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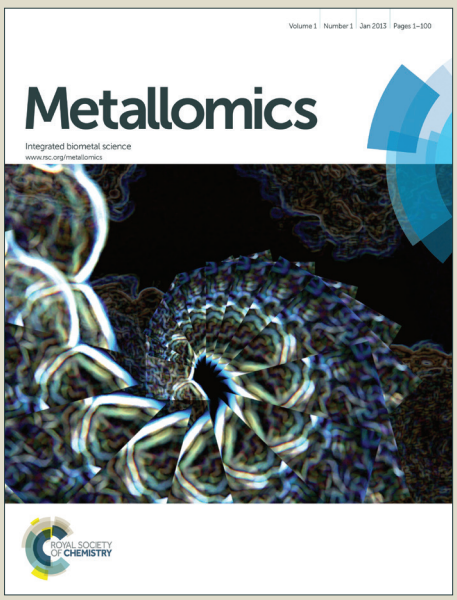
Accepted Manuscript



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Serum metabolomics reveals arsenic exposure disrupted lipid and amino acid metabolism in rat: a step forward in understanding chronic arsenic toxicity

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Running title: Serum metabolomic response to chronic arsenic exposure

Abstract: Chronic arsenic exposure through drinking water threatens public health worldwide. Although its multi-organ toxicities have been reported, the impact of chronic arsenic exposure on metabolic network remains obscure. In this study, male Sprague Dawley rats were exposed to 0.5, 2 or 10 ppm sodium arsenite for three months. An ultra-high performance liquid chromatography/mass spectrometry based metabolomics approach was utilized to unveil the global metabolic response to chronic arsenic exposure in rats. Distinct serum metabolome profiles were found to be associated with the doses. Eighteen differential metabolites were identified, and most of them showed dose-dependent responses to arsenic exposure. Metabolic abnormalities mainly involved with lipid metabolism and amino acid metabolism. The metabolic alterations were further confirmed by hepatic gene expression. Expressions of *cpt2*, *lcat*, *cact*, *crot* and *mtr* were significantly elevated in high dose group. This study provides novel evidence to support the association between arsenic exposure and metabolic disruption, and it contributes to understand the mechanism of chronic arsenic toxicity.

Keywords: arsenic; metabolomics; serum; lipid metabolism; amino acid metabolism

1. Introduction

Millions of individuals worldwide are exposed to inorganic arsenic through drinking water.[1, 2] The toxicity of arsenic has been well documented,[3-6] but the mechanisms are not fully understood. Advances in omics technologies have enabled simultaneous and non-targeted profiling of genes, proteins and metabolites in complex biological matrices.[7] These non-hypothesis-driven omics approaches have been used to identify exposure-specific biomarkers and related pathways. Genomic study of arsenic-exposed Bangladeshi individuals identified genome-wide associations of urinary monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) levels with single nucleaotide polymorphisms (SNP) variants located near the arsenic methylatransferase (As3MT) gene, and one of these variants was associated with risk of skin lesions.[8] Besides, transcriptomics data suggested that prolonged arsenic exposure might elevate the expression of inflammatory molecules and increase atherosclerosis risk in arsenic endemic areas in Taiwan.[9] Moreover, a previous urinary proteomic study identified human α defensin-1 as a biomarker in Taiwan patients with arsenic-induced blackfoot disease.[10]

Transcriptomics and proteomics are now widely used across the biological sciences and provide extensive information regarding the genotype, but they convey limited information about phenotype. This has led to increasing interest in metabolomics, which can capture low molecular weight metabolites that are the closest to phenotype. Metabolomics is believed to be one of the most powerful techniques to study the metabolic alteration associated with the treatment of environmental toxicants.[11, 12]

With regards to arsenic, its adverse effects on steroid receptors and endogenous/exogenous hormone-driven genes have been demonstrated in vivo and in vitro.[13-16] Endocrine disruption is tightly involved with metabolic disruption.[17] Recent epidemiologic studies suggested inorganic arsenic exposure was tightly associated with metabolic syndrome.[18, 19] Aberrant glucocorticoid (GC) / glucocorticoid receptor (GR) signaling between hormones and their cognates is critical checkpoints in mammalian energy homeostasis, and further linked to severe metabolic dysfunction, including obesity, insulin resistance and type 2 diabetes.[20-22] Therefore, we speculate metabolic disruption might be another important mechanism underlying the toxicity of chronic arsenic exposure.

The global metabolic profile response to inorganic arsenic exposure is not well understood. Our previous metabolomics data revealed ambient arsenic exposure disrupted individual's metabolism in the general Chinese population, and identified potential biomarkers with dose-dependent response.[23] Quite recently, metabolomics studies reported the adverse effects of acute arsenic exposure (7 days) and high dose arsenic (50 mg/L) on metabolic response in mice and rat,[24, 25] which provided valuable information of the toxicity and the mode of action of arsenic. However, it should be noted the difference in dose and time may result in varied molecular mechanisms. Acute exposure experiments were limited to mimic chronic arsenic exposure of humans under real environment. Therefore, more metabolomics data of chronic arsenic exposure are urgently required.

The present study is designed to investigate the serum metabolome alterations induced by the chronic arsenic exposure. An ultra-high performance liquid chromatography/mass spectrometry (UHPLC/MS) based metabolomics approach was used to profile metabolome and characterize significantly altered metabolites in the sera of the rats exposed to arsenic. Besides, the expressions of key genes involved in altered metabolism pathway were also examined to support metabolomics results. This study provides more knowledge associated with arsenic-induced metabolic disturbance and leads to a more comprehensive understanding of chronic arsenic toxicity.

2. Materials and Methods

2.1 Chemicals and solvents

Sodium arsenite (purity>98.5%), and formic acid (HPLC grade) were purchased from Acros (Morris Plains, NJ, USA). All standards (purity>95%) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Methanol (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water (18.2 MΩ) was obtained from a Milli-Q system (Beford, MA, USA).

2.2 Animals and dosing

A total of thirty male Sprague Dawley rats aged six weeks (weight 200 ± 10g) were obtained from Shanghai Laboratory Animal Center, China. Animals were housed separately in stainless steel cages and acclimatized for one week before initiation of arsenic exposure. Rats were maintained in an air-conditioned room at the temperature of 26 ± 2 °C, a relative humidity of 50 ± 5%, and a 12 h light/12 h dark cycle. Each

animal had *ad libitum* access to water and a pellet diet. After a 7-day quarantine and acclimatization, all the rats were randomly divided into control and three dose groups. The control group (n=6) was fed with deionized water. The low (n=8), medium (n=8) and high-dose (n=8) groups were administered, respectively, with 0.5, 2 and 10 ppm sodium arsenite, which are comparable to previous reports.[26] All animals were treated humanely and with regard for alleviation of suffering according to the China Animal Welfare legislation.

2.3 Sample collection and preparation

After being treated with arsenic for three months consecutively, the rats were killed by decapitation. Serum was obtained by centrifugation (3500 ×g, 10 min at 4 °C) and frozen at -80 °C before metabolomic analysis. The livers were removed immediately after sacrifice, rinsed with PBS (room temperature), flash frozen in liquid nitrogen and stored at -80 °C.

For the pretreatment of serum samples, a volume of 600 μL cold methanol was added to 200 μL serum and was shaken vigorously, and the mixture was stored for 10 min and subsequently centrifuged at 12,000 ×g for 10 min at 4 °C. The supernatant was filtered through a 0.22 μm syringe filter prior to metabolic profile acquisition.

2.4 Metabolic Profile Acquisition

Serum metabolic profiles were acquired using an ultra-high performance liquid chromatography (UHPLC)/Orbitrap-mass spectrometer (MS) (Thermo, USA). A Kinetex C18 column (150 mm× 2.1 mm, 2.6 μm) was used for chromatographic separation. Sample injection volume was 5 μL. The mobile phase consisted of water

containing 0.1% formic acid (mobile phase A) and methanol containing 0.1% formic acid (mobile phase B). A programmed gradient was used: 5% B increased to 100% B in 16 min and held for 4 min, then decreased to 5% in 0.1 min, and finally maintained at 5% B for 3 min. The mass spectrometer was operated in positive-ion mode with a range of 100-1000 m/z. Spray voltage and cone voltage were 3.5 KV and 35 V, respectively. Heated capillary temperature and source temperature were 380 ^{o}C and 350 ^{o}C, respectively. Curtain and auxiliary gas flow were 60 and 35 L/h, respectively. Serum samples were run in a randomized fashion to avoid possible uncertainties from artifact-related injection order and gradual changes of instrument sensitivity in whole batch runs. A serum quality control (QC) was prepared by pooling and mixing the same volume of each sample. The QC samples and blank samples (pure methanol) were injected at interval of six samples to identify the sample carryover and check for the stability (n=6) during the whole sequence. After data preprocess, there were 691 variables remaining in the dataset. CV values of 71.9% variables were<30%, and the CV values of 55.6% variables were<15%, indicating our method had excellent repeatability and the dataset was worthy for further multi-variate analysis (Fig. S1). To carry out MS/MS mode to identify differentiated metabolites, argon was used as a collision gas, and the collision energy was adjusted from 15 eV to 40 eV for each metabolome.

2.5 Metabolome analysis

UHPLC-MS data were analyzed with the Micromass MarkerLynx applications manager Version 4.1 (Waters, UK). Raw data were deconvoluted, aligned, and

reduced to give a table of mass and retention time pairs with associated intensities for all the detected peaks. The main parameters were set as follows: retention time range 1–15 min, mass range 100–1000, mass tolerance 0.01 Da, masses per retention time 10, minimum intensity 1%, mass window 0.05 Da, retention time window 0.20 min, and noise elimination level 6. The table was normalized to total intensity to correct for the different enrichment factors of serum among individuals. Finally, the processed tables were pareto-scaled and fed to SIMCA-P+ 12 software (Umetrics AB, Uppsala, Sweden) for multivariate statistical analysis. PCA was firstly performed to discover intrinsic treatment-related clusters within the datasets. Following this, PLS-DA was used to improve the group separation and screen differential metabolites. The robustness and validity of the PLS-DA model was tested using a 200-permutation test (Fig. S2). Variable importance in projection (VIP) is an appropriate quantitative statistical parameter ranking the variables according to their ability to discriminate different doses, and variables with VIP> 3 were selected into preset of differential metabolites. The candidates were further tested with the Kruskal-Wallis test (p<0.01).[27] If the overall statistic was significant, the Mann-Whitney test was used to compare the levels of the candidates between control group and each dose group. The candidates which showed significant alteration in at least one dose group were selected as final differential metabolites. All p values shown were two-tailed. The identification of these metabolites followed the procedure described previously,[28] the following databases were used: HMDB (http://www.hmdb.ca/), METLIN

(http://metlin.scripps.edu/), Massbank (http://www.massbank.jp), PubChem (http://ncbi.nim.nih.gov/) and KEGG (http://www.kegg.com/).

2.6 Quantitative real-time PCR

Total RNA samples were extracted from homogenized liver samples using Total RNA Kit I (OMEGA). NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA) and agarose gel electrophoresis were used to measure RNA concentration and purity. Reverse-transcription of cDNA synthesis was performed with 1 μg total RNA using PrimeScript®RT reagent Kit (TaKaRa Bio, Otsu, Japan). Real-time PCR was carried out in a 20 μL final volume and performed in duplicate using SYBR Green Master Mix reagents in a Light cycler 480 detection system (Roche Applied Science, Indianapolis, USA) according to the manufacture protocol. PCR primers were listed in Table 1. The conditions for quantitative PCR were as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. Gene expression levels were normalized to β-actin expression levels. The fold changes of the tested genes were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.7 Statistical analysis

All analyses were conducted using SPSS Version 18.0 (SPSS Inc., Chicago, USA). If data were not normal distributed, Wilcoxon test was carried out to compare between and within groups. Normal distributed data were analyzed using one-way ANOVA; LSD or Tamhane test was used based on homogeneity of variances. Significance was set at $p \leqslant 0.05$.

3. Results

3.1 Multivariate statistical analysis

In PCA score plot (Fig. 1A), the samples of control group were differentiated from those of 2 and 10 ppm groups, but they were not completely separated from those of 0.5 ppm group. A supervised PLS-DA model was further used to discover the difference among groups and screen the differential metabolites associated with arsenic exposure. The corresponding PLS-DA model had a faithful representation of the data and a good cumulative predictive capacity (Fig. 1B). Control group and three dose groups were obviously separated from each other. Moreover, larger variation was observed for 10 ppm group.

3.2 Differential metabolites indicative of arsenic exposure

Differential metabolites are responsible for group separation through the PCA or PLS-DA loadings and variable importance plots (VIP). They are important indicators of mode of action of the pollutants. In this study, differential metabolites were selected according to the workflow described in the experimental part. Specifically, 77 variables had a VIP above 3, and among these variables, eighteen discriminant metabolites were selected, which were involved in lipid, amino acid and nucleotide metabolism (Table 2). Serum levels of all these metabolites did not show significant changes in 0.5 ppm group, but almost all of them altered markedly in 10 ppm group.

The lipid metabolism-related metabolites, lysoPC(20:1), ceramide(d18:0/16:0), octadecenylcarnitine only significantly increased in 10 ppm group. Ceramide(d18:0/14:0), palmitoylcarnitine, sphingosine, phytosphingosine seemed to be more sensitive to arsenic exposure than above mentioned metabolites. They were

significantly elevated in both 5 and 10 ppm groups. In addition, lysoPC(18:0) was significantly increased at 10 ppm group, but there were no significant alterations in 0.5 and 2 ppm groups. Interestingly, carnitine, which plays a key role in fatty acid beta-oxidation, showed an inverted U-shaped curve: it was not altered in 0.5 ppm group, but it significantly increased in 2 ppm group yet significantly declined in 10 ppm group (Fig. 2A).

Besides, six amino acids (i.e. methionine, proline, valine and tyrosine) and their derivatives (i.e. indoleacetaldehyde and pyroglutamic acid) were also identified as differential metabolites (Fig. 2B). Serum levels of methionine, proline and tyrosine markedly decreased in a dose-dependent manner. Valine and indoleacetaldehyde peaked in 0.5 ppm group (not significantly) and significantly decreased in 10 ppm group. Pyroglutamic acid increased in all dose groups, and reached its peak in 2 ppm group. Creatine, uric acid and cytosine did not show significant change in 0.5 and 2 ppm groups, but they significantly decreased in 10 ppm group (Fig. 2B).

3.3 Gene expression

Several lipid metabolism-related key genes, like *cpt1*, *cpt2*, *lcat*, *cact*, *crot*, and amino acid metabolism-involved gene *mtr* were investigated in liver. The expression of *lcat*, *cpt2* increased significantly in 10 ppm group, while they changed little in either 0.5 or 2 ppm group. The expression of *crot* and *mtr* increased in all dose groups as compared with the control (Fig. 3). Hepatic *cact* mRNA expression increased in 2 and 10 ppm group, but decreased in 0.5 ppm group, presenting a U-shaped curve.

4. Discussion

Environmental epidemiology studies have tightly associated chronic arsenic exposure with obesity and metabolic syndrome. However, the influence of chronic arsenic on global metabolic system is not well understood. Toxicometabolomics aims to dig out critical metabolites and affected biological pathways that respond to environmental perturbations using global metabolic profiling technologies. Therefore, it can be utilized to augment our understanding of the toxic mechanisms involved in chronic arsenic exposure. Previous arsenic toxicity studies were conducted using relatively high exposure levels (20-200 ppm). The lowest arsenic dose (0.5 ppm) in our study was within the range of natural environmental exposure levels, and the highest inorganic arsenic dose (10 ppm) was comparable to environmental arsenic levels of 9.9 ppm in the ground water of heavily polluted areas,[26] and it also corresponded to 1/25 of the median lethal dose (LD50) of sodium arsenite through oral administering on rats (41 mg/kg). [29]

One primary objective of our study was to identify potential differentiated metabolites that correlated with the metabolic changes triggered by chronic arsenic exposure, thus discover the disturbed metabolic pathways responsible for arsenic toxicity. Our previous work showed total arsenic levels proportionally increased with doses in a variety of organs (*i.e.* liver, kidney, heart, spleen, lung and pancreas),[30] which indicated the dose-dependent accumulation of arsenic in rats. In metabolomics data analysis, PCA was firstly used to detect potential outliers and determine the variation in the data set. As shown in Fig. 1A, it allows only preliminary discrimination between groups. When there are more than two groups, PLS-DA is more appropriate

to distinguish variability that occurs among groups and within groups. We performed PLS-DA - now routinely used in the field of metabolomics - to explore metabolomics profiles linked with chronic exposure to arsenic. A clear dose-dependent trajectory was observed for control and three dose groups in the developed PLS-DA model, which suggested arsenic-related metabolic perturbations. What's more, further analysis identified chronic arsenic exposure-associated alterations in lipid metabolism, amino acid metabolism and nucleotide metabolism (Fig. 4).

4.1 Lipid metabolism

LysoPC is formed with the help of ecithin–cholesterol acyltransferase (LCAT) in plasma.[31] Along with the elevated expression of hepatic *lcat* in 10 ppm group, the marked increased level of lysoPC (20:1, 18:0) indicated that arsenic exposure may disrupted the transformation process of lysoPCs, which was also supported by previous studies.[24, 32, 33] Recently, another study reported some serum medium and long chain lysoPCs (i.e. lysoPC 14:0, 18:4, 18:0, 20:2) decreased in rats after 10 and 50 ppm arsenic exposure for 6 months.[25] The disparity may due to distinct responses to arsenic toxicity of rats in different life stages. Compared with sexual matured rats, adult ones may have better abilities to mount an effective response to arsenic attack. However, this hypothesis needs to be further studied.

Sphingolipid metabolism, associated with PC homeostasis, plays an important role in integrity of lipid rafts. Thus, increased serum ceramide (d18:0/14:0, d18:0/16:0) and sphinganine in the rats exposed to arsenic might present a disruption of membrane distribution. Moreover, increased level of ceramide is well recognized in relation to

cell apoptosis, oxidative stress and proteolysis,[34, 35] and this could be a mechanism associated with arsenic exposure.

Located at the outer mitochondrial membrane, carnitinepalmitoyltransferase I (CPT I) catalyzes the formation of acylcarnitine, such as palmitoylcarnitine and octadecenylcarnitine. Then, acylcarnitine is converted to long chain acyl-CoA molecules by carnitinepalmitoyltransferase II (CPT II) for β-oxidationand, and it is coupled to free carnitine to translocate back to the cytoplasm by carnitineacylcarnitinetranslocase (CACT) for the next cycle reaction.[36] The elevated expression of *cpt2, cact* in 10 ppm group coincided with increased acylcarnitine synthesis and decreased serum carnitine accumulation. Interestingly, serum carnitine level increased in both 0.5 and 2 ppm groups, and previous studies reported conflicting results about carnitine level change after arsenic exposure,[32, 37] exemplifying the complex effects of arsenic exposure. Furthermore, the non-monotonic dose-response (NMDR) of carnitine metabolism validated endocrine-disrupting effect of arsenic at low doses that are not expected by effects at higher doses and vice versa.[38-40] However, the exact molecular mechanism underlying NMDR of arsenic exposure remained obscure. More efforts are needed to explore the intrinsic causation of low-dose effect of arsenic. CROT, a member of the carnitine/choline acetyltransferase family, involves in lipid metabolism and β-oxidation of C6–C10 chain fatty acids.[41] In our study, *crot* expression was markedly elevated in all dose groups. Recent studies have suggested that epigenetic mechanisms may mediate toxicity resulting from arsenic exposure.[42] For example, rats [43] and mice

[44, 45] exposed to sodium arsenite for several weeks displayed global hepatic DNA hypomethylation, which may explain the up-regulation of *crot* in our study. In contrast with animal findings, DNA methylation increased in human *crot* regulatory elements following a 2 year exposure of up to 1.1 ppm arsenic,[46] and increased methylation tightly correlated with gene silence.[47] The discrepancy demonstrates puzzling mechanisms whereby arsenic may interfere with carnitine metabolism, hence more research should be conducted to unveil hypo- and hypermethylation of specific key genes.

4.2 Amino acid metabolism and nucleotide metabolism

Disruption of amino acid metabolism seems to be a common response to many toxins in animal and human researches, and it suggests a general response to various toxicants exposure rather than specific biological response to a particular toxicant.[48] Still, there are some biomarkers that need to be paid attention on. Methionine can converse into S-adenosylmethionine (SAM) by methionine adenosyltransferase. SAM donates a methyl group to arsenic to form methyl and dimethyl arsenic. In our study, *mtr* expression increased in all dose groups, and this should lead to an increase level of methionine. But this is not the case here. A lowered level of methionine in 2 ppm and 10 ppm groups indicated that higher dose arsenic exposure not only triggered methionine and SAM consumption for arsenic methylation, but also disturb other metabolism pathways that needed methyl donor, such as DNA methylation. A variety of studies support the notion that chronic arsenic exposure caused a significant depletion of SAM in arsenic-transformed cells.[49,50] Meanwhile, the accumulation of

cytosines in 10 ppm group might be related to restoration of modified cytosine so that it can replenish cytosine pools after the methylated DNA is cut off and resynthesized.[51] Monoamine neurotransmitters such as dopamine (DA) and norepinephrine (NE) are synthesized from tyrosine, which can ameliorate the working memory deficit.[52] The lower level of tyrosine in this study provided the evidence to support deficiency in neurotransmission as the pathophysiology of depression and other neuronal defects upon arsenic exposure.[53, 54] Uric acid is the final oxidative product of purine nucleotide metabolism. Previous studies showed that uric acid levels in plasma and urine in rats declined after chronically exposed to arsenic,[55] which supported our result and proved that arsenic exposure intervened purine metabolic processes.

4.3 Comparison of differential metabolites between our study and previous studies

It has been confirmed by this study and previous studies that the short-term and long-term exposure to arsenic disrupted serum metabolome in rats and mice.[24, 25] However, the alterations of some differential metabolites are contradicted by each other study (Table S1). For example, lysoPCs were observed to be elevated by arsenic exposure in our study and Garc á-Sevillano *et al.* study,[24] but they were reported to decrease in Wang *et al.* study.[25] It should be noted that it is difficult to reconcile opposing results found in various reports. The diversity of model systems, treatment protocols, start and end points used by different research groups to assay arsenic-induced metabolome alteration contribute to the uncertainty.

5. Conclusion

A non-targeted metabolomics approach was used to investigate serum metabolic characteristics of rats chronically exposed to arsenic. Eighteen differential metabolites in relation to arsenic exposure were selected. Metabolic abnormalities upon arsenic exposure were mainly revealed as disruption of lipid metabolism and amino acid metabolism. Rats in 2 and 10 ppm groups were intimately associated with lysolipid, sphingolipid, fatty acid beta-oxidation and amino acid metabolic abnormalities, which were further confirmed by the results of related hepatic genes expression. The present study provides new evidences to understand the mechanism of chronic arsenic toxicity, and it helps to clarify the effect of chronic low-level arsenic exposure in humans. However, the preliminary findings of this study should be confirmed by large epidemiological studies.

Acknowledgements

This work was financially supported by NSFC foundation (21407143) and Knowledge Innovation Program of the Chinese Academy of Sciences (IUEZD201401).

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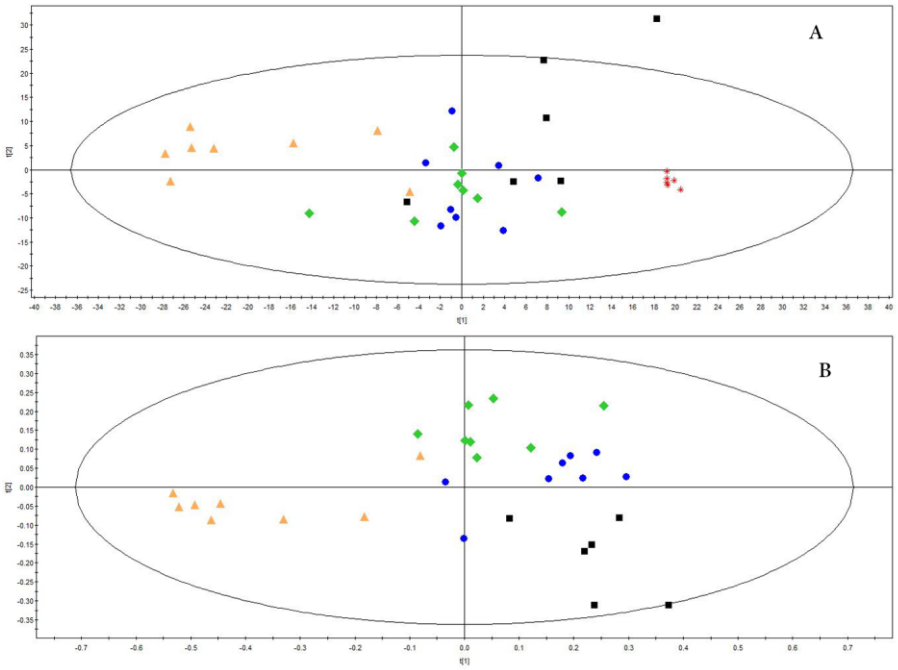
Appendices

Figure 1. Scoring plots of PCA (A) and PLS-DA (B) analysis. ■ control, ∗QC, ● 0.5 ppm, ♦ 2 ppm, ▲ 10 ppm.

Figure 2. Arsenic-induced disruption of metabolome homeostasis in rats. A: lipid metabolism, B: amino acid metabolism and nucleotide metabolism. All the data were expressed as mean ±standard deviation (SD). *p< 0.05,**p< 0.01.

Figure 3. Effects of arsenic on mRNA level of selected genes involved in lipid metabolism and amino acid metabolism. All the data were expressed as mean ± standard deviation (SD). *p< 0.05, **p< 0.01.

Figure 4. A schematic representation of the reactions of differential metabolites.



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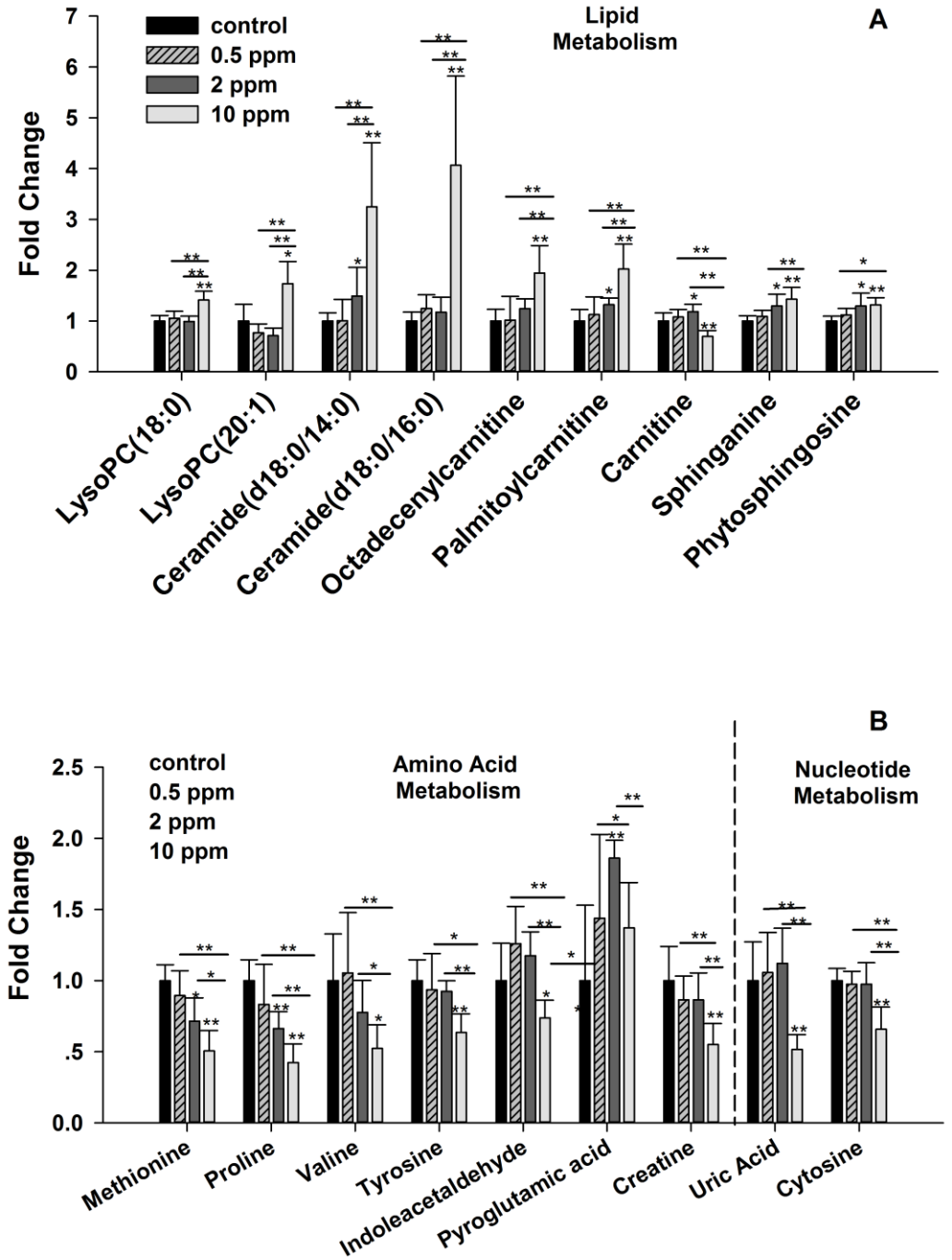

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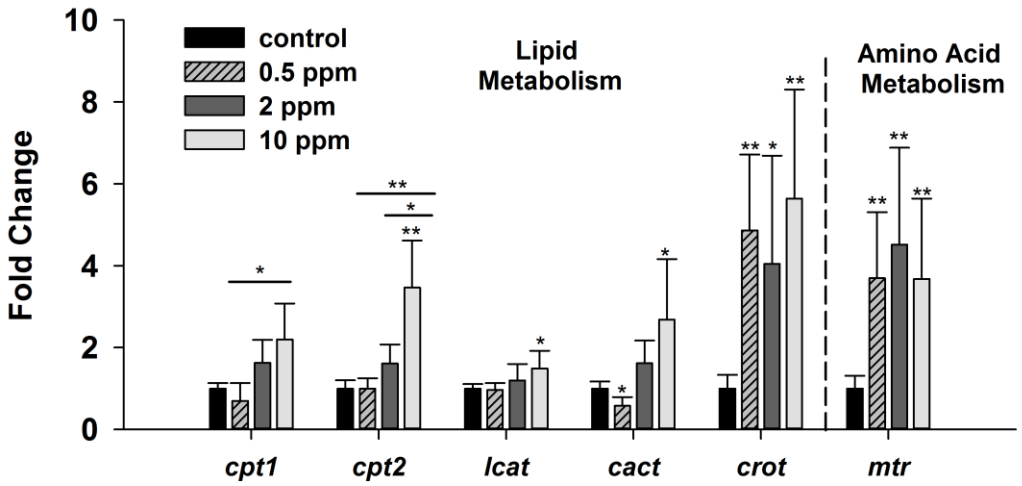

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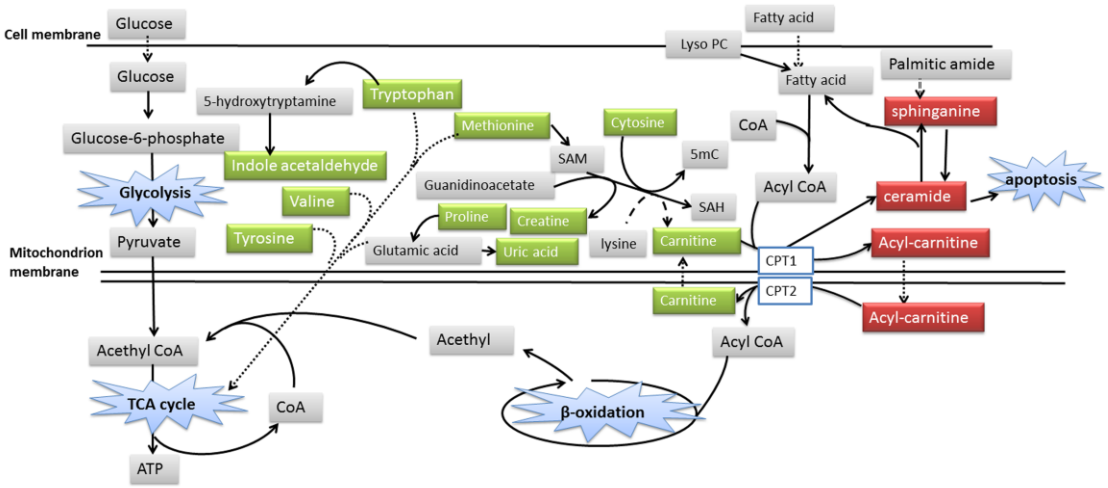

Figure 4. A schematic representation of the reactions of differential metabolites.

Table 1. Primers sequences used for real-time PCR analysis.

Gene Name	Gene Symbol		Primer(5'-3')	Target Size (bp)
Beta-actin	*β-actin*	*Forward*	*CCCATCTATGAGGGTTACGC*	150
		Reverse	TTTAATGTCACGCACGATTTC	
Carnitinepalmitoyltransferase 1	*cpt1*	*Forward*	ATCCACCATTCCACTCTGCT	107
		Reverse	TGTGCCTGCTGTCCTTGATA	
Carnitinepalmitoyltransferase 2	*cpt2*	*Forward*	CTGTCCACCAGCACTCTGAA	111
		Reverse	GCAACCTATCCAGTCATCGT	
Ecithin–cholesterol acyltransferase	*lcat*	*Forward*	CTCCTTCTGGCTCCTCAATG	171
		Reverse	TCCTCTGTCTTTCGGTAGCAC	
Carnitine O-octanoyltransferase	*crot*	*Forward*	AGACGGAAGGGAGATGGAAG	168
		Reverse	AAGATGTGAAGGTAGATGCTGCT	
Mitochondrial carnitine / acylcarnitine carrier protein	*cact*	*Forward*	TTCTCCACTGCTGCTCCTG	100
		Reverse	CCTGTCTGCTCCCATTCAG	
5-methyltetrahydrofolate-homocysteine methyltransferase	*mtr*	*Forward*	GGTTCGGTTGAAGAAGAGGA	112
		Reverse	TATTACAGCCCAGCACCACA	

Table 2. Identified differential metabolites in rat serum.

Super-pathway	Sub-pathway	Biochemical name	Chemical Formula	VIP	Kruskal-Wallis Test
Lipid metabolism	Lysolipid metabolism	LysoPC(18:0)	$C_{26}H_{54}NO_7P$	15.14	0.002
		LysoPC(20:1)	$C_{28}H_{56}NO_7P$	4.03	0.001
	Sphingolipid metabolism	Sphinganine	$C_{18}H_{39}NO_2$	7.73	0.001
		Phytosphingosine	$C_{18}H_{39}NO_3$	7.24	0.008
		Ceramide(d18:0/14:0)	$C_{32}H_{65}NO_3$	4.7	<0.001
		Ceramide(d18:0/16:0)	$C_{34}H_{69}NO_3$	4.03	0.001
	Fatty acid beta-oxidation	Carnitine	$C_7H_{15}NO_3$	5.3	<0.001
		Palmitoylcarnitine	$C_{23}H_{45}NO_4$	4.96	<0.001
		Octadecenylcarnitine	$C_{25}H_{47}NO_4$	3.72	0.002
Amino acid metabolism	Methionine metabolism / betaine metabolism /glycine and serine metabolism	Methionine	$C_5H_{11}NO_2S$	5.67	0.001
	Arginine and proline metabolism	Proline	$C_5H_9NO_2$	4.9	0.001
	Valine, leucine and isoleucine degradation / propanoate metabolism	Valine	$C_5H_{11}NO_2$	4.7	0.006
	Phenylalanine and tyrosine metabolism / catecholamine biosynthesis	Tyrosine	$C_9H_{11}NO_3$	4.41	0.005
	Tryptophan metabolism	Indoleacetaldehyde	$C_{10}H_9NO$	3.21	0.001
	Glutathione metabolism	Pyroglutamic acid	$C_5H_7NO_3$	5.09	0.003

	Arginine and proline metabolism / glycine, serine and threonine metabolism	Creatine	$C_7H_{15}NO_3$	4.23	0.005
Nucleotide metabolism	Purine metabolism	Uric acid	$C_5H_4N_4O_3$	5.99	0.001
	Cytosine metabolism	Cytosine	$C_4H_5N_3O$	4.31	0.004