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Urinary $^1$H NMR metabolomics profile of Italian citizens exposed to background levels of arsenic: a (pre)cautionary tale

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ABSTRACT

Objectives: Arsenic is a toxic metal ubiquitous in the environment and in daily life items. Long-term arsenic exposure is associated with severe adverse health effects involving various target organs. It would be useful to investigate the existence of metabolic alterations associated with lifestyle and/or with the environment. For this purpose, we studied the correlation between urinary arsenic levels and urinary proton nuclear magnetic resonance spectroscopy ($^1$H NMR) metabolomics profiles in a non-occupationally nor environmentally arsenic exposed general population.

Methods: Urine samples were collected from 86 healthy subjects. Total and non-alimentary urinary arsenic (U-naAs) levels, namely the sum of arsenite, arsenate, monomethylarsonate and dimethylarsinate, were measured and $^1$H NMR analysis was performed. Orthogonal Projection to Latent Structures was applied to explore the correlation between the metabolomics profiles and U-naAs levels.

Results: Despite the extremely low U-naAs levels (mean value = 6.13 ± 3.17 μg/g creatinine) of our studied population a urinary metabolomics profile related to arsenic was identified.

Conclusion: The identified profile could represent a fingerprint of early arsenic biological effect and could be used in further studies as an indicator of susceptibility, also in subjects exposed to a low arsenic dose, with implications in occupational health, toxicology, and public health.

Introduction

Arsenic (As), a naturally occurring element abundant in the earth crust, is a toxic metal ubiquitous in the environment (Nearing et al. 2014). Many As compounds can dissolve in water, thus, As contamination of groundwater is a major concern for public health. Humans are exposed to As through ingestion of contaminated water and food, smoking, occupational and environmental pollution (National Research Council, 2015). Arsenic is classified by the International Agency for Research on Cancer as a carcinogen (Group 1) for bladder, lung, and skin, while DMA and MMA are classified as possibly carcinogenic for humans (Group 2B) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). The mechanism of damage is not fully understood and seems to involve biological pathways implicated in oxidative stress, DNA fragmentation, gene expression, induced apoptosis, and deregulation of ion channels (Alamolhodaei et al. 2015).

Due to the severity of As related adverse health effects and to the large number of worldwide exposed subjects, the study of its toxicity still represents a topic of particular concern for public and occupational health. Recently, the National Health and Nutrition Examination Survey reported on the effect of the Environmental Protection Agency maximum contaminant level on As exposure in the USA from 2003 to 2014, showing that the lowering of As threshold level from 50 to 10 μg/L in public water system was directly correlated with the decrease in urinary DMA levels in the general USA population (Nigra et al. 2017). This decrease, actually achievable by several means (Ali et al. 2011, Ali et al. 2014, Ali 2018), was discussed in light of the new 2006 threshold values challenged by the recent proposal of deregulation for contaminant levels in drinking water advanced by the USA administration (Landrigan 2017).

In this context, the knowledge of early subclinical effects related to As exposure could help to better understand the
complex toxicological mechanisms that may produce damage to human health, and, as a consequence, could contribute to the identification of health-based exposure limits both for workers and for the general population, and, moreover, for health surveillance programmes monitoring (Alessio et al. 2007).

A new approach in the study of metals toxicology is represented by metabolomics which, by means of the qualitative-quantitative analysis of low molecular weight metabolites within a cell, tissue, or biological fluid, gives a global holistic overview of the metabolic status of an organism in response to various stimuli, such as disease, genetic variation, environmental, occupational, and lifestyle factors (Dunn et al. 2011, Campagna et al. 2016, Vermeulen 2017). As-induced metabolomic perturbations have been observed in animal models as a consequence of long term and acute high dose exposure (Wei et al. 2009, Huang et al. 2013, Garcia-Sevillano et al. 2014a, 2014b, Wang et al. 2014). Several recent studies reported on the use of metabolomics to investigate the effect of long-term exposure to low doses of As on the biological profile of environmentally or occupationally exposed individuals (Shen et al. 2013, Dudka et al. 2014, Zhang et al. 2014, Garcia-Sevillano et al. 2015, Laine et al. 2017). Metabolomics studies are commonly performed on urine samples, due to the ease of their collection and to the possibility of a direct correlation between urinary As levels and the As-exposure metabolomics signature.

Due to the widespread presence of As in nature and in daily life items, it would be useful to identify possible metabolic alterations merely associated with lifestyle and/or with the environment to be considered as subclinical early biological effects.

The aim of this study was to investigate whether general background As exposure may affect the urinary metabolomics patterns in a human cohort living in a non-contaminated area and without occupational exposure to As. For this purpose, we studied a population group that represents what it is normally considered as a non-exposed control group, using Proton Nuclear Magnetic Resonance (1H NMR)-based metabolomics to study the correlation between urinary metabolomics profiles and urinary non-alimentary As (U-naAs) levels, which is the sum of all As hazardous forms, namely arsenite (iAsIII), arsenate (iAsV), MMA, and DMA.

Clinical significance

- Chronic As exposure has a pathogenic concentration-related activity. Urinary As concentration is employed as a biomarker of exposure.
- Epidemiological studies provided data on the severe adverse health effects of high As levels in drinkable water, but data on low/very low dose exposure are still lacking.
- Studies focussing on ‘personalized’ susceptibility may confirm, or exclude, a putative causal role of a lifelong As exposure in the onset or development of diseases in which its causative role has been already proven.
- Urinary metabolomics profile seems to be useful to monitor, at individual level, the biological effects of low/very low As exposure, even in a preclinical scenario.

Materials and methods

Study population and sample collection

The study protocol was notified to the Independent Ethical Committee of the Azienda Ospedaliero-Universitaria of Cagliari. All participating subjects provided written informed consent prior to participation. A cross-sectional study was performed collecting data during the annual workplace health surveillance programme conducted by the Occupational Medicine Department of the University of Cagliari, Italy, from October 2014 to December 2014. Eighty-six healthy male workers, the overall workforce of a logistic support company for safety in communication and flight were enrolled (Figure 1). Only male workers were recruited since no female subjects were present in this specific workplace. All subjects were in force from at least three years and they all agreed to participate in the study. Demographic data and lifestyle information, including age, Body Mass Index (BMI), alcohol intake, smoking habits, physical recreational activity, and health history were recorded for each...
participant. Furthermore, clinical parameters, including systolic and diastolic pressure, heart rate, glycaemia, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), serum and urine creatinine were recorded. Exclusion criteria were the existence of chronic pathologies and the detection of drugs metabolites in urine that can generate an alteration of spectra. During the annual workplace health surveillance, first-morning urine samples were collected from fasted subjects, mixed with sodium azide 0.01% (NaA₉, Sigma-Aldrich, Milan, Italy), in order to avoid bacterial growth, and immediately stored at −80 °C.

**Urinary as levels detection**

Urinary concentrations of total (U-tAs) and non-alimentary As (U-naAs), the latter corresponding to the sum of total inorganic ions (iAs³⁺ and iAs⁵⁺), MMA and DMA, were determined using a Varian Spectra AA300 Atomic Absorption Spectrophotometer, following a previously reported method (Subramanian 1989). U-tAs and U-naAs calibration lines were obtained by using standard in matrix of Institut National de Santé Publique, Quebec, Canada. Detection limits were as follows: U-tAs = 0.05 µg/L and U-naAs = 0.40 µg/L. The results were adjusted for urine creatinine.

**¹H NMR sample preparation and analysis**

For ¹H NMR analysis, 1 mL of thawed urine was centrifuged at 12,000 rpm for 10 min at 4 °C. 630 µL of the supernatant were mixed with 70 µL of a 1.5 M phosphate buffer solution (pH 7.4) in D₂O (99.9%, Cambridge Isotope Laboratories Inc., Tewksbury, MA) containing the internal standard sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TSP, 98 atom % D, Sigma-Aldrich, Milan, Italy) at a 0.59 mM final concentration, and 650 µL of the obtained solution were transferred into a 5 mm NMR tube.

¹H NMR experiments were performed using a Varian UNITY INOVA 500 spectrometer operating at 499.839 MHz for proton (Agilent Technologies, Santa Clara, CA). ¹H NMR spectra were recorded using a 1D-NOESY pulse sequence for water suppression with a mixing time of 1 ms and a recycle time of 3.5 s. Spectra were acquired at 300 K with a spectral width of 6000 Hz, a 90° pulse, and 128 scans. Before Fourier transformation, the Free Induction Decays (FID) were zero-filled to 64K and an exponential weighting function was applied with a line-broadening factor of 0.5 Hz. All spectra were imported in the MestReNova software (version 9.0; Mestrelab Research S.L., Santiago de Compostela, Spain), phased, and baseline corrected. Chemical shifts were referred to the TSP single resonance at 0.00 ppm. Assignment of NMR resonances was based mainly on literature data (Bouatra et al. 2013). In this phase, ten subjects were excluded due to the detection of drug metabolites in urine. Thus, the analysis was conducted in a selected population of 76 subjects.

**Statistical data analysis**

¹H NMR spectra were reduced into consecutive integrated spectral regions (bins) of equal width (0.02 ppm) from 0.72 to 9.4 ppm. The spectral region between 4.52 and 6.20 ppm was excluded from the analysis because it showed artefacts arising from water signal suppression and broad urea resonance. Bins corresponding to drug signals (mannitol and paracetamol) were also removed in all the spectra. In order to minimize the effects of variable concentration among different samples, the integrated area within each bin was normalized to a constant sum of 100 for each spectrum. A total of 270 variables (bins) that represent the spectral profile for each sample were obtained. Multivariate statistical data analysis was performed by SIMCA 13 (Umetrics, Umea, Sweden). The unsupervised principal component analysis (PCA) was used to detect outliers and recognize particular trends in the collected data. The supervised Projection to Latent Structures (PLS) regression and its Orthogonal (OPLS) implementation (Eriksson et al. 2013) were applied to model the effect of U-naAs on the urinary metabolome. The model quality was evaluated based on the residuals (R²X, R²Y) and the model predictive ability parameter (Q²Y) determined through the default leave 1/7th out cross-validation. Models were further validated through the permutation test on the response (500 random permutations), satisfying the following conditions: Q²Y(cum) and R²Y(cum) for the original model should be larger than all the results for the permuted models, and the y-intercept of the Q²Y(cum) should be negative. PLS-VIP-based selection was applied to select the most important variables in data modelling. Prior to performing the analysis, the spectral data were mean centred and Pareto scaled.

Clinical data and urinary As were expressed as mean±standard deviation (SD) or median and interquartile range (IQR), in case of normality violation. The Kolmogorov–Smirnov test was applied for normality analysis of the parameters. Spearman correlation tests were used to determine the strength of the relationships between U-naAs and demographic, lifestyle, and clinical variables. Bonferroni correction was used for multiple comparisons adjustment and a p-value of 0.005 was considered as the cut-off point for statistical significance. Data analysis was performed by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

**Results**

The demographic and lifestyle characteristics and the clinical parameters of the selected study population are reported in Table 1. All subjects involved in the study were healthy individuals.

Total As (U-tAs) and non-alimentary As (U-naAs) concentrations were measured for all participants, and are reported in Table 2. U-naAs represents the sum of iAs³⁺, iAs⁵⁺, MMA, and DMA, which are the major metabolites present in urines after exposure to inorganic As, and are commonly determined to assess occupational or environmental exposure.
In our study group, U-naAs was significantly correlated with U-tAs \((p < 0.0001)\), while no significant correlations were observed with age, BMI, HR, systolic and diastolic blood pressure, glycaemia, AST, ALT, GGT, serum creatinine, smoking, and alcohol intake.

\(^1\)H NMR experiments were performed on all the urine samples. Several low molecular weight metabolites were identified, including amino acids, organic acids, small organic compounds, osmolytes, and sugars. After the exclusion of ten subjects for the aforementioned reasons, spectral data of selected subjects were submitted to multivariate statistical analysis. At first, an exploratory unsupervised PCA was performed in order to investigate the sample characteristics and visualize trends and outliers. The first two principal components (PCs) accounted for 31% of the variance. In the PCA score scatter plot of the urine samples, no strong outliers neither sample clustering associated with U-naAs were observed (Figure 2).

A supervised OPLS model was then applied to study any As-related metabolic modifications. Specifically, \(^1\)H NMR spectral data were correlated with U-naAs as the outcome. Figure 3(a) shows the OPLS score plot where urine samples with increasing U-naAs values are located along the predictive component (x-axis). The quality of the model was evaluated on the basis of the fitness ability \((R^2_Y = 0.69)\) and the prediction ability \((Q^2_Y = 0.59)\). The model was further validated performing the permutations test on the corresponding PLS model \((n = 500\) permutations, y-intercept of the \(Q^2_Y(\text{cum}) = -0.357\); Figure 3(b)). The variable importance on the projection (VIP) value of each variable was calculated to evaluate its contribution to the model. Based on PLS-VIP selection (using the threshold VIP \(> 1\)), 51 variables contributed the most to data modelling and can be considered as the urinary metabolomics signature correlated with U-naAs levels. Figure 3(c) shows the loading column plot of the variables (i.e. the urinary metabolites) along the predictive

| Table 1. Demographic, lifestyle, and clinical parameters of the study population. |
|---------------------------------|-------------|----------------|----------------|----------------|
| **Demographic data**           | n (%)       | Median (min–max) | Mean (SD)      |
| Age (years)                    | 37 (20–63)  | 37.74 (11.3)     |                |
| BMI (kg/m^2)                   | 24.8 (20.1–35.2) | 25.13 (2.63)    |                |
| Smoking                        |             |                 |                |
| Never                          | 36 (47%)    |                 |                |
| Past                           | 21 (28%)    |                 |                |
| Current                        | 15 (20%)    |                 |                |
| Missing                        | 4 (5%)      |                 |                |
| Alcohol intake                 |             |                 |                |
| 0 unit                         | 21 (28%)    |                 |                |
| 1 unit                         | 27 (36%)    |                 |                |
| 2 units                        | 23 (30%)    |                 |                |
| Missing                        | 5 (6%)      |                 |                |

**Clinical data**

<table>
<thead>
<tr>
<th></th>
<th>Median (min–max)</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (f/m)</td>
<td>64 (44–88)</td>
<td>64 (11.71)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>120 (100–160)</td>
<td>123.19 (12.14)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>80 (60–110)</td>
<td>77.67 (9.44)</td>
</tr>
<tr>
<td>Glycaemia (mg/dL)</td>
<td>87.5 (59–134)</td>
<td>89.14 (11.32)</td>
</tr>
<tr>
<td>AST (UI/L)</td>
<td>23 (14–211)</td>
<td>27.81 (22.37)</td>
</tr>
<tr>
<td>ALT (UI/L)</td>
<td>21 (8–103)</td>
<td>27.12 (17.88)</td>
</tr>
<tr>
<td>GGT (UI/L)</td>
<td>20 (8–136)</td>
<td>27.24 (21.45)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.02 (0.05–1.60)</td>
<td></td>
</tr>
<tr>
<td>Urine creatinine (mg/dL)</td>
<td>1.30 (0.2–3.1)</td>
<td></td>
</tr>
</tbody>
</table>

Urinary arsenic concentrations were corrected with creatinine values to compensate variations in urine dilution.

| Table 2. Urinary arsenic concentration (µg/g creatinine) of the study population. |
|---------------------------------|-------------|----------------|-------------|
|                                | Mean (SD)  | Min    | 25% tile  | Median | 75% tile | Max  |
| U-tAs                           | 34.13 ± 42.91 | 2.44  | 10.90     | 17.46  | 37.85     | 270.0 |
| U-naAs                          | 6.13 ± 3.17  | 1.31  | 3.85      | 5.34   | 7.73      | 17.33 |

In our study group, U-naAs was significantly correlated with U-tAs \((p < 0.0001)\), while no significant correlations were observed with age, BMI, HR, systolic and diastolic blood pressure, glycaemia, AST, ALT, GGT, serum creatinine, smoking, and alcohol intake.

\(^1\)H NMR experiments were performed on all the urine samples. Several low molecular weight metabolites were identified, including amino acids, organic acids, small organic compounds, osmolytes, and sugars. After the exclusion of ten subjects for the aforementioned reasons, spectral data of selected subjects were submitted to multivariate statistical analysis. At first, an exploratory unsupervised PCA was performed in order to investigate the sample characteristics and visualize trends and outliers. The first two principal components (PCs) accounted for 31% of the variance. In the PCA score scatter plot of the urine samples, no strong outliers neither sample clustering associated with U-naAs were observed (Figure 2).

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component, which are correlated with U-naAs levels and listed in Table 3. In particular, trimethylamine N-oxide (TMAO), taurine, citrate, hippurate, dimethylamine (DMA), acetate, 3-hydroxy-butyrate, cis-aconitate, acetone, 3-hydroxy-isovalerate, and 2-oxoglutarate are positively correlated with U-naAs levels, whereas creatinine, lactate, N-acetyl-groups, glutamine, choline and its derivatives are negatively correlated.

Figure 3. OPLS model with U-naAs as the outcome. (a) Score scatter plot with the samples coloured on the basis of the U-naAs interquartile values (blue = 1st, light blue = 2nd, green = 3rd, red = 4th quartile). (b) Permutation test of the corresponding PLS model (n = 500 random permutations). (c) Loading column plot of variables (spectral data, metabolites) along the predictive component t[1].
Table 3. List of metabolites correlated to U-naAs levels obtained from the OPLS loading plot.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAO</td>
<td>Inversely correlated</td>
</tr>
<tr>
<td>Taurine</td>
<td>Creatinine</td>
</tr>
<tr>
<td>Citrate</td>
<td>Lactate</td>
</tr>
<tr>
<td>Hippurate</td>
<td>N-acetyl groups</td>
</tr>
<tr>
<td>DMA</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Acetate</td>
<td>Choline and its derivatives</td>
</tr>
<tr>
<td>3-Hydroxy-butyrate</td>
<td></td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxy-isovalerate</td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

Animal and human studies performed on individuals exposed either too high or low As dose showed a response at a molecular level to this toxic stress, with alteration of the metabolite content in the organisms, that is mainly due to impaired energy production and ion regulation. Our purpose was to evaluate whether As may affect particular metabolic pathways inducing perturbation of specific urinary metabolites also at a very low dose of exposure.

The median values of U-tAs and U-naAs in the study sample were 17.46 and 5.34 μg/g creatinine, respectively. These values are comparable with those expected in Italian general populations (Società Italiana Valori di Riferimento 2005), and lower than the levels detected in similar studies conducted in non-occupationally exposed populations, where the median values were 40.03 and 27.6 μg/g creatinine for U-tAs and U-naAs, respectively (Zhang et al. 2014).

Our results showed a correlation between the global urinary metabolic profile and U-naAs concentration, even if this latter is extremely low (Table 3). Most of the identified metabolites were amino acids and organic acids that play important roles in various biochemical processes. Some of them showed a positive trend, i.e. they increase with increasing U-naAs, while others showed an opposite trend. The decreasing trend of choline with increasing U-naAs levels associated with the opposite behaviour of some of its major metabolites, such as DMA and TMAO, suggest disturbances in choline metabolism that may be related to As-induced membrane toxicity (Huang et al. 2013). The observed increase of taurine urinary levels with increasing U-naAs may suggest its active role against oxidative damage related to As exposure, since taurine plays a critical role in anti-inflammation, osmoregulation, stabilization of cell membranes, and protects from oxidative stress (García-Sevillano et al. 2014a, 2014b). This mechanism was already proposed in response to toxic substances such as lead and cadmium (García-Sevillano et al. 2014a). The observed decrease of urinary lactate acid with increasing U-naAs, together with the increase of TCA cycle intermediates (namely citric, cis-aconitic, and α-ketoglutaric acid), suggests a shift of pyruvate metabolism towards the formation of acetyl-CoA. The excess of acetyl-CoA can be further converted to acetate and ketone bodies. These results are consistent with previous reports showing that high levels of citrate in liver, kidney, and plasma are related to As exposure (García-Sevillano et al. 2014a).

Moreover, a positive correlation of acetate with As and other metals has been repeatedly reported in the literature, and proposed as a biomarker of exposure (Huang et al. 2013, García-Sevillano et al. 2015). Increased hippurate urinary levels are indicative of oxidative stress following As exposure, as reported in a previous study on a Chinese population screened for environmental As exposure (Zhang et al. 2014).

The overall urinary metabolomics profile described here is consistent with the one identified in a rat model treated with realgar (a medicament that contain over 90% of tetraarsenic tetrarsulfide) (Wei et al. 2009), where perturbation of TCA cycle together with increased TMAO, taurine, ketone bodies, and choline, and decreased hippurate and lactate levels, were correlated to As exposure. Similar modifications have also been described in a neonatal population delivered by women living in areas with elevated As levels in drinking water (BEAR cohort), and related to prenatal As exposure (Laine et al. 2017).

To the best of our knowledge, this is the first metabonomics study on the effects of background As exposure on the metabolism of an Italian population. Our results, in agreement with previous reports, provide evidence for the existence of a metabolomics profile associated with urinary As levels even in a non-exposed population. We have identified a metabolic pattern of early biological effect and/or of susceptibility that could be very useful in further screening of the general population. Furthermore, our results suggest a possible causative role of As even at extremely low levels on the alteration of some metabolic pathways. This aspect is remarkable considering the lack of a precise hazardous threshold for As exposure, particularly for carcinogenesis (Abernathy et al. 1996, Cohen et al. 2016), and enforces the questionability of safe As exposure limits. This issue represents a particular concern in As exposure scenario, due to the large number of exposed individuals worldwide and to the severity of As related adverse health effects. In this perspective, the identification of early biological effect biomarkers may enlighten new insights in exposed populations (even to low or very low dose) so to achieve personalized risk assessment, and to adopt preventive strategies, based on the hypothesis of a continuum of effects from an early detectable effect towards subclinical and clinical manifestations as already shown in the behaviour of several toxic metals (Alessio et al. 2007). Accordingly, preventive actions aimed at reducing the critical concentration (Ali et al. 2011, 2014, Ali 2018), being it the one able to cause the earliest alteration in the most sensitive cells, will protect more effectively the entire organism. If an association between internal dose increase and early effects appearance may be postulated, the progression of the former, being responsible for wider and wider alterations to various target organs, may pave the path for subclinical damages up to an overt clinical intoxication.

The lack of information on the precise adverse effects induced by the critical dose, the knowledge of early biological effects on a target organ, and the large number of exposed subjects have to be considered, applying the precautionary principle, enough to identify an As threshold
value. In the last century, the failure in applying this approach, such as for tetraethyl lead in gasoline, misled health policy makers, causing severe health consequences for the general population.

Some limitations affect our findings. First, we focussed only on the cumulative value of U-naAs (represented by the pool of inorganic As, MMA and DMA, which are the only pathological species reported in literature). Furthermore, our sample was limited being the experiment designed as a proof of concept, but despite this, the 1H NMR approach allowed us to identify a metabolomics profile related to early subclinical effects and/or critical effects related to background As in non-exposed healthy individuals. Moreover, urinary As was only measured in male subjects at a single time point and not in a longitudinal way. This approach may be justified by our principal interest, which was to investigate the possible effects of background As exposure in a real-world scenario, and by the explorative nature of this study. Finally, the lack of a control group is justified by the unfeasibility to collect samples from urinary As-free individuals.

Conclusion
The main result of our study is the suggestion of a metabolomics effect of background As exposure on the human urinary profile. Intriguingly, the main metabolites involved in the early body response are the same described either in the animal models of high dose As exposure and in the environmentally exposed human beings, suggesting a common pathway activation to As in a dose-independent way. If these preliminary results will be confirmed by analysing a wider dataset or by performing a longitudinal analysis, a major concern may arise from the choice of a legal definition of a ‘safe’ As threshold level in the environment. The history of the delays in appropriate risk management policies should lead to strongly consider the precautionary principle in the definition of As exposure limit values, even if the international political trend seems to privilege a ‘no-threshold’ approach.

Further studies are warranted in order to increase the knowledge of As toxicology by means of ‘omics’ sciences by studying the association between As-related metabolic pattern alterations and early subclinical adverse health effects in selected national cohorts and/or in target organs.

Author contributions
MC and EdA conceptualized the study and contributed to the overall interpretation of the data. LIL, RP, AN, and IP collected the data. EL performed the NMR experiments. EL, LIL, RP, and AN performed the data analysis and interpretation. All authors contributed to the manuscript writing and approved the submitted version of the manuscript.

Disclosure statement
No potential conflict of interest was reported by the authors.

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