

Metabolomic study in plasma, liver and kidney of mice exposed to inorganic arsenic based on mass spectrometry

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Abstract The mechanism of arsenic toxicity still remains unclear, although enzymatic inhibition, impaired antioxidants metabolism and oxidative stress may play a role. The toxicological effects of trivalent inorganic arsenic on laboratory mouse *Mus musculus* after oral administration (3 mg/kg body weight/day) were investigated along 12 days, using a metabolomic approach based on direct infusion mass spectrometry to polar and lipophilic extracts from different organs and fluids (liver, kidney, and plasma). Positive and negative acquisition modes (ESI⁺/ESI⁻) were used throughout the experiments. The most significant endogenous metabolites affected by exposure were traced by partial least square-discriminant analysis and confirmed by tandem mass spectrometry (MS/MS) and gas chromatography coupled to MS. In this work, the toxic effect of arsenic has been related with important metabolic pathways, such as energy metabolism (e.g., glycolysis, Krebs cycle), amino acids metabolism,

choline metabolism, methionine cycle, and degradation of membrane phospholipids (cell apoptosis). In addition, this work illustrates the high reliability of mass spectrometry based on a metabolomic approach to study the biochemical effects induced by metal exposure.

Keywords Arsenic · ICP-MS · Metabolomics · Metals · *Mus musculus* · Mass spectrometry

Introduction

Arsenic (As) is one of the most widely studied elements in relation to mammal toxicity. This element is a widespread pollutant in the environment found in water, soil, and air from natural and anthropogenic sources, occurring in both inorganic and organic chemical forms, which strongly differ in terms of toxicity, accumulation, and involvement of arsenic respect to metabolism of living organisms [1, 2]. Inorganic forms of arsenic (iAs) are highly toxic [arsenite (iAs^{III}) and arsenate(iAs^V)] (LD₅₀ for mice are 4.5 mg kg⁻¹ per body weight and per day (bw/d) and 14–18 mg kg⁻¹ for the arsenite and arsenate by subcutaneous injection, respectively), producing a wide range of adverse health effects, but this toxicity decreases in the methylated forms [methylarsonic acid (MA^V) and dimethylarsinic acid (DMA^V)] (LD₅₀ for mice are 1,800 and 1,200 mg kg⁻¹ for the MA^V and DMA^V by subcutaneous injection, respectively), which are considered moderately toxic and can be excreted in urine after iAs ingestion; however, other organic species such as arsenobetaine (AB) (LD₅₀ for mice is 10,000 mg kg⁻¹ by subcutaneous injection), arsenocholine (AsC), and arsenosugars are regarded as innocuous [3–5]. In recent decades, the biomethylation of iAs has been considered as a detoxification mechanism, but methylated trivalent arsenicals, such as methylarsonite (MA^{III}) and dimethylarsinite (DMA^{III}), are toxic intermediates that arise

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during biomethylation process of arsenic in mammals and have been recently identified as cancer promoters [6–8]. Otherwise, pentavalent arsenicals are significantly less toxic than their equivalent trivalent forms [9, 10]. On the other hand, it has been recently demonstrated that arsenic species have high affinity for thiol-containing residues of proteins [11], especially the trivalent form due to its higher reactivity, as a consequence increase the number of proteins involved, such as tubulin and actin [12], hemoglobin [13, 14], metallothionein [15], galectin-I, thioredoxin peroxidase II [16], and others. This fact results in alterations of their biological functions (i.e., enzyme inhibition and metabolic pathways) that markedly contribute to arsenic carcinogenesis [17, 18]. Additionally, it is well known that iAs^{III} can perturb the metabolism of selenium at environmentally relevant doses [19], which could also be related with its mechanism of arsenic carcinogenesis [20].

In some areas, drinking water containing high levels of inorganic arsenic and industrial pollution are major sources of exposure to iAs for population through oral ingestion. This is an important problem for more than 200 million people in many countries chronically exposed to iAs through contaminated drinking water. Once ingested the soluble forms of As are readily absorbed from the gastrointestinal tract to bloodstream and distributed to organs. iAs^V can be metabolized by consecutive reduction to iAs^{III} and oxidative biomethylation that convert the element to methylarsenic (MA^V) and dimethylarsenic (DMA^V), which contain arsenic in a pentavalent oxidation state [21]. Human arsenic exposure is related to several health problems such as liver damage and cardiovascular disease [22] and has been demonstrated the capacity of iAs^{III} and MA^V to cross the blood–brain barrier [23, 24]. Moreover, a number of epidemiological studies have shown the association between the presence of arsenic in drinking water and the risk of cancer (skin, lung, bladder, liver, and kidney) [25–28], although the mechanisms involved in these carcinogenic processes and toxic effects caused by inorganic arsenic remain still unclear.

The metabolome is the set of low molecular mass compounds (typically <1,000 Da) present in biological systems [29]. Metabolomics is the analysis of the metabolome under certain endogenous (physiological) or exogenous (exposure) conditions. Metabolomics refers to the measurement of the entirety of metabolites in a cell [30], whereas metabolomics describes “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [31, 32]. Two disciplines have been highlighted by Nicholson as a necessary complement to metabolomics: analytical chemistry and chemometrics [32]. Metabolomic approaches have been developed in many areas of biomedical research including toxicology [33] because they allow for the detection of metabolic response to chemical exposure with the purpose of finding

biomarkers about the actual physiological status of living organisms. For this reason, metabolomics plays a key role to understand the results of complex biological processes, such as metal exposure experiments [34]. The main analytical techniques that are employed for metabolomic studies are based on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). MS is also a major technique for molecular identification of unknown metabolites, especially through the use of tandem MS/MS experiments for fragment ion studies using direct infusion [35–38]. The metabolic fingerprint of biofluid or tissues extracts obtained by MS or NMR-based methods contain few hundreds to thousands signals related to both genetic and environmental contributions. MS approaches used in metabolomics normally requires a previous separation of metabolic components using either gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) to avoid traditional isobaric interferences affecting direct infusion. However, a number of studies have proposed the use of direct infusion to the mass spectrometer (DIMS) without any previous separation using high-resolution mass analyzers, such as hybrid systems triple quadrupole-time-of-flight (QqQ-TOF) [34, 38, 39] or Orbitrap [40]. In this sense, DIMS exhibits several advantages in metabolomics such as high-throughput screening capability, fast analysis, and wider nontarget metabolite coverage than chromatographic and electrophoretic couplings, since there is not exclusion of compounds associated to the separation step. For these reasons, DIMS is recommended to be a fast diagnostic method for evaluating the toxicological effects of metals, while LC-MS is necessary when comprehensive screening of biomarkers is required [41]. Considering that a much higher throughput can be obtained without a chromatographic step, DIMS could be developed as a fast metabolomic approach to evaluate the possible alterations in metabolic cycles caused by toxic metals [41]. On the other hand, ability of GC-MS to measure key primary metabolites, such as aminoacids, organic acids, or carbohydrates, involved in essential pathways in the metabolism of organisms (glycolysis, Krebs cycle) makes it an interesting alternative in toxicology and, in particular, in the discovery of potential biomarkers.

A crucial step in metabolomics is to get a suitable sample extraction to recover as many metabolites as possible present in the biological fluid or tissue. Most of strategies consider the use of multiplexed extractions under certain conditions for selective recovery of different families or classes of metabolites, such as lipids [42], or water-soluble metabolites [43], related to certain potential biomarkers in the mass range of typically between 50 and 1,000 m/z . On the other hand, complete extraction of metabolites is one current weak point in metabolomic studies, being necessary the development of extraction procedures for both polar and nonpolar metabolites from matrix to get a nontargeted multi-metabolite analysis to assess about alterations related to the effects of arsenic in mice.

The aim of the present study is to determine the biochemical alterations in the metabolic pathways of mice subjected to acute exposure to arsenic during 12 days by oral administration using mass spectrometry complemented by PLS-DA. For this purpose, DIMS has been applied, in both positive and negative ionization modes, to liver, kidney, and plasma of mice (*Mus musculus*) to obtain metabolic information. Subsequently, metabolites were identified using MS/MS experiments and a metabolomics database. Additionally, a complementary metabolomic approach based on GC-MS was applied to quantify and validate the metabolic pathways altered by DIMS in plasma from mice affected by acute doses of inorganic arsenic during 12 days of exposure.

Experimental

Standard solutions and reagents

As₂O₃ (99.995 %, trace metals basis) used to prepare acute doses for oral administration was supplied by Sigma-Aldrich (Steinheim, Germany). All solvents used for sample preparation were of optima grade for mass spectrometry. Methanol and chloroform were purchased from Fisher Chemical (Geel, Belgium), while ethanol, pyridine, and formic acid were supplied by Sigma-Aldrich. Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Derivatizing agents, methoxylamine hydrochloride, and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 1 % (*w/v*) trimethylchlorosilane (TMCS), were obtained from Sigma-Aldrich. Alanine, valine, isoleucine, proline, glycine, serine, threonine, glutamic acid, phenylalanine, fructose, galactose, glucose, tyrosine, tryptophan, urea, aspartic acid, glutamine, cholesterol, α -ketoglutarate, isocitric acid, citric acid, lactic acid, and uric acid, purchased from Sigma-Aldrich, were used as standard substances in gas chromatographic quantification.

Apparatus

A cryogenic homogenizer SPEX SamplePrep (Freezer/Mills 6770) was used to prepare tissue homogenates. The extraction was carried out with a pellet mixer (VWR, UK). Metabolomic experiments were performed by direct infusion in a high resolution mass spectrometer QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using the electrospray ionization (ESI) source. Gas chromatography analysis were performed in a Trace GC ULTRA gas chromatograph coupled to an ion trap mass spectrometer detector ITQ900, both from Thermo Fisher Scientific (Bremen, Germany), using a Factor Four capillary column VF-5MS 30 m \times 0.25 mm ID, with 0.25 μ m of film thickness (Varian).

Animals and exposure protocol

M. musculus (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Thirty-two mice of 7 weeks of age were fed ad libitum with conventional pellets containing an arsenic concentration $<0.35 \mu\text{g g}^{-1}$. The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature in the range of 25–30 °C and a 12-h light–dark cycle) prior to start exposure experiments. *M. musculus* mice were divided into two groups, one used as control and the other subjected to As(III) (in the form As₂O₃) exposure, using an oral administration of 100 μ L of a solution containing 3 mg of As per kilogram of body weight per day during a total period of 12 days. The control mice were subjected to oral administration of 100 μ L of ultrapure water with 0.9 % (*w/v*) NaCl per day for 12 days. The acute oral arsenite dose of 3 mg kg⁻¹ bw/day was selected taking into account the LD₅₀ for mice (42 mg kg⁻¹), which was not exceed over experiment the overall exposure experiment (12 days).

Eight mice of each group were killed on the sixth day from the beginning of the experience and again on the last day of the exposure experiment to assess the effect of arsenic and the diet. Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture, dissected using a ceramic scalpel, and finally, the organs were transferred rapidly to dry ice. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9 % (*w/v*) NaCl solution in individual Petri dish, drained on a clean absorbent paper, frozen in liquid nitrogen, and stored at –80 °C until they were used for extracts preparation. Plasma collection was carried out by centrifugation (4,000 \times g, 30 min, 4 °C) after addition of heparin (ANTICLOT) as anticoagulant. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

Analytical procedures

Sample treatment for DI-ESI(\pm)-QTOF-MS analysis First of all, individual organs were disrupted by cryogenic homogenization. Sample preparation of individual tissues for metabolomic analysis based on DI-ESI(\pm)-QTOF-MS was carried out in two-step. Firstly, polar metabolites were extracted with a mixture of (1:1, *v/v*) methanol/water by adding 500 μ L to 50 mg of tissue in an Eppendorf tube followed by vigorous vortex shaking during 5 min. Then, the cells were disrupted using a pellet mixer (2 min) under low temperature, followed by centrifugation at 4,000 \times g for 10 min at 4 °C. The supernatant was carefully collected and transferred to another Eppendorf. The pellet was homogenized again, using a pellet mixer for 2 min with 100 μ L of (1:1, *v/v*) methanol/water mixture, then it was centrifuged at the same conditions

described above and the pellet was kept for further treatment. After that, both supernatants were combined (600 μL), and the resulting polar extract was taken to dryness under nitrogen stream and stored to $-80\text{ }^\circ\text{C}$ until analysis. The pellet was extracted with 500 μL of a mixture (2:1, v/v) chloroform/methanol, using a pellet mixer (2 min), to extract lipophilic metabolites and centrifuged at the same conditions described above. Finally, the resulting supernatant was taken to dryness under nitrogen stream and stored to $-80\text{ }^\circ\text{C}$ until analysis.

For DI-ESI(\pm)-QTOF-MS of plasma samples, proteins were removed from blood plasma by adding 400 μL of methanol/ethanol mixture (4:1, v/v) to 100 μL of plasma in an Eppendorf tube followed by vigorous vortex shaking for 5 min at room temperature and centrifugation at $4,000\times g$ for 10 min at $4\text{ }^\circ\text{C}$. The supernatant was carefully collected avoiding contamination with the precipitated proteins, transferred to another Eppendorf tube, and the resulting supernatant was taken to dryness under nitrogen stream for storage at to $-80\text{ }^\circ\text{C}$ until analysis. The pellet was homogenized again, with 200 μL of a mixture of (2:1, v/v) chloroform/methanol, using a pellet mixer (2 min), to extract lipophilic metabolites and centrifuged ($10,000\times g$ at $4\text{ }^\circ\text{C}$ for 10 min). Finally, the resulting supernatant was taken to dryness under nitrogen stream and stored to $-80\text{ }^\circ\text{C}$ until analysis.

The polar extracts were reconstituted to 200 μL of a mixture of methanol/water (4:1, v/v) and lipophilic extracts were reconstituted to 200 μL of (1:1) chloroform/water mixture before the analysis by ESI-MS. For data acquisitions from positive ionization, 0.1 % (v/v) formic acid was added to polar extract and 30 mM of ammonium acetate to lipophilic extract. In the case of negative ionization, intact extracts were directly infused to the mass spectrometer.

Sample treatment for GC-MS analysis Sample preparation before GC-MS analysis should be performed carefully to ensure the reliability of metabolomic analysis. Plasma was thawed at $4\text{ }^\circ\text{C}$ and vortex-mixed before use. For the extraction of metabolites from plasma, 100 μL of plasma samples was mixed with 400 μL of 1:1 methanol/ethanol mixture in an Eppendorf tube and vortexed for 5 min at room temperature, followed by centrifugation at $4,000\times g$ for 10 min at $4\text{ }^\circ\text{C}$. The supernatant was transferred to another Eppendorf tube and dried under nitrogen stream. Plasma metabolites are not volatile and a previous derivatization step is mandatory. To this end, they were derivatized following a previously published procedure [44]. Briefly, all dried samples were derivatized with 50 μL of methoxylamine hydrochloride solution (20 mg mL^{-1} in pyridine) at $70\text{ }^\circ\text{C}$ for 40 min for protection of carbonyl groups by methoximation, followed by treatment with 50 μL MSTFA with 1 % (w/v) of TMCS at $50\text{ }^\circ\text{C}$ for 40 min for derivatization of primary amines and primary and secondary hydroxy groups in the case of MTSFA. In addition, TMCS aids in the derivatization of amides and secondary

amines and hindered hydroxy groups, as a previously published elsewhere with some modifications [44]. Finally, the derivatized samples were vortex-mixed for 2 min before GC analysis, centrifuged at $4,000\times g$ for 5 min and supernatant collected for analysis.

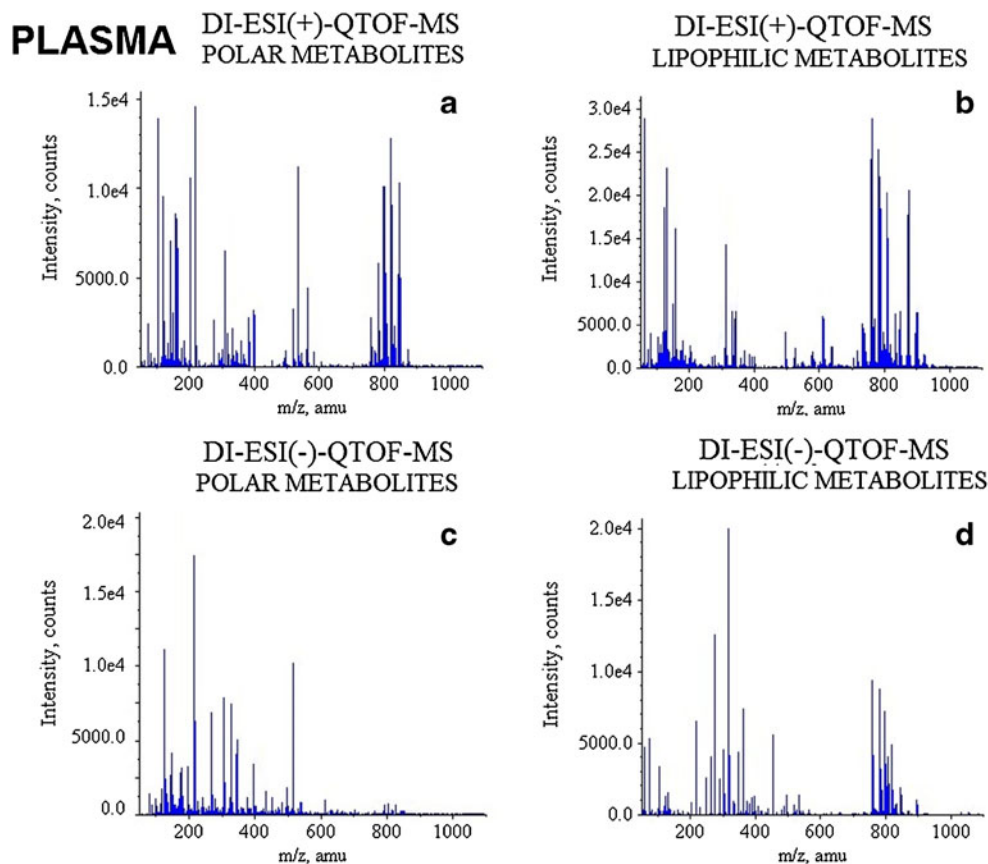
Analysis of samples by DI-ESI-QTOF-MS Metabolomic experiments of liver, kidney, and plasma extracts from mice exposed to arsenic were performed by DIMS using an ESI. The parameters for hybrid analyzer QqQ-TOF system were optimized to obtain the higher sensitivity with minimal fragmentation of molecular ions, both in positive and negative ion modes. To acquire MS/MS spectra, nitrogen was used as collision gas (Table 1).

The extraction of metabolites from organs and plasma were carried out as described in the sample treatment section. Both extracts were analyzed in positive (ESI $^+$) and negative (ESI $^-$) ion modes resulting different profiles in a wide spectral range (m/z 50–1,100). Figure 1 shows the typical spectrum of mice plasma from control group (exposed to 0.9 % NaCl), after 6 days from the beginning of the experience, obtained by DI-ESI(\pm)-QTOF-MS. When these extracts were analyzed in positive ion mode (ESI $^+$), metabolic profiles exhibited peaks in a wide range, including signals in the m/z interval 50–1,100. Both extracts show similar mass profiles, although several important differences can be remarked. In polar extracts, a large number of signals can be observed, with different intensities in the m/z range 50–400; in addition, several clusters of peaks at higher m/z values can also be remarked, Fig. 1a, probably associated to structurally related compounds, such as lyso-phosphatidylcholines (LPCs, m/z 400–600) and phosphatidylcholines (PCs, m/z 750–900). In lipophilic extracts (Fig 1b), peak clusters at m/z above 500 can also be observed, being remarkable that the number of peaks in these clusters and their intensities increase. Alternatively, the analysis of

Table 1 Operating conditions for DI-ESI-QqQ-TOF-MS

DI-ESI(\pm)-QTOF-MS conditions		
Flow	5 $\mu\text{L min}^{-1}$	
Acquisition time	0.2 min (1.005 s per scan)	
m/z range	50–1,100	
Curtain gas (N_2)	1.13 L min^{-1}	
Nebulizer gas (N_2)	1.56 L min^{-1}	
Source temperature	60 $^\circ\text{C}$	
	Acquisition mode	
	Positive	Negative
Ion spray voltage (IS)	3500 V	–4,000 V
Declustering potential (DP)	60 V	–100 V
Focusing potential (FP)	250 V	–250 V
Ion energy (IE)	2.0 V	–2.0 V
Channel electron multiplier (CEM)	2,400 V	2,400 V

Fig. 1 Mass spectra from plasma extracts of control mice. **(a)** ESI⁺ spectrum of polar metabolites. **(b)** ESI⁺ spectrum of lipophilic metabolites. **(c)** ESI⁻ spectrum of polar metabolites. **(d)** ESI⁻ spectrum of lipophilic metabolites



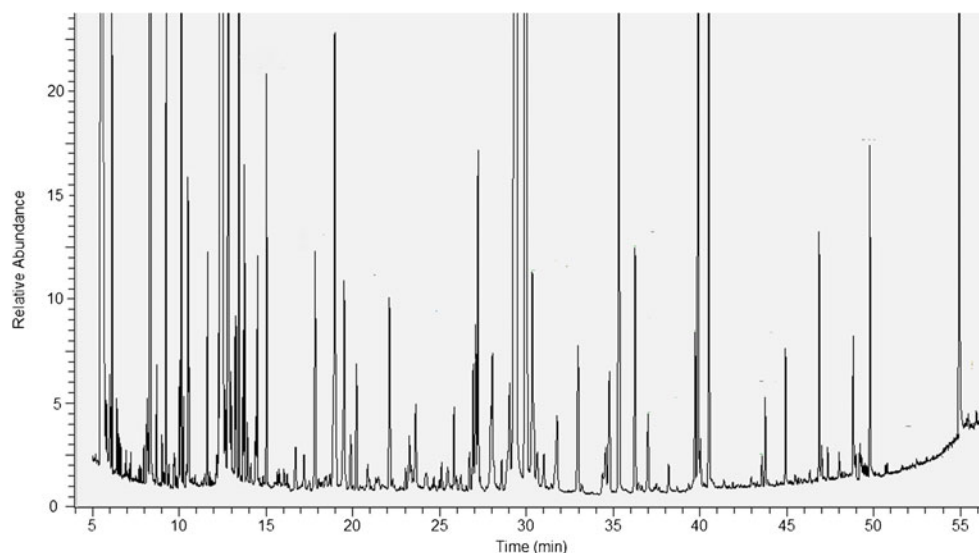
these extracts in negative mode (ESI⁻) gave completely different profiles (Fig 1c, d), which only exhibit signals with high sensitivity at m/z values under 500 in both extracts. On the other hand, high molecular mass metabolites (m/z 500–1,000) show a very low intensity, but the same manner as in ESI⁺, their intensity increase in the second extraction step. These results confirm the complementarity of the extraction procedure and molecule ionization mode to get a high diversity of signals.

Analysis of samples by GC-MS Chromatography was performed on a Factor Four capillary column VF-5MS 30 m × 0.25 mm ID, with 0.25 μm of film thickness (Varian). The injector temperature was kept at 280 °C. Helium carrier gas was used at a constant flow rate of 1 mL/min. To acquire a good separation, the column temperature was initially maintained at 60 °C for 5 min, and then increased from 60 to 140 °C at a rate of 7 °C/min for 4 min. Then, the column temperature was increased to 180 °C at 5 °C/min for another 6 min. Finally, the temperature was increased to 280 °C at 5 °C/min, and held for 2 min. For mass spectrometry detection, ionization was carried out by electronic impact (EI) with a voltage of 70 eV, using full scan mode in the m/z range of 35–650, with an ion source temperature of 200 °C. Typical GC-MS total ion current chromatograms from control mice

plasma (exposed to 0.9 % NaCl), after 6 days from the beginning of the experience are shown in Fig. 2. For the analysis, 1 μL of sample was injected in splitless mode. The identification of endogenous metabolites was based on comparison with the corresponding standards according to their retention times and mass spectra characteristics, complementarily, searching on NIST Mass Spectral Library (NIST 02) was used.

Data analysis Markerview™ software (Applied Biosystems) was employed to filter the mass spectrometric results. Statistical data analysis (Partial least squares discriminant analysis (PLS-DA) were performed by means of statistical software packages SIMCA-P™ (version 11.5, published by UMetrics AB, Umeå, Sweden). PLS-DA is a partial least squares regression of a set Y of binary variables describing the categories of a categorical variable on a set X of predictor variables. It is a compromise between the usual discriminant analysis and a discriminant analysis on the significant principal components of the predictor variables [45]. The data were processed in order to find differences between mice groups submitted to different exposure time and to trace the metabolites altered by arsenic for later identification by their molecular mass and fragments in MS/MS experiments. In addition, altered metabolites were characterized using different

Fig. 2 Typical chromatographic profile obtained from control mice plasma



databases of metabolomics based on DI-ESI-QqQ-TOF/MS, such as Human Metabolome Database (<http://www.hmdb.ca>), METLIN (<http://metlin.scripps.edu>), and Mass Bank (<http://www.massbank.jp>). In GC-MS analysis, identification of metabolites was performed using the NIST Mass Spectral Library (NIST 02).

Results

Metabolomic study of liver from *Mus musculus* mice under arsenic exposure by DI-ESI(±)-QTOF-MS

In order to discriminate between exposure groups, a PLS-DA was performed employing the intensities of the m/z signals in polar and lipophilic liver extracts, using positive and negative modes (Fig. 4). The models allow a good classification of the samples into two groups, which are showed by the respective scores plots (Fig. 3). To identify which variables were responsible for this separation, the variable influence on the projection (VIP) parameter was used. VIP is a weighted sum of squares of the PLS-DA weight, which indicates the importance of the variable to the whole model. VIP coefficients reflect the relative importance of each X variable for each X variate in the prediction model. VIP coefficients thus represent the importance of each X variable in fitting both the X - and Y -variates, since the Y -variates are predicted from the X -variates. VIP allows to classify the X -variables according to their explanatory power of Y . Predictors with large VIP, larger than 1, are the most relevant for explaining Y . Thus, it is possible to select variables with the most significant contribution in discriminating between metabolomic profiles corresponding to groups of exposure against controls. Only metabolites with $VIP > 2$ have been considered good biomarkers. Such as strict

criterion was set because of the large number of variables or metabolites in the mass spectra (Fig. 1). The t test was performed in succession, and the variables without significant differences between the exposed mice and the controls ($p > 0.01$) were eliminated. Goodness of the model was measured through the $R^2 Y$ and Q^2 values, provided by the software (indicative of class separation and predictive power of the model, respectively). These parameters are ranged between 0 and 1 and indicate the variance explained by the model for all the data analyzed (R^2), and this variance in a test set by cross-validation (Q^2). In particular, for all the analysis performed, the values of $R^2 Y$ (cum) and Q^2 (cum) of the combined model are 0.90–0.99 and 0.8–0.95, respectively, indicating that a combination of datasets between groups provides the best classification and prediction. Mostly altered metabolites have been obtained in polar extracts from liver tissue (Table 2), but a number of metabolites were found in lipophilic extracts, which demonstrates the complementarity of using both extracts. Application of both ionization modes is also an important and critical point.

Metabolomic study of kidney from *Mus musculus* mice under arsenic exposure by DI-ESI(±)-QTOF-MS

The application of the metabolomic approach to kidney tissue, using MS signals in a wide m/z range (m/z 50–1100), allows discrimination between control and exposed mice (12th day) using polar metabolites with positive and negative acquisition modes (Fig. 4). The involvement of kidney in excretion and detoxification of water soluble metabolites and its relationship to blood plasma composition provides to these results an especial interest in health and biomedical fields. As it can be seen in Table 3, an important number of polar metabolites are altered under inorganic arsenic exposure.

LIVER

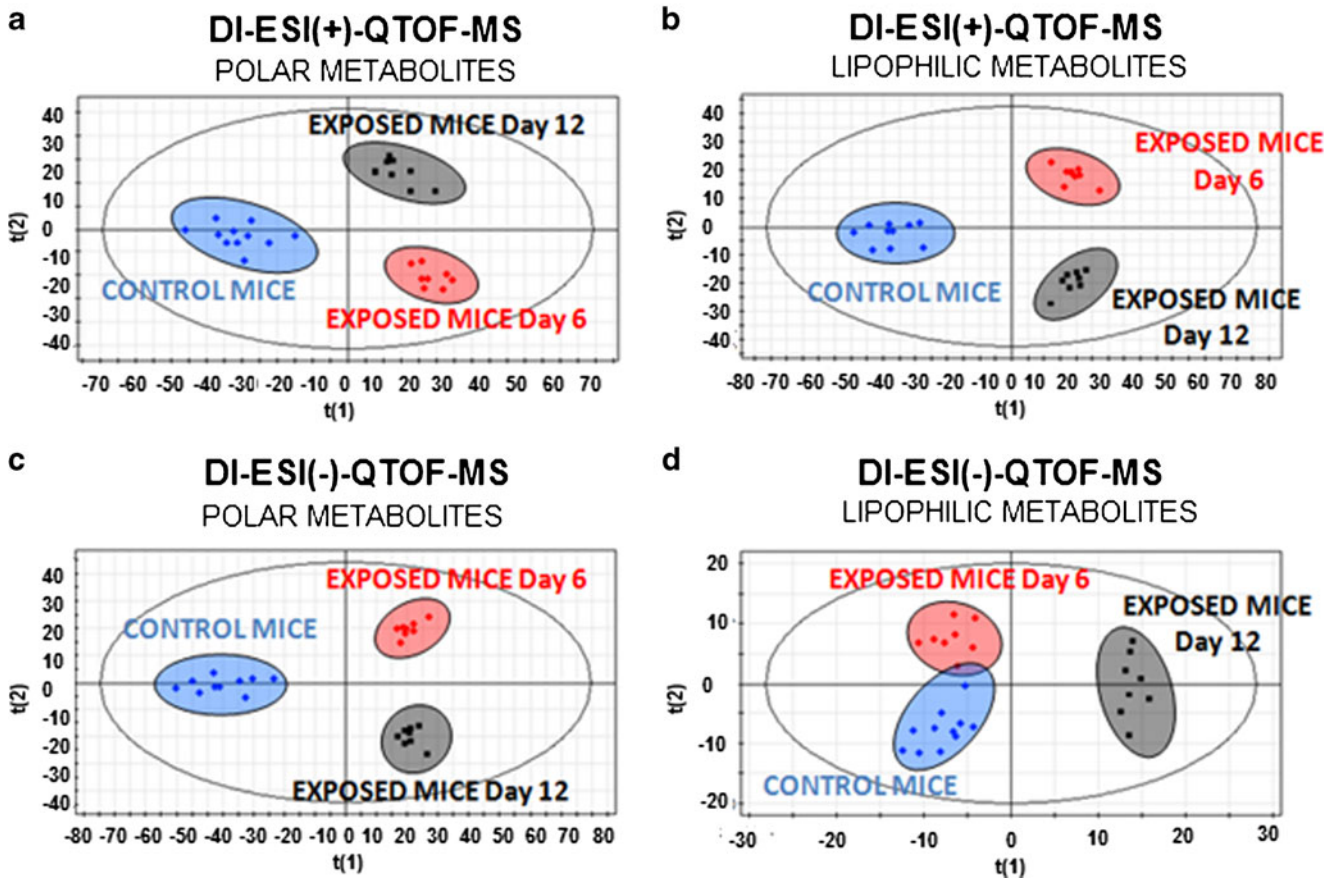


Fig 3 Scores plots of PLS-DA for $ES1^+$ and $ES1^-$ ionization modes of polar and lipophilic mice liver extracts exposed to inorganic arsenic during 12 days

Table 2 Altered metabolites from liver of mice (*Mus musculus*) exposed to arsenic (As_2O_3) during 12 days of acute exposure

Altered metabolites	<i>m/z</i>	Extract	Altered metabolites	<i>m/z</i>	Extract
Taurine ↑	124.01 (H^-)	Polar	MA^V ↑	141.05 (H^+)	Polar
Methionine ↑	150.05 (H^+)	Polar	DMA^V ↑	139.04 (H^+)	Polar
Arginine ↓	175.09 (H^+)	Polar	Glutamic acid ↑	147.05 (H^+)	Polar
Betaine ↑	118.08 (H^+)	Polar	Carnitine ↓	162.10 (H^+)	Polar and lipophilic
Cysteine ↑	122.02 (H^+)	Polar	Choline ↑	104.09 (H^+)	Polar and lipophilic
Homocysteine ↓	136.03 (H^+)	Polar	Phosphatidylcholines (PC) ↓	700-850	Polar and Lipophilic
Tryptophan ↓	205.09 (H^+)	Polar	Pyruvate ↓	89.02 (H^+)	Lipophilic
Glyceraldehyde-3-phosphate ↓	168.99 (H^-)	Polar	Pipecolic acid ↑	130.08 (H^+)	Lipophilic
Phosphorylcholine ↑	185.07 (H^+)	Polar	Arachidonic acid ↑	303.24 (H^-)	Lipophilic
Citric acid ↑	193.03 (H^+)	Polar	Oleic acid ↑	281.25 (H^-)	Lipophilic
Glutathione ↓	308.08 (H^+)	Polar	Linoleic acid ↑	279.25 (H^-)	Lipophilic
Glucose ↓	203.05 (Na^+)	Polar	Lyso-phosphatidylcholines (Lyso-PC) ↑	450-600	Polar and lipophilic
Xanthine ↑	153.03 (H^+)	Polar	Diglycerides ↑	600-700	Lipophilic
Hypoxanthine ↑	137.04 (H^+)	Polar	Triglycerides ↑	850-950	Lipophilic

Variations compared to control mice: ↑ increasing signal intensity, ↓ decreasing signal intensity

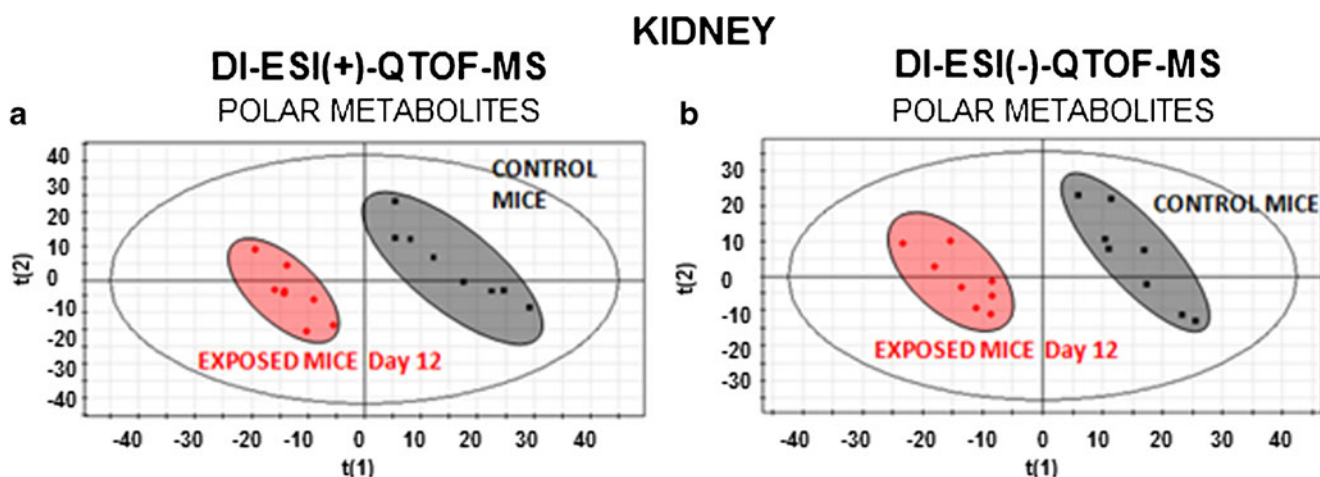


Fig 4 Scores plots of PLS-DA for ESI^+ of polar and lipophilic metabolites from mice kidney tissue exposed to inorganic arsenic during 12 days

Metabolomic study of plasma from *Mus musculus* mice under arsenic exposure by DI-ESI(\pm)-QTOF-MS

The application of PLS-DA to mass spectra signals from polar and lipophilic metabolites of plasma samples establish a slight separation between exposed and control mice for a wide range of peaks (m/z 50–1,100) using positive and negative acquisition mode (Fig. 5). In this case, the values of R^2Y (cum) and Q^2 (cum) of the combined model are 0.90–0.99 and 0.80–0.90, respectively. Several polar metabolites are altered in plasma under inorganic arsenic exposure (see Table 4).

Quantification of altered metabolites in plasma from *Mus musculus* mice under arsenic exposure by GC-MS

GC-MS was applied to confirm and quantify some of the altered metabolites traced by DI-ESI(\pm)-QTOF-MS. Additionally, using GC-MS, it is possible to analyze others metabolites nonionized using ESI ionization source. For this purpose, three derivatizing reagents were used for plasma samples analysis in order to get as much metabolic information as possible. Plasma metabolic profiles of five mice plasma of each group of exposure were obtained with the method

previously described in experimental section. The concentration of mice plasma metabolites are shown in Table 5.

Discussion

Perturbations in energy metabolism

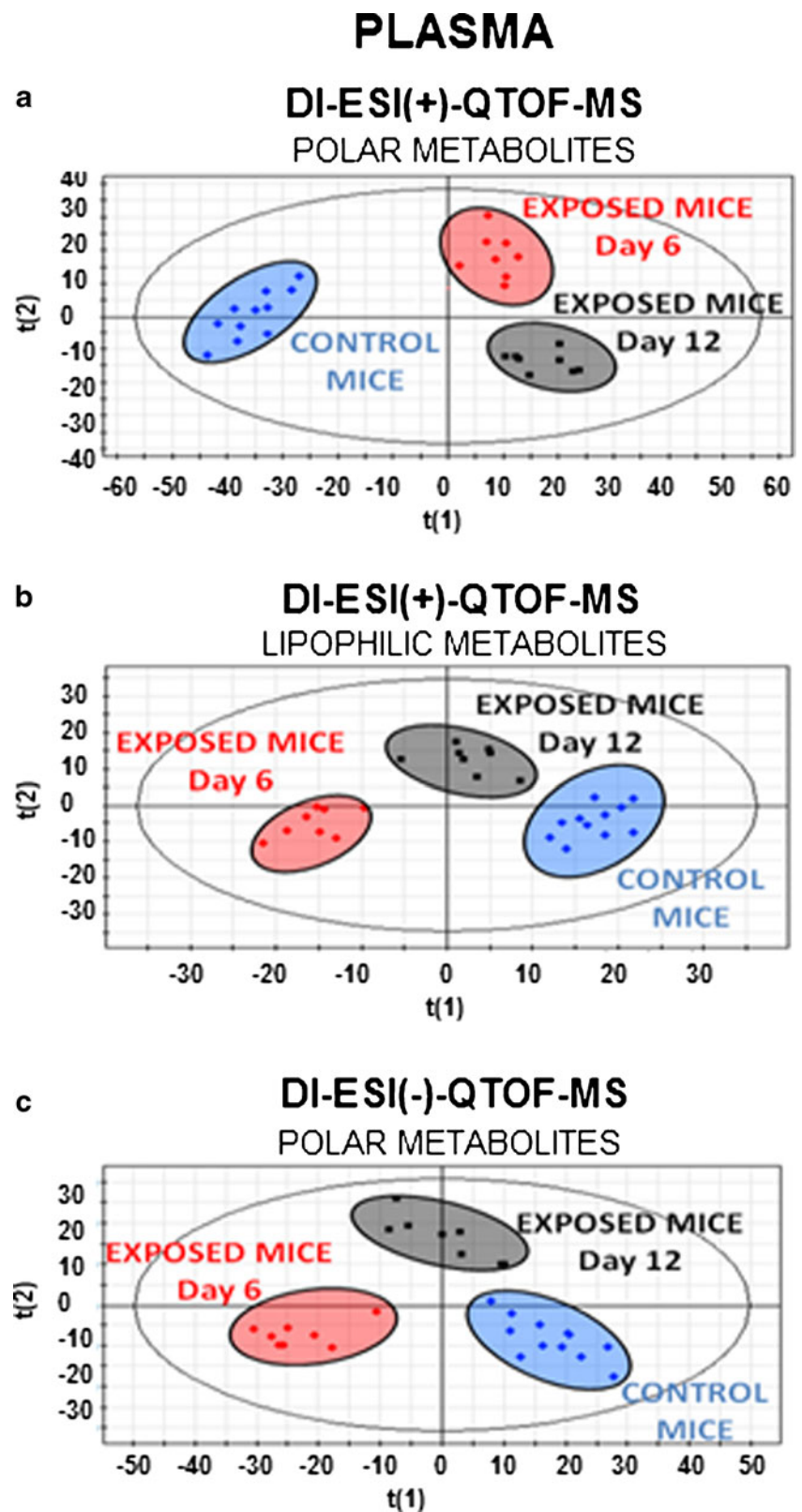
Intermediate metabolites in energy metabolism such as glucose, glyceraldehyde-3-phosphate, pyruvate, and citric acid are altered under inorganic arsenic exposure. The levels of these compounds decrease in liver and plasma according to results obtained by DI-ESI(\pm)-QTOF-MS (Tables 2 and 4). These results are confirmed by GC-MS analysis of plasma (Table 5), which shows decreased concentrations of lactic acid and alanine and increased concentrations of isocitric acid, α -ketoglutarate, glutamic acid, and citric acid. This fact can be related to perturbations in carbohydrate metabolism (Fig. 6A), and is consistent with the depletion of glucose, reported by Szinicz and Forth [46], as toxic effect of arsenic in rats. Other authors [47, 48] have also reported significant depletion of glucose in blood and liver of guinea pigs after arsenic exposure. The decreased level of pyruvate in liver, as end product of glycolysis cycle, supports this idea (Table 2), which is also borne by the formation of acetyl-CoA via pyruvate

Table 3 Altered metabolites from kidney of mice (*Mus musculus*) exposed to arsenic (As_2O_3) during 12 days of acute exposure

Altered metabolites	m/z	Extract	Altered metabolites	m/z	Extract
Taurine \uparrow	124.01 (H^+)	Polar	Phosphorylcholine \uparrow	185.07 (H^+)	Polar
Arginine \downarrow	175.11 (H^+)	Polar	Citric acid \uparrow	193.03 (H^+)	Polar
Methionine \uparrow	150.05 (H^+)	Polar	DMA $^{\vee}$ \uparrow	139.04 (H^+)	Polar
Choline \uparrow	104.09 (H^+)	Polar	Betaine aldehyde \uparrow	102.09 (H^+)	Polar
Phosphatidylcholines \downarrow	700–850	Polar	Uric acid \uparrow	169.05 (H^+)	Polar
Creatinine \uparrow	114.06 (H^+)	Polar			

Variations compared to control mice: \uparrow increasing signal intensity, \downarrow decreasing signal intensity

Fig 5 Scores plots of PLS-DA for ESI⁺ of polar and lipophilic metabolites from mice plasma exposed to inorganic arsenic during 12 days



metabolism (Fig. 6A). The main function of acetyl-CoA is to convey carbon atoms within the acetyl group to the citric acid cycle (Krebs cycle) to be oxidized for

energy production. As intermediate compound in the Krebs cycle, high levels of citric acid have been observed along arsenic exposure in liver, kidney, and

Table 4 Altered metabolites from plasma of mice (*Mus musculus*) exposed to arsenic (As₂O₃) during 12 days of acute exposure

Altered metabolites	<i>m/z</i>	Extract	Altered metabolites	<i>m/z</i>	Extract
Taurine ↑	124.01 (H ⁻)	Polar	Acetyl-carnitine ↑	204.11 (H ⁺)	Polar
Arginine ↓	175.11 (H ⁺)	Polar	Glutamic acid ↑	147.05 (H ⁺)	Polar
Methionine ↑	150.05 (H ⁺)	Polar	Creatine ↑	132.07 (H ⁺)	Polar
Choline ↑	104.09 (H ⁺)	Polar	Homocysteine ↓	136.03 (H ⁺)	Polar
Phosphorylcholine ↑	185.07 (H ⁺)	Polar	Phosphatidylcholines (PC) ↓	700–850	Polar and Lipophilic
MA ^V ↑	141.05 (H ⁺)	Polar	Lyso-phosphatidylcholines (Lyso-PC) ↑	450–600	Polar and Lipophilic
DMA ^V ↑	139.04 (H ⁺)	Polar	Diglycerides ↑	600–700	Lipophilic
Citric acid ↑	193.03 (H ⁺)	Polar	Triglycerides ↑	850–950	Lipophilic
Glutathione ↓	308.04 (H ⁺)	Polar	Pyruvate ↓	89.02 (H ⁺)	Lipophilic
Glucose ↓	203.05 (Na ⁺)	Polar			

Variations compared to control mice: ↑ increasing signal intensity, ↓ decreasing signal intensity

plasma (see Tables 2, 3, and 4, respectively). The increased concentration of citric acid, isocitric acid, α-ketoglutarate, and glutamic acid observed in plasma confirm this hypothesis (see Table 5).

On the other hand, an increased presence of lipids (free fatty acids, triacylglycerols, diacylglycerols, and LPCs) was observed in liver and plasma (see Tables 2 and 4, respectively) as a result of apoptosis induced by arsenic (Fig. 7B). This fact

Table 5 Quantification of mice plasma metabolites (*Mus musculus*) exposed to arsenic (As₂O₃) by GC-MS during 12 days of acute exposure

Plasma metabolites	PLASMA			
	Retention time (min)	Control mice (<i>n</i> =5) Mean±SD	As exposed mice day 6 (<i>n</i> =5) Mean±SD	As exposed mice day 12 (<i>n</i> =5) Mean±SD
Lactic acid (mmol L ⁻¹)	5.6	1.66±0.11	1.31±0.12	1.09±0.19
Alanine (mmol L ⁻¹)	6.0	0.326±0.021	0.303±0.024	0.254±0.032
Valine (mmol L ⁻¹)	11.6	0.216±0.042	0.198±0.021	0.222±0.032
Urea (mmol L ⁻¹)	12.5	360±12	344±21	377±29
Isoleucine (mmol L ⁻¹)	13.2	0.0681±0.009	0.0702±0.013	0.0669±0.015
Proline (mmol L ⁻¹)	13.3	0.142±0.005	0.151±0.007	0.138±0.012
Glycine (mmol L ⁻¹)	13.4	0.184±0.009	0.211±0.012	0.224±0.018
Serine (mmol L ⁻¹)	14.5	0.0982±0.003	0.0922±0.007	0.0102±0.01
Threonine (mmol L ⁻¹)	15.0	0.129±0.008	0.121±0.011	0.131±0.014
Aspartic acid (mmol L ⁻¹)	17.2	0.086±0.009	0.081±0.012	0.089±0.008
Glutamine (mmol L ⁻¹)	22.1	0.648±0.021	0.618±0.033	0.651±0.018
Glutamic acid (mmol L ⁻¹)	23.1	0.134±0.08	0.148±0.12	0.175±0.21
α-Ketoglutarate (mmol L ⁻¹)	23.5	0.144±0.007	0.172±0.012	0.204±0.021
Phenylalanine (mmol L ⁻¹)	25.5	0.0562±0.006	0.0548±0.004	0.0553±0.002
Isocitric acid (mmol L ⁻¹)	25.9	0.512±0.021	0.554±0.015	0.589±0.033
Citric acid (mmol L ⁻¹)	27.2	3.21±0.24	3.52±0.32	3.84±0.36
Fructose (mmol L ⁻¹)	27.9	0.935±0.033	0.902±0.024	0.942±0.045
Galactose (mmol L ⁻¹)	29.0	0.125±0.013	0.117±0.016	0.114±0.021
Glucose (mmol L ⁻¹)	29.9	8.24±0.47	7.85±0.68	6.85±0.89
Tyrosine (mmol L ⁻¹)	31.0	0.115±0.012	0.105±0.016	0.121±0.015
Uric acid (mmol L ⁻¹)	37.0	0.142±0.006	0.168±0.01	0.192±0.02
Tryptophan (mmol L ⁻¹)	38.1	0.094±0.011	0.087±0.009	0.101±0.01
Cholesterol (mmol L ⁻¹)	54.6	1.26±0.05	1.52±0.09	1.73±0.08

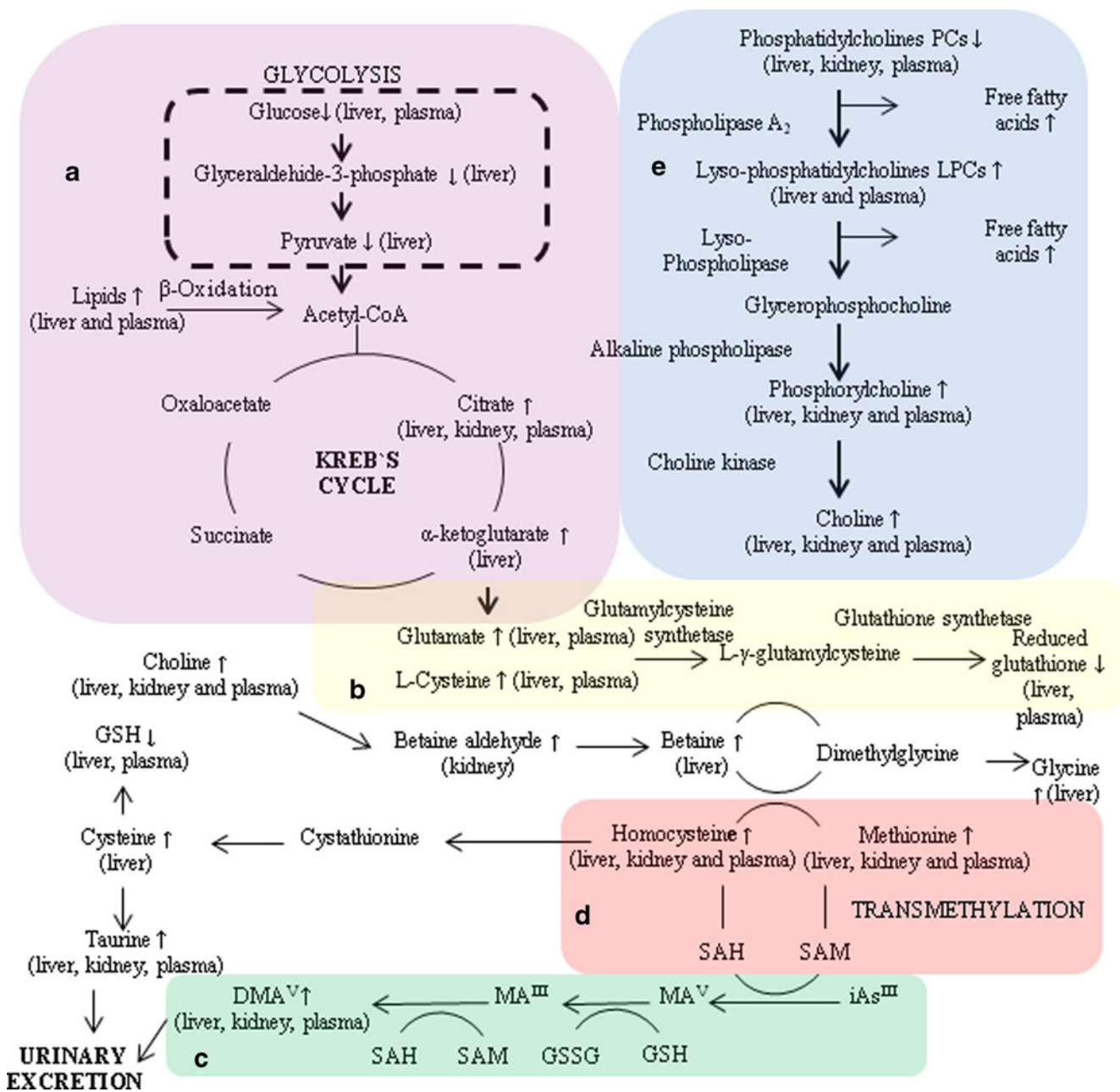


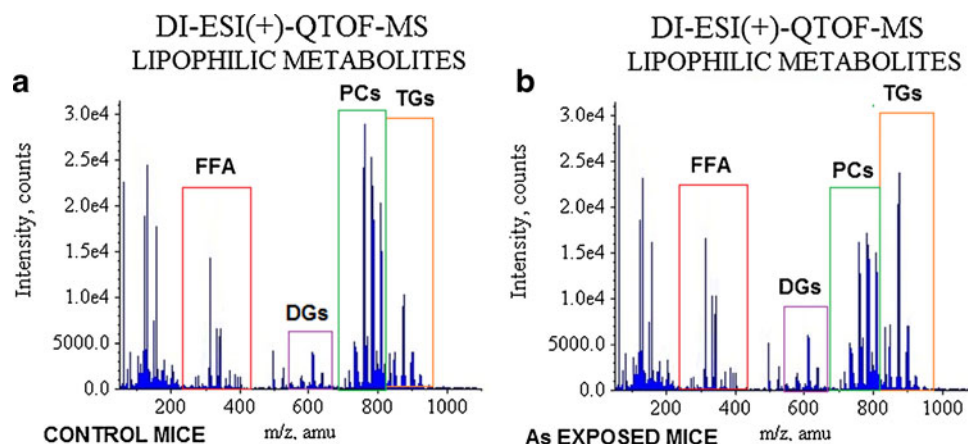
Fig 6 Schematic representation of the energy metabolic pathways (a), synthesis of reduced glutathione (b), biomethylation of inorganic arsenic (c), alterations in methionine cycle (transmethylation) (d), and breakdown of membrane phospholipids

has been observed in rat glioma and under effect of realgar undergoing apoptosis [33, 36]. In addition, increased levels of carnitine in liver (Table 2) as well as acetyl-carnitine in plasma (Table 4) are observed along the exposure time. Carnitine is required in living cells for the transport of fatty acids from the cytosol into the mitochondria during the breakdown of lipids for the generation of metabolic energy by β-oxidation. Therefore, this metabolite is also related to energy metabolism.

Perturbations in amino acid metabolism

Alterations of glucose and amino acid concentrations seem to be a common response to many toxins in several species and are likely indicative of a general response to xenobiotics exposure rather than specific biological response to a particular toxicant [49]. However, other metabolites altered under arsenic exposure were the aminoacids arginine, tryptophan, cysteine, glutamic acid, and methionine, some of them with

Fig 7 ESI⁺ mass spectra of pool of plasma lipophilic extracts (m/z 50–1,100) from (a) control mice and (b) exposed mice to inorganic arsenic during 12 days



antioxidant activity such as arginine [50] and tryptophan [51]. In the exposure experiment, decreased levels of arginine have been observed in liver, kidney, and plasma (see Table 2, 3, 4, and 5, respectively), and tryptophan in liver (see Table 2).

On the other hand, decreased levels of glutathione in liver and plasma and correlative increase in amino acids cysteine and glutamic acid (see Table 2, 4, and 5) can be observed. Glutathione can be biosynthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine (Fig. 6B), and in reduced form, GSH, is an antioxidant compound that can protect cell from free radicals, which may play an important role against arsenic carcinogenesis, due to their activity as electron donors in arsenic methylation (Fig. 6C) [52]. Therefore, glutathione level decreases as a consequence of oxidative stress caused by arsenic and correlatively increases the presence of L-cysteine and L-glutamic acid to contribute to glutathione production. In addition, it is well known that iAs^{III} inhibits the activity of GSSG reductase enzyme [53].

In addition, methionine, involved in the biosynthesis of cysteine, carnitine, taurine, lecithin, phosphatidylcholine, and other phospholipids, increases in liver, kidney, and plasma (see Table 3, 4, and 5, respectively). The role of methionine in arsenic methylation is important due to the conversion of methionine into S-adenosylmethionine (SAM) by methionine adenosyltransferase. SAM donates a methyl group to arsenic to form methyl and dimethyl arsenic (Fig. 6D) [54], which also contributes to the rise in the concentration of methionine during arsenic exposure.

Degradation of membrane phospholipids

The increased levels of choline containing metabolites, such as choline and phosphorylcholine, were detected in liver and plasma principally after arsenic intake and have been associated with drug induced disruption of cell membrane [55]. One of the most important families of altered metabolites found in our study was phosphatidylcholines whose levels decrease in

exposed mice. The majority of phospholipids in cellular membranes consist of glycerophospholipids such as phosphatidylcholine (PC), the most abundant phospholipids component in all the lipoprotein classes with levels ranging from 60 to 80 % of total phospholipids [56]. PC is involved in pathological mechanisms and crucial cellular functions such as signal transduction, apoptosis, necrosis, and protein sorting [57–59]. LPCs are produced by degradation of PC under enzymatic action of phospholipase A2, which releases one of the fatty acid groups (Fig. 6E). LPC is rapidly hydrolyzed to glycerophosphocholine and, finally, phosphorylcholine and choline.

The results show that intensities in MS spectra from different PC family compounds decreased under inorganic arsenic exposure in the m/z range of 700–850 in polar and lipophilic extracts using positive mode (ESI⁺) (Fig. 7). Secondly, LPC (m/z 450–600) increased in exposed mice in polar extract using positive mode of acquisition (Fig. 7). These results demonstrate breakdown of membrane phospholipids (cell apoptosis), which is corroborated by the increase in free fatty acids in the lipophilic extract from liver ESI⁺ and ESI⁻ analysis (Fig. 7), such as pipecolic, arachidonic, oleic, linoleic, gamma-linolenic, docosahexanoic, piranaric, and lauric acids, as a consequence of PCs degradation (Fig. 6E).

Alterations in degradation choline pathway and methionine cycle

Degradation of phospholipids from cell membranes leads to accumulation of choline in plasma and tissues. As a consequence, perturbations in the pathway of choline degradation can be observed, which is shown by the increased levels of betaine aldehyde in kidney (Table 3). This metabolite is an intermediate compound in the oxidation of choline to betaine, reaction catalyzed by the enzyme choline monooxygenase. Betaine is a source of methyl groups required for methylation of inorganic arsenic and constitutes a detoxification mechanism for this element (Fig. 6D); for this reason, the betaine

level increases in liver extract (Table 2). Therefore, the level of methionine increases in liver, kidney, and plasma of exposed mice, as previously mentioned, and correlatively the level of homocysteine, biosynthesized from methionine, that is recycled back to methionine completing the cycle.

In addition, changes in taurine levels in liver, kidney, and plasma can be observed (see Table 2, 4 and 5, respectively). Taurine has many important metabolic roles, such as conjugation of bile acids, antioxidation, osmoregulation, membrane stabilization, and modulation of calcium signaling. It also acts as an antioxidant and protects against toxicity of a number of substances (such as lead and cadmium) [60, 61]. Moreover, the opposite alteration of taurine and GSH has been related by GSH, which is a competitive metabolic product with taurine from cysteine. This fact suggests that the biosynthesis of GSH from cysteine was blocked, and the cysteine was diverted to produce taurine. The same results have been observed by other authors when realgar (As_4S_4) is administered to rats [33].

Induction of cell apoptosis and vascular risk

Perturbations in triglycerides and diglycerides levels in response to arsenic can also be observed (Fig. 7). Perturbations in triglycerides levels have previously been demonstrated in both bank voles under arsenic exposure [62] and rats under cadmium exposure [63]. In addition, increased biosynthesis of triglycerides and diacylglycerol has been found in apoptotic KB cells [64]. However, the mechanism for the relationship between increased lipids metabolites and cell apoptosis still remains unclear.

On the other hand, arsenic exposure has been correlated with a large number of cardiovascular diseases. In our study, we found slightly increased levels of di- and triglycerides in liver and plasma of exposed mice (see Table 2 and 5, respectively) that, together with the large amount of free fatty acid previously commented, suggest a situation of hyperlipidemia, which constitutes one of the most important vascular risk factors. Cardiovascular diseases such as hypertension, QT prolongation, peripheral arterial disease, atherosclerosis, impaired microcirculation, coronary heart disease, and stroke are well recognized in relation to dose and duration of arsenic exposure [65, 66]. Similar results have been obtained in renal tissue of exposed bank vole to cadmium [55].

Other metabolites

Other metabolites altered under arsenic exposure were also observed. Increased levels of xanthine and hypoxanthine were found in liver tissue from mice exposed to inorganic arsenic (see Table 2). Xanthine is a product generated in degradation pathway of purine. Xanthine is formed by oxidation of hypoxanthine by xanthine oxidase, an enzyme that generates

reactive oxygen species [67]. This enzyme can further catalyze the oxidation of xanthine to uric acid [68]; for this reason, it is reasonable to expect that the observed altered levels of uric acid in kidney in arsenic exposed mice. In mammals, uric acid is the final oxidation (breakdown) product of purine metabolism and is excreted in urine [69]. For this reason, uric acid may be a marker of oxidative stress [70], and may have a potential therapeutic role as an antioxidant [69]. Thus, elevated levels of uric acid could be associated with a protective response caused by oxidative stress. In addition, increased concentration of uric acid is obtained in plasma by GC-MS (see Table 5). On the other hand, creatine is not an essential nutrient, as it is biosynthesis in the body from L-arginine, glycine, and L-methionine. It is reasonable the high levels of plasma creatine as a consequence of transmethylation perturbation due to increased choline levels (see Table 4). Moreover, creatine functions as part of the cell's energy shuttle from mitochondria to the cytosol. In addition, creatine is excreted as creatinine in the urine. The increased levels of creatinine in kidney support this idea (see Table 3).

Conclusions

Mass spectrometry has been proven to be an efficient technique to investigate the biochemical effects of trivalent inorganic arsenic. The time-dependent changes in endogenous metabolites by oral administration of arsenic were identified by PLS-DA method. Mass spectrometry analysis of polar and lipophilic metabolites of liver, kidney tissues, and plasma by DI-ESI(\pm)-QTOF-MS using positive and negative modes of acquisition highlighted a number of perturbations in the endogenous metabolites profiles, which could be explained to arsenic induced biochemical pathways alterations. In addition, complementarily of gas chromatography coupled to mass spectrometry with DI-ESI(\pm)-QTOF-MS has been demonstrated. The toxicity mechanism of arsenic still remains unclear, although enzymatic inhibition, impaired antioxidant metabolism, and oxidative stress, may play a role. In this work, the toxic effect of arsenic has been related in some metabolic pathways, such as energy metabolism (e.g., glycolysis, Krebs cycle), amino acid metabolism, choline metabolism, methionine cycle (transmethylation), purine metabolism, and degradation of membrane phospholipids (cell apoptosis). In addition, analysis of polar and lipophilic extracts using positive and negative mode (ESI⁺/ESI⁻) allows the study and characterization of a great number of possible biomarkers. Additionally, although DIMS had comparable classification and prediction capability to LC-MS, as reported by other authors. LC-MS method is necessary when comprehensive screening of biomarkers is required. For this reason, a complementary chromatography separation based on reverse phase and hydrophilic interaction chromatography using

UPLC-ESI-QqQ-MS will be applied in the future. In this way, changes in the metabolome that were apparent after an exposure to 6 versus 12 days of exposure can be discussed, which would have been very useful from a biochemical point of view. In summary, mass spectrometry technique-based metabolomics approach can be employed to investigate the toxicological effects of different metals and other xenobiotics in the future using experiment exposure.

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