the levels of UCd and U-NAG were significantly different. All participants provided informed consent according to institutional guidelines, and both university and CDC ethics committees approved this study.

Table 1. Demographic Characteristics of 94 Individuals in This Study[a]

characteristics	UCd of <5 μg/L	UCd of ≥5 μg/L	*p* value
number of subjects	56	38	
female	56	38	
smoker	0	0	
age (years)			0.072
range	45.2−70.2	44.5−69.4	
mean ± SD	57.11 ± 7.11	53.21 ± 7.23	
local living (years)			0.292
range	21−70	22−69	
mean ± SD	46.14 ± 13.27	42.92 ± 15.18	
UCd (μg/L)			<0.001
range	0.20−4.67	5.07−68.67	
mean ± SD	1.98 ± 1.13	13.07 ± 11.97	
UCd (μg/g of creatinine)	2.92 ± 2.38	12.83 ± 10.66	<0.001
U-NAG (U/g of creatinine)	5.15 ± 2.87	8.36 ± 4.28	<0.001
BMI (kg/m^2)	22.35 ± 3.13	21.47 ± 2.58	0.144

[a]Age, UCd, U-NAG, and BMI were expressed as the mean ± SD. SD denotes standard deviation.

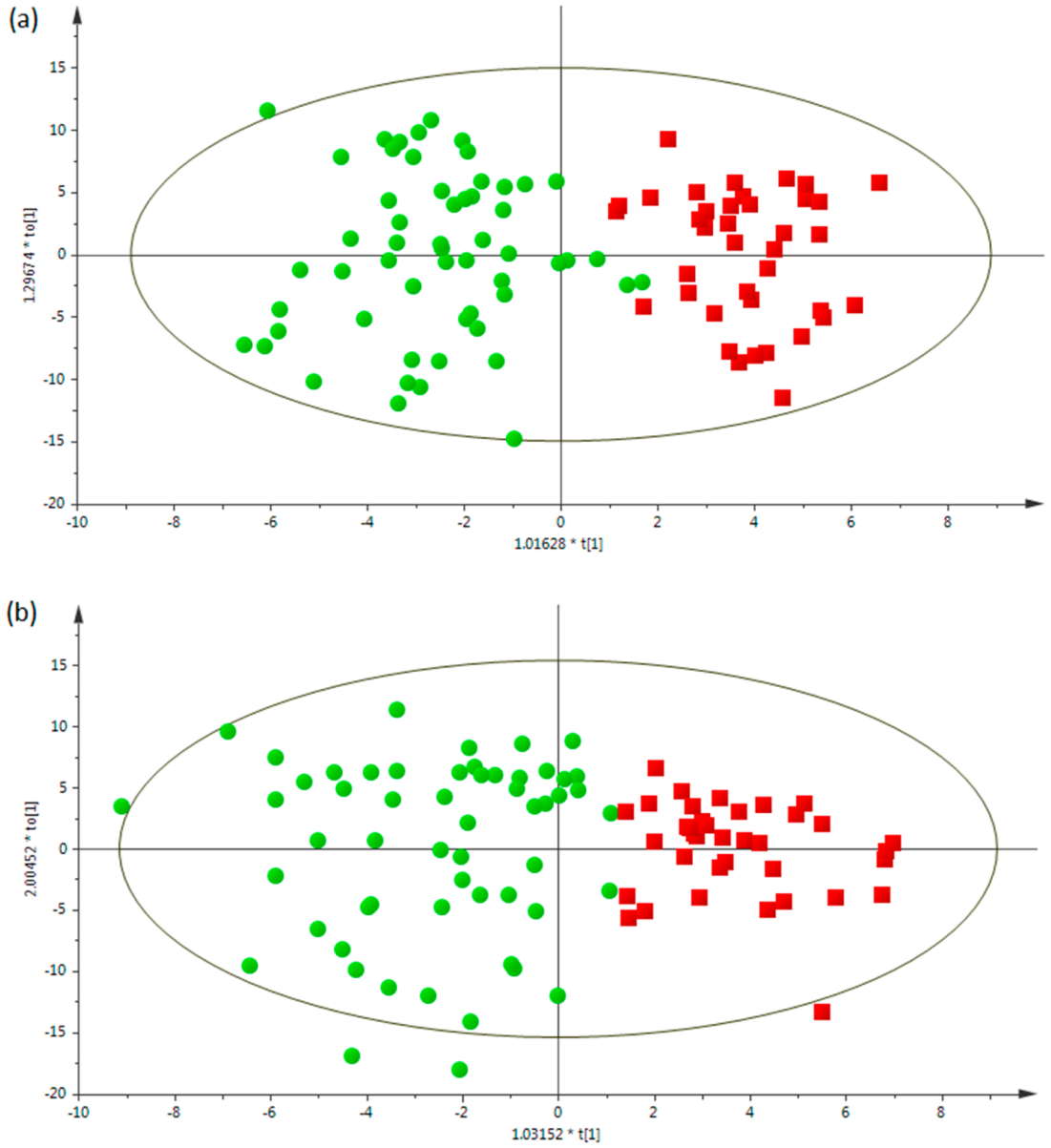

Figure 1. OPLS-DA score plots of the urinary metabolic profiles obtained from 56 low (UCd < 5 μg/L; green circles) and 38 high (UCd ≥ 5 μg/L; red squares) Cd exposure volunteers: (a) LC−MS data (R^2Y = 0.791; Q^2 = 0.307) and (b) GC−MS data (R^2Y = 0.702; Q^2 = 0.218).

Sample Preparations. A 100 μL sample of urine was diluted with 300 μL of cold MeOH, containing 10 μg/mL 9-fluorenylmethoxycarbonyl (FMOC)−glycine as an internal standard. The mixture was shaken vigorously for 30 s. After centrifugation at 14 000 rpm for 10 min at 4 °C, the supernatant fraction was collected and divided into two parts: one (100 μL) for LC−MS analysis and the other one (10 μL) for GC−MS analysis. For GC−MS analysis, the 10 μL supernatant was dried under nitrogen and further derivatized with methoxyamine (50 μL/mL in pyridine) and subsequent trimethylsilylation with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA). The pooled quality control (QC) sample was prepared by mixing equal amounts (10 μL) of urine samples from all subjects before sample processing. The QC sample was analyzed at the beginning, the end, and randomly throughout the whole assay for both LC−MS and GC−MS measurements, to evaluate the stability of analytical performance.

LC−MS. LC−MS analysis was performed on an Agilent 1290 ultrahigh-pressure liquid chromatography system (Waldbronn, Germany) coupled to a 6540 Q-ToF mass detector equipped with an electrospray ionization source. The separation was performed on an Agilent rapid resolution HT Zorbax SB-C18 column (2.1 × 50 mm, 1.8 μm, Aglient) at a column temperature of 50 °C. The mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient program was as follows: 0−9 min, 5−45% B; 9−12 min, 45−100% B; 12−13 min, 100% B; and 13−16 min, 100−5% B. The flow rate was set at 0.4 mL/min. A 10 μL sample was loaded for each individual analysis. The mass spectra were acquired from m/z 100 to 1000 in positive-ion mode. The ion spray voltage was set at 4000 V, and the heated capillary temperature was maintained at 350 °C. The drying gas and nebulizer nitrogen gas flow rates were 12.0 L/min and 50 psi, respectively. The collision energy in the tandem

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Table 2. Biomarkers Identified by GC−MS and LC−MS

	metabolite	VIP	*t* test *p* value	FDR *p* value	class	trend[b]
GC−MS	urea	1.21	0.0104	0.0469	aliphatic acyclic compounds	up
	pyroglutamic acid[a]	1.08	0.0053	0.0435	aliphatic heterocyclic compounds	up
	myo-inositol	1.28	0.0002	0.0069	aliphatic heterocyclic compounds	up
	4-hydroxyhippuric acid	1.45	0.0006	0.0161	amino acids and derivatives	up
	L-tyrosine[a]	1.01	0.0058	0.0447	amino acids and derivatives	up
	hydroxyproline dipeptide	1.26	0.0021	0.0253	amino acids and derivatives	up
	hippurate[a]	1.52	0.0183	0.0454	amino acids and derivatives	up
	pseudouridine	1.15	0.0001	0.0084	carbohydrates and carbohydrate conjugates	up
	gluconate	1.48	0.0013	0.0193	carbohydrates and carbohydrate conjugates	up
	D-galactose	1.06	0.0010	0.0293	carbohydrates and carbohydrate conjugates	up
	cis-aconitate	1.23	0.0001	0.0106	organic acids and derivatives	up
LC−MS	pyroglutamic acid[a]	1.19	0.0004	0.0079	aliphatic heterocyclic compounds	up
	creatinine	1.10	0.0099	0.0418	aliphatic heterocyclic compounds	up
	N-methyl-L-histidine	1.20	0.0020	0.0131	amino acids and derivatives	up
	L-glutamine	1.04	0.0005	0.0113	amino acids and derivatives	up
	phenylacetylglutamine	1.18	0.0007	0.0008	amino acids and derivatives	up
	creatine	1.48	0.0002	0.0105	amino acids and derivatives	up
	L-tyrosine[a]	1.07	0.0007	0.0157	amino acids and derivatives	up
	hippurate[a]	1.55	0.0004	0.0078	amino acids and derivatives	up
	L-thyronine	1.47	0.0036	0.0284	amino acids and derivatives	down
	L-cystine	1.12	0.0022	0.0189	amino acids and derivatives	up
	xanthine	1.59	0.0031	0.0218	aromatic heterocyclic compounds	up
	L-histidinol	1.15	0.0083	0.0451	aromatic heterocyclic compounds	up
	deoxyadenosine monophosphate (dAMP)	1.15	0.0018	0.0291	nucleosides, nucleotides, and analogues	up
	1-methyladenosine	1.13	0.0001	0.0073	nucleosides, nucleotides, and analogues	up
	taurine	1.11	0.0001	0.0066	organic acids and derivatives	up
	17-α-hydroxyprogesterone	1.03	0.0039	0.0418	steroids and steroid derivatives	up
	corticosterone	1.07	0.0007	0.0133	steroids and steroid derivatives	up
	estrone	1.07	0.0033	0.0264	steroids and steroid derivatives	up
	tetrahydrocortisone	1.21	0.0009	0.0153	steroids and steroid derivatives	up

[a]The metabolites were identified both by GC−MS and LC−MS. [b]The trend means that the metabolite is up- and downregulated in individuals under long-term environmental Cd exposure.

mass spectrometry (MS/MS) mode was set to 10, 20, or 40 V, respectively.

GC−MS. GC−MS analysis was performed on an Agilent 7683 series injector (Agilent, Santa Clara, CA) coupled to an Agilent 6890 series gas chromatograph system and a 5973 mass selective detector (MSD) (Agilent, Santa Clara, CA). A fused-silica capillary column HP-5MSI (30 m × 0.25 mm inner diameter and 0.25 μm film thickness) was used. The injector was kept at 250 °C. A 1 μL sample was splitless-injected for each individual analysis. Helium was used as the carrier gas, with a constant flow rate of 1 mL/min through the column. The GC oven temperature was maintained at 70 °C for 1 min, then increased to 250 °C at a rate of 10 °C/min, further increased at 25 °C/min to 300 °C, and held for 6 min. The transfer line temperature was kept at 280 °C. Detection was achieved using MS in electron impact mode (70 eV) and full-scan monitoring (m/z 50−550). The temperature of the ion source was set at 230 °C, and the quadrupole was set at 150 °C.

Data Analysis. The spectral data were exported as mzData (LC−MS) and NetCDF (GC−MS) files and processed by the open-source software MZmine version 2.2 for peak detection, peak alignment, and peak area normalization in each data set. The preprocessed metabolomic data were inputted into SIMCA-P software version 13.0 (Umetrics AB, Umeå, Sweden) for multivariate statistical analysis, including principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Univariate statistical tests were performed using Student's *t* test of SPSS 16.0 (SPSS, Inc., Chicago, IL). *p* values less than 0.05 were considered significant. The false discovery rate (FDR) method by Benjamini and Yekutieli was used to correct for multiple hypothesis testing and reduce false positives.

RESULTS

Reliability of Measurement Methods. The stability of LC-MS and GC-MS analytical systems for the large-scale sample analysis was examined and evaluated using the pool QC sample.[23] First, the spectral data of QC samples were analyzed by PCA together with all urine samples collected. The QC samples were tightly clustered in the PCA score plot (see Figures S1 and S2 of the Supporting Information). In parallel to using PCA analysis for qualifying the overall performance of the methods, variations of retention time, mass accuracy, and peak area of the peaks across all QC samples were calculated: the retention time shift was less than 0.2 min; the mass accuracy deviation was less than 5 mDa; and the relative standard deviations (RSDs) of peak areas were below 20%. We thus concluded that both LC−MS and GC−MS analytical methods provided measurement stability and reliability for the duration of the analysis sequence. We did not observe the significant interference caused by urea during GC−MS analysis, although urease was not applied to process the urine sample in this study.

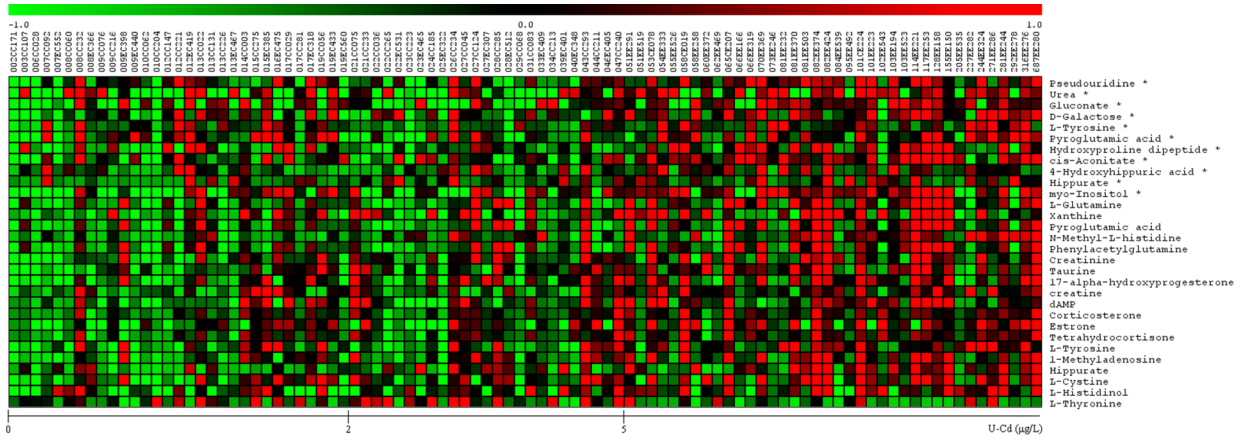

Figure 2. Heat map of 27 identified differential metabolites from both LC−MS and GC−MS analyses. Each row shows the peak area for a specific metabolite after mean centering and unit variance scaling of the data. Each column shows the urinary metabolic profile of volunteers. (∗) These metabolites were from GC−MS analysis. dAMP = deoxyadenosine monophosphate.

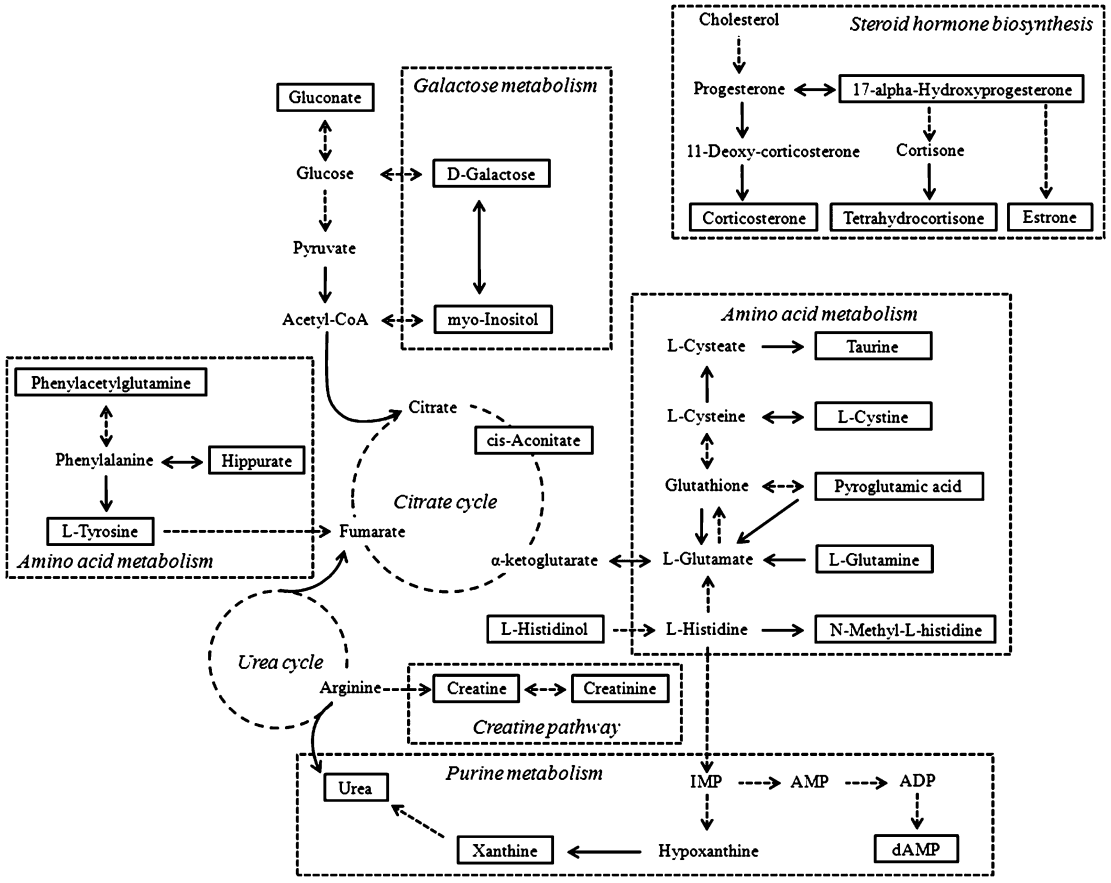

Figure 3. Major upregulated metabolites (in the black squares) and pathways under long-term environmental Cd pollution. Black arrows indicate a direct relationship between two metabolites, and dotted arrows indicate indirect relationships between two metabolites. IMP, inosine monophosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; and dAMP, deoxyadenosine monophosphate.

Global Urinary Metabolic Changes of Different Levels of Cd Exposure. To investigate the global urinary metabolite shifts in humans with chronic environmental Cd exposure, urine samples from 56 individuals with UCd of ≥5 μg/L were first analyzed, blinded with 38 individuals with UCd of <5 μg/L by LC-Q-ToF-MS and GC−MS. Using our optimized spectral data analysis protocol, 412 and 281 peaks were extracted from LC−MS and GC−MS data, respectively. We examined the variances of the metabolic profile between the two groups by supervised multivariate OPLS-DA to achieve the maximum separation.[24]

The OPLS-DA scatter plot (Figure 1a) showed that the UCd of ≥5 μg/L group could be clearly separated from the UCd of <5 μg/L group based on the 412 peaks detected by LC−MS. The quality of the OPLS-DA model was first evaluated by R^2Y (cum) and Q^2 (cum) parameters, which indicated the fitness and prediction capability, respectively. Using five latent variables, the OPLS-DA model showed a R^2Y (cum) value of

0.791 and Q^2 (cum) value of 0.307. Moreover, statistical validation of the corresponding PLS-DA model (permutation tests with 100 iterations; see Figure S3 of the Supporting Information) was carried out to investigate overfitting of data in the OPLS-DA model. The Q^2-intercept value (−0.013) for the corresponding PLS-DA model was lower than 0.05, which strongly indicated that the OPLS-DA model was statistically valid. The variable importance in the projection (VIP) value of each peak was calculated to indicate its contribution of the X variables to the classification. On the basis of the VIP threshold (VIP of >1.0) in OPLS-DA and $p < 0.05$ in Student's t test, 109 peaks responsible for the class separation were considered as potential biomarkers of Cd exposure.

The OPLS-DA was applied to GC−MS data as well, which also showed a trend of separation between low and high Cd exposure subjects (Figure 1b). The R^2Y (cum) and Q^2 (cum) values were 0.702 and 0.218, respectively. The Q^2-intercept value is −0.036, which suggests that our model does not overfit. On the basis of the VIP threshold and p value, 41 peaks responsible for the class separation were considered as potential biomarkers.

Biomarker Identification and Pathway Analysis. The structure identification of differential metabolites was based on our previously described strategy,[21,25] and commercial standards were not applied in this study. Finally, 27 biomarkers were identified: 19 through LC−MS analysis and 11 by GC−MS analysis, 3 of which were common to both methods. The identified metabolites are listed in Table 2 (see Tables S1 and S2 of the Supporting Information). It is interesting to note that all of the metabolites were upregulated with Cd exposure, except L-thyronine. The reason for this is not known; however, this may reflect the fact that Cd could have affected the renal function and led to poor filtration and reabsorption efficiency. The relative average normalized quantities of the identified metabolites in the UCd of >5 μg/L group compared to those in the UCd of <5 μg/L group were plotted in a heat map (Figure 2) using the MeV software.

On the basis of the knowledge of these identified metabolites and the online database of metabolic pathways (KEGG PATHWAY database, http://www.genome.jp/kegg/), a map of the environmental Cd-exposure-related metabolic correlation network was constructed (Figure 3). Affected metabolites were found to belong to amino acid metabolism (L-glutamine, L-cystine, L-tyrosine, *N*-methyl-L-histidine, L-histidinol, taurine, phenylacetylglutamine, hippurate, and pyroglutamic acid), galactose metabolism (D-galactose and *myo*-inositol), purine metabolism [xanthine, urea, and deoxyadenosine monophosphate (dAMP)], creatine pathway (creatine and creatinine), and steroid hormone biosynthesis (17-α-hydroxyprogesterone, tetrahydrocortisone, estrone, and corticosterone).

Early Metabolic Changes with Low Cd Exposure. Our terminal objective was to discover the early biomarkers of chronic environmental Cd exposure to assess Cd toxicity. In other words, we aimed to determine if there were any significant metabolic changes that have already shown up in the individuals with UCd of <5 μg/L. To have roughly the same number of subjects in the study groups, the limit of UCd of 2 μg/L was arbitrarily used to investigate the differences. The characteristics of the two groups are summarized in Table 3.

The mean levels of 27 metabolites in these two groups (UCd of <2 μg/L and UCd of 2−5 μg/L) were calculated and compared (see Table S3 of the Supporting Information). The significance of these metabolites was expressed using Student's t test (p value less than 0.05 was considered significant). We found that the level of *N*-methyl-L-histidine among those with UCd of 2−5 μg/L had already significantly upregulated when compared to those with UCd of <2 μg/L. Moreover, there was no significant difference between those with UCd of 2−5 μg/L and those with UCd of ≥5 μg/L. More importantly, it showed an earlier change than the commonly used biomarker for tubular dysfunction, U-NAG. The tubular enzyme, NAG, only started to show a significant release in individuals with UCd of ≥5 μg/L. The altered expression among the three groups of different UCd levels for *N*-methyl-L-histidine and U-NAG are illustrated in Figure 4. The result indicated that *N*-methyl-L-histidine could be an early marker for Cd exposure.

Table 3. Characteristics of 56 Individuals with UCd below 5 μg/L[a]

characteristics	UCd of <2 μg/L	UCd of 2−5 μg/L	p value
number of subjects	31	25	
female	31	25	
smoker	0	0	
age (years)			0.063
range	46.2−68.4	45.2−70.2	
mean ± SD	58.68 ± 7.09	55.16 ± 6.77	
local living (years)			0.158
range	22−68	24−70	
mean ± SD	48.45 ± 11.90	43.28 ± 14.53	
UCd (μg/L)			<0.001
range	0.20−1.99	2.07−4.67	
mean ± SD	1.16 ± 0.47	3.00 ± 0.85	
UCd (μg/g of creatinine)	1.83 ± 1.03	4.27 ± 2.86	<0.001
U-NAG (U/g of creatinine)	4.91 ± 2.95	5.45 ± 2.80	0.483
BMI (kg/m^2)	22.42 ± 3.02	22.25 ± 3.31	0.842

[a]Age, UCd, U-NAG, and BMI were expressed as the mean ± SD. SD denotes standard deviation.

DISCUSSION

Our present study has succeeded in unveiling a wealth of information linking a number of urinary metabolites with chronic Cd exposure. According to the Cadmium Geochemical Map of China,[26] there are several provinces in southern China with high geological Cd; the average Cd concentration in the Earth's crust of these areas is about 0.4 mg/kg, with some up to 0.7 mg/kg. Some areas have also been developed for mining, smelting, and other industrial activities for decades; thus, local residents are likely to have potential exposure to Cd.[27] Nevertheless, until to date, there is no systematic investigation on urinary metabolic changes in humans with long-term environmental Cd exposure. Because tobacco is one of the major sources of Cd exposure, in this study, we therefore selected never smokers to avoid the influence of a known environmental confounding factor. It is noted that, similar to many villages in China, the men are working in large cities and the majority of the residents are females and children; therefore, only females were recruited in this study. In addition, the study subjects have resided in their respective villages for over 20 years, with minimum exposure to other pollutants (e.g., lead and copper) and second-hand cigarettes. Our first objective was therefore primarily to differentiate whether there is a change of urinary metabolic features among female villagers with UCd levels that exceed and are below 5 μg/L based on the current WHO guideline. Moreover, we also hoped

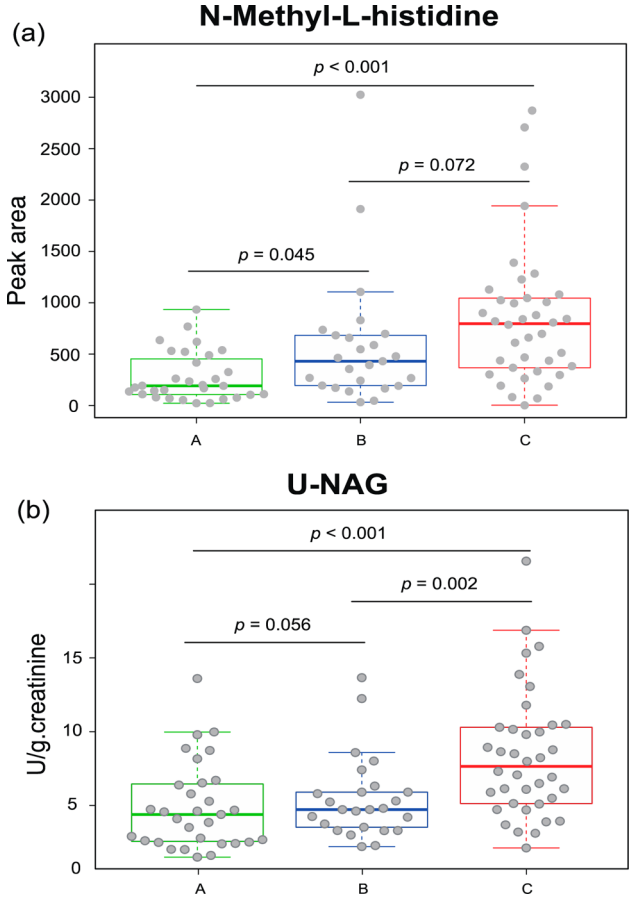

Figure 4. Beeswarm boxplots demonstrated altered expression of *N*-methyl-L-histidine and U-NAG in those individuals with different UCd levels: A, UCd of <2 μg/L (n = 31); B, UCd of 2−5 μg/L (n = 25); and C, UCd of ≥5 μg/L (n = 38).

to identify whether there are urinary metabolite changes in the individuals with a UCd level below 5 μg/L, to assist in assessment of Cd toxicity.

Suitability of Urinary Creatinine Adjustment for UCd Determination. The UCd concentration tends to reflect the amount of Cd in the kidney and has also been regarded as a marker to assess chronic Cd exposure, which is expressed as either creatinine-corrected (μg/g of creatinine) or uncorrected (μg/L).[28] Creatinine correction is the most commonly used method to compensate for variation caused by urine dilution when a urinary biomarker is analyzed.[29] However, numerous recent studies have demonstrated that the uncorrected UCd concentration was found to be more appropriate for studying nephrotoxicity or kidney dysfunction.[30] In this study, we calculated Spearman's correlation coefficient between the urinary metabolic profile and UCd level (creatinine-corrected and uncorrected Cd concentrations, respectively). It was found that the uncorrected UCd concentration showed closer association with the urinary metabolic profile (with higher r_s and lower p values) than the creatinine-corrected UCd concentration (see Figure S4 of the Supporting Information). This observation confirms the earlier findings and suggested that excretion of urinary creatinine itself is a reflection of kidney dysfunction and, hence, should not be used to normalize for kidney function measurements. Therefore, the uncorrected UCd concentration (μg/L) was used in the subsequent data analysis.

Nephrotoxic Effect under Long-Term Environmental Cd Exposure. It is well-established that exposure to high levels of Cd may cause multiple organ damage, such as kidney, liver, lung, testis, brain, and bone.[31,32] Data from our study demonstrated that the renal system of exposed individuals were affected, and this is well-reflected by the detection of increased metabolites related to renal dysfunction. Creatinine is a waste product formed by the dehydration of muscle creatine, and the serum creatinine level is widely used as an index of renal function in clinical practice.[33] The increased urinary creatinine level suggests potential kidney dysfunction. Hippurate is a toxin that accumulates in serum during chronic renal failure, which plays a role in inhibition of both plasma protein binding and organic anion secretion by the kidney.[34] Urea constitutes the major form of waste nitrogen and is usually excreted by the kidney in the urine. High levels of urea and hippurate in urine indicate chronic kidney malfunction.[35] The detection of these two metabolites in urine may thus reflect lower nephron efficiency. Xanthine is a purine and is normally converted to uric acid (the waste product of proteins found in the blood). Excessive amounts of xanthine in the urine may be associated with formation of xanthine uroliths (urinary tract stones) because of its low solubility in water.[36]

Cd-Induced Oxidative Stress. Cd is unable to generate free radicals directly; nevertheless, mounting evidence has shown that Cd alters antioxidant defense systems and increases production of cellular reactive oxygen species (ROS).[37] Our earlier study has demonstrated that Cd could cause change of mitochondrial membrane potential, which led to cell damage.[38] Herein, our current observation also indicated that Cd-induced oxidative stress might be the primary driver for the physiological effect to the kidney. Pyroglutamic acid is a cyclized derivative of L-glutamate and could elicit oxidative stress.[39] Its elevated level may be associated with the alterations in glutathione and glutamine metabolism. Dityrosine is a stable cross-linked product of L-tyrosine and has been used as an important biomarker for oxidative stress.[40] Although dityrosine was not identified in our study, we found that the level of L-tyrosine was significantly increased. This may be the result of lower nephron resorption efficiency. Our present study was also in support of the earlier finding by Ellis et al. on a population living near the point source of cadmium pollution. The study used ^{1}H NMR and identified six urinary metabolites that were associated with cadmium exposure. These metabolites were generally associated with oxidative stress.[19] In addition, similar to their findings, both creatinine and creatine were found elevated with increased Cd exposure in our study.

On the other hand, it was proposed that, during long-term Cd exposure, self-adaptation mechanisms are induced to prevent Cd-induced oxidative stress.[41] One of the most important antioxidant mechanisms is to upregulate the levels of antioxidant metabolites. In this regard, glutathione is one of the most important intracellular antioxidant cellular molecules,[42] although it was not noted to be significantly increased in the urine in this study. Nevertheless, we did observe increased levels of some amino acids that could assist in protecting kidney from oxidative damage caused by Cd toxicity. For instance, the level of L-glutamine increased under long-term Cd exposure, which is a precursor of glutathione, and may protect kidney from oxidative stress by maintaining high levels of glutathione.[43] Similarly, taurine and L-cystine, which are sulfur-containing amino acids, are also increased. Taurine plays a critical role in anti-inflammation and against oxidative stress.[44]

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It scavenges ROS in the kidney glomerulus and is critical for osmoregulation at the renal medulla.[45] L-Cystine is an oxidized dimeric form of L-cysteine, which could increase intracellular glutathione levels.[46] The increased level of L-cystine is also reminiscent of cystinuria, where inadequate cystine resorption occurs at the proximal convoluted tubules.[47] When both elevated taurine and cystine levels are considered together, we postulate that a reduction in resorption capability might be a result of long-term Cd exposure. We also found increased levels of L-histidinol and *N*-methyl-L-histidine. A previous study suggested that histidine-related compounds are potent endogenous antioxidants in the brain and muscles.[48] They also act as a peroxyl radical scavenger to protect protein fragmentation.[49,50] In addition, endogenous steroid estrogens are believed to protect the kidney against oxidative damage in both men and women in human and animal studies.[51] In this study, we observed that four steroid estrogens, including 17-α-hydroxyprogesterone, corticosterone, tetrahydrocortisone, and estrone, were increased under chronic Cd exposure. This finding is in line with the earlier observation that UCd levels were associated with higher concentrations of testosterone and total estradiol in 1262 healthy individuals that participated in the U.S. National Health and Nutrition Examination Survey.[52] A similar observation was also reported among Japanese women with UCd of >3 μg/g of creatinine than in those with less than 2 μg/g of creatinine.[53] Henson and Chedrese elaborated upon the endocrine disruptive effect of Cd, where progesterone is a key estrogen affected by Cd exposure.[54] On the basis of the steroid synthesis pathway, these estrogens are dependent upon progesterone as a synthesis precursor (Figure 3). Hence, the changes of these estrogens are likely to be a consequence of altered progesterone levels, which could be due to Cd exposure.

Early Biomarker for Long-Term Cd Exposure. It was proposed that the initial sign of Cd-induced kidney dysfunction is the renal tubular system, usually detected as an increased excretion of urinary enzyme NAG, which has been commonly used as an early marker for the tubular damage caused by Cd toxicity.[55] In the present study, we noted that the urinary *N*-methyl-L-histidine level showed close correlation with the UCd level, with Spearman's correlation coefficient of $r_s = 0.531$ ($p < 0.001$) better than that of U-NAG ($r_s = 0.491$; $p < 0.001$) (see Table S4 of the Supporting Information). Furthermore, the receiver operating characteristic (ROC) curve revealed that *N*-methyl-L-histidine showed a better discriminatory power to predict case status. In comparison to U-NAG (0.606), the area under the curve (AUC) based on *N*-methyl-L-histidine is 0.68 (see Figure S5 of the Supporting Information). More importantly, urinary *N*-methyl-L-histidine started to elevate among individuals with a UCd concentration of $\geq$2 μg/L (Figure 4). This finding tends to suggest that *N*-methyl-L-histidine appears to be a more sensitive biomarker of renal function than U-NAG. Interestingly, histidine-related compounds have been reported in previous studies as response markers in a non-vertebrate (earthworms, *Lumbricus rubellus*) to copper toxicity.[56,57] Our salient finding of elevated urinary *N*-methyl-L-histidine could be an additional biomarker to assess renal dysfunction caused by Cd toxicity. However, the limitation of this study should be acknowledged, because the specific role of this metabolite on renal function is yet to be elucidated, and its usage for biomonitoring also needs to be confirmed by subsequent epidemiological investigations.

It is worth mentioning that, although the same approach and techniques have been used for this and our recent study on urinary metabolites among subjects with chronic kidney disease (CKD),[58] the metabolic pathways affected however appear to be rather different. In the present study, oxidative, amino acids, creatine, and steroid hormone biosynthesis pathways were found affected. Whereas, for CKD, a vastly different set of metabolites was affected; in particular, many of the uremic toxins, such as indoxyl sulfate, *p*-crysulfate, and indoacetic acids, were detected in high concentrations among the CKD patients. This may reflect that the mechanisms that led to the two diseases are different.

This study represents the first systematic investigation of urinary metabolic changes in humans with chronic environmental Cd exposure using two complementary and sensitive MS-based metabolomic platforms. As a result, 27 significantly changed metabolites were identified in those individuals with UCd level of >5 μg/L. It was found that metabolites related to amino acid metabolism, galactose metabolism, purine metabolism creatine pathway, and steroid hormone biosynthesis were significantly increased. In comparison to U-NAG, *N*-methyl-L-histidine shows a good correlation to the UCd level and also tends to elevate at a lower exposure level and could be an additional biomarker for long-term environmental Cd exposure. In brief, our results demonstrated that an integrated GC−MS and LC−MS metabolomic platform is useful for revealing various urinary metabolic changes of environmental Cd exposure and also offer a better understanding of the mechanisms of the Cd-induced nephrotoxic effect.

■ ASSOCIATED CONTENT

Supporting Information

PCA score plot of all urine samples (black dot) and QC (red dot) samples based on LC−MS data ($R^2X = 0.681$; $Q^2 = 0.276$) (Figure S1), PCA score plot of all urine samples (black dot) and QC (red dot) samples based on GC−MS data ($R^2X = 0.545$; $Q^2 = 0.406$) (Figure S2), validation plot obtained from permutation test ($n = 100$) for the corresponding PLS-DA model: (A) LC−MS data with $R^2 = 0.454$ and $Q^2 = -0.013$ and (B) GC−MS data with $R^2 = 0.312$ and $Q^2 = -0.036$ (Q^2-intercept value less than 0.05 indicating that there was no overfitting in the model) (Figure S3), association between the urinary metabolic profile and UCd level: (a) Spearman's correlation coefficient calculated on the basis of LC−MS and GC−MS data, respectively, and (b) *p* values of the correlation coefficient (Figure S4), area under ROC curve for U-NAG and *N*-methyl-L-histidine (Figure S5), information of 11 biomarkers identified by GC−MS (Table S1), information of 19 biomarkers identified by LC−MS (Table S2), mean levels of 27 metabolites in those individuals with different UCd levels (Table S3), and Spearman's correlation coefficients between UCd, *N*-methyl-L-histidine, and U-NAG (Table S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Anetor, J. I. Rising environmental cadmium levels in developing countries: Threat to genome stability and health. *Niger. J. Physiol. Sci.* **2012**, *27* (2), 103−115.

(2) Bernard, A. Renal dysfunction induced by cadmium: Biomarkers of critical effects. *BioMetals* **2004**, *17* (5), 519−523.

(3) Liang, Y.; Lei, L.; Nilsson, J.; Li, H.; Nordberg, M.; Bernard, A.; Nordberg, G. F.; Bergdahl, I. A.; Jin, T. Renal function after reduction in cadmium exposure: An 8-year follow-up of residents in cadmium-polluted areas. *Environ. Health Perspect.* **2012**, *120* (2), 223−228.

(4) Satarug, S.; Garrett, S. H.; Sens, M. A.; Sens, D. A. Cadmium, environmental exposure, and health outcomes. *Environ. Health Perspect.* **2010**, *118* (2), 182−190.

(5) Chaumont, A.; Voisin, C.; Deumer, G.; Haufroid, V.; Annesi-Maesano, I.; Roels, H.; Thijs, L.; Staessen, J.; Bernard, A. Associations of urinary cadmium with age and urinary proteins: Further evidence of physiological variations unrelated to metal accumulation and toxicity. *Environ. Health Perspect.* **2013**, *121* (9), 1047−1053.

(6) Ikeda, M.; Moriguchi, J.; Sakuragi, S.; Ohashi, F. Association of past diseases with levels of cadmium and tubular dysfunction markers in urine of adult women in non-polluted areas in Japan. *Int. Arch. Occup. Environ. Health* **2013**, *86* (3), 343−355.

(7) Gallagher, C. M.; Kovach, J. S.; Meliker, J. R. Urinary cadmium and osteoporosis in U.S. women ≥50 years of age: NHANES 1988−1994 and 1999−2004. *Environ. Health Perspect.* **2008**, *116* (10), 1338−1343.

(8) Lin, Y. S.; Caffrey, J. L.; Chang, M. H.; Dowling, N.; Lin, J. W. Cigarette smoking, cadmium exposure, and zinc intake on obstructive lung disorder. *Respir. Res.* **2010**, *11*, 53.

(9) Huff, J.; Lunn, R. M.; Waalkes, M. P.; Tomatis, L.; Infante, P. F. Cadmium-induced cancers in animals and in humans. *Int. J. Occup. Environ. Health* **2007**, *13* (2), 202−212.

(10) Adams, S. V.; Newcomb, P. A. Urinary cadmium as a marker of exposure in epidemiological studies. *Environ. Health Perspect.* **2013**, *121* (10), A296.

(11) World Health Organization (WHO). Cadmium. *International Programme on Chemical Safety: Environmental Health Criteria (EHC)*; WHO: Geneva, Switzerland, 1992; Vol. *134*.

(12) Frost, S.; Ho, M. Cadmium poisoning: A case study of gold peak batteries in China. *Corp. Soc. Responsib. Environ. Manage.* **2005**, *12* (4), 220−226.

(13) Zhang, G.; Lindars, E.; Chao, Z.; Bai, Y.; Spickett, J. Biological monitoring of cadmium exposed workers in a nickel−cadmium battery factory in China. *J. Occup. Health* **2002**, *44* (1), 15−21.

(14) Järup, L.; Berglund, M.; Elinder, C. G.; Nordberg, G.; Vanter, M. Health effects of cadmium exposure—A review of the literature and a risk estimate. *Scand. J. Work, Environ. Health* **1998**, 1−51.

(15) Cui, L.; Lee, Y. H.; Kumar, Y.; Xu, F.; Lu, K.; Ooi, E. E.; Tannenbaum, S. R.; Ong, C. N. Serum metabolome and lipidome changes in adult patients with primary dengue infection. *PLoS Neglected Trop. Dis.* **2013**, *7* (8), e2373.

(16) Lu, Y.; Wang, C.; Chen, Z.; Zhao, H.; Chen, J.; Liu, X.; Kwan, Y.; Lin, H.; Ngai, S. Serum metabolomics for the diagnosis and classification of myasthenia gravis. *Metabolomics* **2012**, *8* (4), 704−713.

(17) Lankadurai, B. P.; Nagato, E. G.; Simpson, M. J. Environmental metabolomics: An emerging approach to study organism responses to environmental stressors. *Environ. Rev.* **2013**, *21* (3), 180−205.

(18) Bundy, J. G.; Davey, M. P.; Viant, M. R. Environmental metabolomics: A critical review and future perspectives. *Metabolomics* **2009**, *5* (1), 3−21.

(19) Ellis, J. K.; Athersuch, T. J.; Thomas, L. D.; Teichert, F.; Pérez-Trujillo, M.; Svendsen, C.; Spurgeon, D. J.; Singh, R.; Järup, L.; Bundy, J. G. Metabolic profiling detects early effects of environmental and lifestyle exposure to cadmium in a human population. *BMC Med.* **2012**, *10* (1), 61.

(20) Suvagandha, D.; Nishijo, M.; Swaddiwudhipong, W.; Honda, R.; Ohse, M.; Kuhara, T.; Nakagawa, H.; Ruangyuttikarn, W. A biomarker found in cadmium exposed residents of Thailand by metabolome analysis. *Int. J. Environ. Res. Public Health* **2014**, *11* (4), 3661−3677.

(21) Lu, Y.; Lam, H.; Pi, E.; Zhan, Q.; Tsai, S.; Wang, C.; Kwan, Y.; Ngai, S. Comparative metabolomics in *Glycine max* and *Glycine soja* under salt stress to reveal the phenotypes of their offspring. *J. Agric. Food Chem.* **2013**, *61* (36), 8711−8721.

(22) Xu, F.; Zou, L.; Liu, Y.; Zhang, Z.; Ong, C. N. Enhancement of the capabilities of liquid chromatography−mass spectrometry with derivatization: General principles and applications. *Mass Spectrom. Rev.* **2011**, *30* (6), 1143−1172.

(23) Gika, H. G.; Theodoridis, G. A.; Wingate, J. E.; Wilson, I. D. Within-day reproducibility of an HPLC−MS-based method for metabonomic analysis: Application to human urine. *J. Proteome Res.* **2007**, *6* (8), 3291−3303.

(24) Wagner, S.; Scholz, K.; Donegan, M.; Burton, L.; Wingate, J.; Völkel, W. Metabonomics and biomarker discovery: LC−MS metabolic profiling and constant neutral loss scanning combined with multivariate data analysis for mercapturic acid analysis. *Anal. Chem.* **2006**, *78* (4), 1296−1305.

(25) Wen, T.; Gao, L.; Wen, Z.; Wu, C.; Tan, C. S.; Toh, W. Z.; Ong, C. N. Exploratory investigation of plasma metabolomics in human lung adenocarcinoma. *Mol. Biosyst.* **2013**, *9* (9), 2370−2378.

(26) Li, J.; Wu, G. *Atlas of the Ecological Environmental Geochemistry of China*; Geological Publishing House: Beijing, China, 1999.

(27) Wang, M.; Liu, X.; Wang, Z. Studies on national surveillance system for food contaminations and foodborne diseases in China. *Chin. J. Food Hyg.* **2006**, *6*, 002.

(28) Paschal, D.; Burt, V.; Caudill, S.; Gunter, E.; Pirkle, J.; Sampson, E.; Miller, D.; Jackson, R. Exposure of the US population aged 6 years and older to cadmium: 1988−1994. *Arch. Environ. Contam. Toxicol.* **2000**, *38* (3), 377−383.

(29) Waikar, S. S.; Sabbisetti, V. S.; Bonventre, J. V. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. *Kidney Int.* **2010**, *78* (5), 486−494.

(30) Miller, R. C.; Brindle, E.; Holman, D. J.; Shofer, J.; Klein, N. A.; Soules, M. R.; O'Connor, K. A. Comparison of specific gravity and creatinine for normalizing urinary reproductive hormone concentrations. *Clin. Chem.* **2004**, *50* (5), 924−932.

(31) Kanter, M.; Aksu, B.; Akpolat, M.; Tarladacalisir, Y. T.; Aktas, C.; Uysal, H. Vitamin E protects against oxidative damage caused by cadmium in the blood of rats. *Eur. J. Gen. Med.* **2009**, *6* (3), 154−160.

(32) Kataranovski, M.; Janković, S.; Kataranovski, D.; Stosić, J.; Bogojević, D. Gender differences in acute cadmium-induced systemi inflammation in rats. *Biomed. Environ. Sci.* **2009**, *22* (1), 1−7.

(33) Perrone, R. D.; Madias, N. E.; Levey, A. S. Serum creatinine as an index of renal function: New insights into old concepts. *Clin. Chem.* **1992**, *38* (10), 1933−1953.

(34) Deguchi, T.; Takemoto, M.; Uehara, N.; Lindup, W. E.; Suenaga, A.; Otagiri, M. Renal clearance of endogenous hippurate correlates with expression levels of renal organic anion transporters in uremic rats. *J. Pharmacol. Exp. Ther.* **2005**, *314* (2), 932−938.

(35) Boelaert, J.; Lynen, F.; Glorieux, G.; Eloot, S.; Van Landschoot, M.; Waterloos, M. A.; Sandra, P.; Vanholder, R. A novel UPLC−MS−MS method for simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients. *Anal. Bioanal. Chem.* **2013**, *405* (6), 1937−1947.

(36) Gargah, T.; Essid, A.; Labassi, A.; Hamzaoui, M.; Lakhoua, M. Xanthine urolithiasis. *Saudi J. Kidney Dis. Transplant.* **2010**, *21* (2), 328.
(37) Cuypers, A.; Plusquin, M.; Remans, T.; Jozefczak, M.; Keunen, E.; Gielen, H.; Opdenakker, K.; Nair, A. R.; Munters, E.; Artois, T. J. Cadmium stress: An oxidative challenge. *BioMetals* **2010**, *23* (5), 927−940.
(38) Yang, C.; Shen, H.; Shen, Y.; Zhuang, Z.; Ong, C. N. Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (MRC-5 cells). *Environ. Health Perspect.* **1997**, *105* (7), 712−714.
(39) Pederzolli, C. D.; Sgaravatti, Â. M.; Braum, C. A.; Prestes, C. C.; Zorzi, G. K.; Sgarbi, M. B.; Wyse, A. T.; Wannmacher, C. M.; Wajner, M.; Dutra-Filho, C. S. 5-Oxoproline reduces non-enzymatic antioxidant defenses *in vitro* in rat brain. *Metab. Brain Dis.* **2007**, *22* (1), 51−65.
(40) DiMarco, T.; Giulivi, C. Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples. *Mass Spectrom. Rev.* **2007**, *26* (1), 108−120.
(41) Liu, J.; Qu, W.; Kadiiska, M. B. Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicol. Appl. Pharmacol.* **2009**, *238* (3), 209−214.
(42) Lobo, V.; Patil, A.; Phatak, A.; Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.* **2010**, *4* (8), 118.
(43) Amores-Sánchez, M. I.; Medina, M. Á. Glutamine, as a precursor of glutathione, and oxidative stress. *Mol. Genet. Metab.* **1999**, *67* (2), 100−105.
(44) Chen, G.; Nan, C.; Tian, J.; Jean-Charles, P.; Li, Y.; Weissbach, H.; Huang, X. Protective effects of taurine against oxidative stress in the heart of MsrA knockout mice. *J. Cell. Biochem.* **2012**, *113* (11), 3559−3566.
(45) Chesney, R. W.; Han, X.; Patters, A. B. Taurine and the renal system. *J. Biomed. Sci.* **2010**, *17* (Supplement 1), S4.
(46) Gukasyan, H. J.; Kannan, R.; Lee, V. H.; Kim, K.-J. Regulation of L-cystine transport and intracellular GSH level by a nitric oxide donor in primary cultured rabbit conjunctival epithelial cell layers. *Invest. Ophthalmol. Visual Sci.* **2003**, *44* (3), 1202−1210.
(47) Chillaron, J.; Font-Llitjos, M.; Fort, J.; Zorzano, A.; Goldfarb, D. S.; Nunes, V.; Palacin, M. Pathophysiology and treatment of cystinuria. *Nat. Rev. Nephrol.* **2010**, *6* (7), 424−434.
(48) Wu, H.; Shiau, C.; Chen, H.; Chiou, T. Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *J. Food Drug Anal.* **2003**, *11* (2), 148−153.
(49) Kang, J. H.; Kim, K. S.; Choi, S. Y.; Kwon, H. Y.; Won, M. H. Oxidative modification of human ceruloplasmin by peroxyl radicals. *Biochim. Biophys. Acta* **2001**, *1568* (1), 30−36.
(50) Shi, X.; Dalal, N.; Kasprzak, K. S. Enhanced generation of hydroxyl radical and sulfur trioxide anion radical from oxidation of sodium sulfite, nickel(II) sulfite, and nickel subsulfide in the presence of nickel(II) complexes. *Environ. Health Perspect.* **1994**, *102* (Supplement 3), 91.
(51) Yi, S.; Selvin, E.; Rohrmann, S.; Basaria, S.; Menke, A.; Rifai, N.; Guallar, E.; Platz, E. A.; Astor, B. Endogenous sex steroid hormones and measures of chronic kidney disease (CKD) in a nationally representative sample of men. *Clin. Endocrinol.* **2009**, *71* (2), 246−252.
(52) Menke, A.; Guallar, E.; Shiels, M. S.; Rohrmann, S.; Basaria, S.; Rifai, N.; Nelson, W. G.; Platz, E. A. The association of urinary cadmium with sex steroid hormone concentrations in a general population sample of US adult men. *BMC Public Health* **2008**, *8* (1), 72.
(53) Nagata, C.; Nagao, Y.; Shibuya, C.; Kashiki, Y.; Shimizu, H. Urinary cadmium and serum levels of estrogens and androgens in postmenopausal Japanese women. *Cancer Epidemiol., Biomarkers Prev.* **2005**, *14* (3), 705−708.
(54) Henson, M. C.; Chedrese, P. J. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Exp. Biol. Med.* **2004**, *229* (5), 383−392.
(55) Järup, L.; Hellström, L.; Alfvén, T.; Carlsson, M. D.; Grubb, A.; Persson, B.; Pettersson, C.; Spång, G.; Schütz, A.; Elinder, C. G. Low level exposure to cadmium and early kidney damage: The OSCAR study. *Occup. Environ. Med.* **2000**, *57* (10), 668−672.
(56) Bundy, J. G.; Keun, H. C.; Sidhu, J. K.; Spurgeon, D. J.; Svendsen, C.; Kille, P.; Morgan, A. J. Metabolic profile biomarkers of metal contamination in a sentinel terrestrial species are applicable across multiple sites. *Environ. Sci. Technol.* **2007**, *41* (12), 4458−4464.
(57) Bundy, J. G.; Sidhu, J. K.; Rana, F.; Spurgeon, D. J.; Svendsen, C.; Wren, J. F.; Stürzenbaum, S. R.; Morgan, A. J.; Kille, P. 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. *BMC Biol.* **2008**, *6*, 25.
(58) Ng, D.; Salim, A.; Liu, Y.; Zou, L.; Xu, F.; Huang, S.; Leong, H.; Ong, C. A metabolomic study of low estimated GFR in non-proteinuric type 2 diabetes mellitus. *Diabetologia* **2012**, *55* (2), 499−508.