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Metal environmental contamination within different human exposure context- specific and non-specific biomarkers

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Abstract

The quality of the environment that we live in is fundamental to our well-being. Although stringent measures have been put into place during the last few decades, exposure to high levels of persistent pollutants such as metal mixtures is commonly encountered by the general population especially in industrialized countries. The aim of this work was to evaluate how metal pollution in contaminated areas is reflected in terms of biomarkers of exposure and effect in human sub-populations living in distinct non-occupational environmental contexts. Thus, four Portuguese sub-populations living in different areas of Portugal were studied: i- the exposure of each member of these sub-populations to lead (Pb), manganese (Mn) and arsenic (As) was evaluated by determining metal levels in urine; ii- biochemical changes were assessed, determining the levels of urinary metabolites of heme biosynthesis; iii- the capability of combinations of these biological markers to predict the context of exposure of each subject was tested, as to develop a tool to identify adverse health effects in these environmentally exposed populations.

Combinations of BMs in populations co-exposed to the metals under study, the heme precursors ALA and porphyrins in urine, were predictive of contexts of environmental exposures, with 88% of the studied subjects correctly identified as to their sub-population origin. The use of non-specific BMs may show not only the exposure to Pb, Mn and As, but also reflect the effects on health induced by the total environmental mixture, including unknown components.

Keywords

metal environmental contamination; human metal exposure; biomarkers of effect; ALA and porphyrins in urine

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1. Introduction:

1.1 Environmental Quality, Health and Well Being:

The quality of the environment and lifestyle is fundamental to our well-being (UNEP 2018). We live in an ever-changing world, where metal exposure is in part natural, but in large part may be secondary to anthropogenic activities, such as agriculture, mining, urban and industrial development. The Earth's ecosystems have changed more rapidly since the second half of the twentieth century, and all ecosystems have been significantly transformed through human actions (Blais et al. 2015; Corvalan et al. 2005).

Environmental contamination occurs when a particular substance is present where it would not normally occur, or at concentrations above natural levels.

Four large groups of contaminants were established, taking into account their negative effects on human health: inorganic and organic substances, radioactive elements and microorganisms (Chapman 2007; Ribeiro 2013). Over the last several years, concern about environmental pollution by metals has grown, as well as the apprehension with public health. Environmental contamination in industrialized countries by heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg) and the metalloid arsenic (As) is largely the consequence of past emissions by nonferrous industries. In urban atmospheres, heavy metals are mainly derived from industrial activities (mining, smelting, and fossil fuel combustion), traffic emissions (vehicle exhausts and the products of wear from tires, brake linings, and bearings), and natural sources (minerals, fires in forest, oceans and volcanoes). The total amount of heavy metals in the environment is commonly used as the main or only evaluation criteria in assessing pollution conditions (Li et al., 2013). Although stringent measures and controls have been put into place during the last decades, high levels of these persistent contaminants remain in the soils and aquatic sediments, and therefore also in the food chain, with possible adverse consequences of chronic environmental exposure to the populations living in these contaminated areas (de Burbure et al. 2006).

Several metals are vital for human health and are referred to as essential metals; other metals and metalloids have no physiological functions (non-essential) and can cause health problems (Rocha et al. 2016; Silva 2014; Tchounwou et al. 2012). Essential metals may also be toxic when exposures exceed optimal levels. Heavy metals have been reported to affect cellular organelles and components, such as cell membrane, mitochondria, lysosome, endoplasmic reticulum, nuclei, and enzymes involved in metabolism, detoxification and damage repair. (Casarett and Doull's 2013; Tchounwou et al. 2012).

Most of our knowledge concerning the health effects of toxic metals stems from studies conducted on populations with relatively high exposure, commonly to individual metals in industry or in heavily contaminated environments. However, in industrialized countries exposure to metal mixtures is most commonly encountered by the general population (Andrade et al. 2015; de Burbure et al. 2006; Li et al. 2013). Furthermore, urbanization may also represent an important source of disparity in metal exposures. A scarce number of studies has addressed the effects of chronic low environmental metal mixture exposures and in most cases these studies have merely focused on the evaluation of metal levels in

biological samples (Nowak 1998). Indeed, a huge gap exists concerning the evaluation of changes in levels of biomarkers of effect that may reflect *in vivo* metal interactions in human populations.

The metals studied herein were lead (Pb), manganese (Mn) and arsenic (As). Arsenic and Pb, all three belonging, according to Csavina et al. (2012), to the list of the six most “threatening” metals in the environment. They are also in the priority lists of the European Environmental Agency (EEA 2013) and the Agency for Toxic Substances and Disease Registry (ATSDR 2017). These lists are based on the toxicity of the chemical and on the potential for exposure from air, water or soil contamination. These two metals have no known nutritional or beneficial effects on human health but are ubiquitous in nature and present in air, water and soil, thus exposure is not readily preventable (Goyer 2004). Concerning Mn, it is one of the most abundant and widely distributed metal in nature. Although an essential metal, in humans, the neurological damage induced by excessive Mn exposure has been well documented (Pinsino et al. 2012; ATSDR 2012).

1.3 Lead, Manganese and Arsenic- Mechanisms of Toxicity and Biomarkers:

A biomarker (BM) serves to assess the exposure to chemicals and/or detect induced toxic effects, preferentially in a reversible stage, before the installation of pathological alterations. Therefore, BMs can be used as predictive tools for improving public health (Amorim, 2003; Prista and Sousa Uva, 2003; Fowler 2012).

The use of BMs of exposure can allow to link the external exposure to a chemical to the internal dose (Atkinson et al. 2001; Fowler 2012). Blood Pb concentration is the most widely used parameter for general clinical and public health surveillance, with measurements of urinary Pb levels also used to assess Pb exposure (ATSDR 2007a). Measurements of blood As reflect recent exposures or exposures to high As levels. Urinary As levels have been mostly used as BM of exposure in epidemiological and occupational studies (ATSDR 2007b). Concerning Mn, its levels in hair, blood or urine have been used as BMs of exposure, but their suitability for such surveillance remains controversial (ATSDR 2012; Cowan et al. 2009). Serum was also used to assess the permissible exposure level to Mn in workers exposed to Mn dioxide (Roels et al. 1992). Urinary Mn (Mn-U) has limited utility on an individual basis for exposure assessment, yet its inclusion in surveillance programs should be considered. Given that on a group basis, Mn-U seems partly influenced by recent exposure, its determination may detect changes in environmental pollution and a time trend for the risk of overexposure (Roels et al. 1992).

Biomarkers of effect are molecular tools that can serve to identify changes or effects occurring in the organism due to exposure to a given toxicant or stressor (Bernard 2008). Several BMs of effect induced by metals have been investigated, so far. The hematological system is an important target for the toxic action of metals that affect heme biosynthesis pathway by interfering with various enzymes (Andrade et al. 2015; Andrade et al. 2017; Ng et al. 2005). Disturbances in heme synthesis are generally characterized by excessive accumulation and excretion of heme precursors, delta-aminolevulinic acid (ALA) and porphyrins (Andrade et al. 2013; Andrade et al. 2015; Ng et al. 2005; Schauder et al. 2010).

Exposure to Pb increases ALA synthase (ALAS) activity and inhibits ALA dehydratase (ALAD) activity, leading to augmented urinary excretion of ALA (ALA-U) (Makino et al. 2000; Prista and Sousa Uva 2003). Mn can also impair enzymes linked to heme synthesis, inhibiting ALAD in the liver and erythrocytes, thereby increasing ALA levels in the blood and consequently ALA-U (Nascimento et al. 2016). It has also been shown that As have influence on porphyrins levels (Ng et al. 2005), with studies associating augmented urinary porphyrins excretion with increased concentrations of As in urine (As-U) (Marchiset-Ferlay et al. 2011).

Given the above information, the levels of heme precursors in urine are not specific BMs of effect, since changes in their levels can be induced by different metals or even other chemicals (Marks 1985; Okuno et al. 1991).

To date, a plethora of studies have relied on single chemicals or at most on binary mixtures (Kossowska et al. 2013). However: 1) Metals are often introduced into the environment as mixtures (ATSDR 2004; Fairbrother et al. 2007) and the degree of urbanization/industrialization of each environmental context likely modulates the qualitative and quantitative composition of these mixtures. It is exceedingly difficult to evaluate their composition, as the heterogeneity of atmospheric deposition of metals in a given geographic region is influenced in different ways by anthropogenic activities (Puente et al. 2013). 2) Human individual variability is a major obstacle for BMs research, due to differences in the metabolism of the toxic concerned, increasing the potential for misclassification of exposures (Mayeux 2004). In fact, if “one size does not fit all” (Friedrich et al. 2014; Zenner 2017) there is a need for BMs for human exposures to pollutants, which can be applied on an individual basis. 3) A BM alone insufficiently reflects the exposure to a mixture of chemicals; rather, a combination of BMs with multivariate techniques should increase their predictive power (Rachakonda et al. 2004; Schulte and Hauser 2012; Robin et al. 2013). 4) Even when used in panels, specific BMs will not encompass all the diversity of components present in mixtures of environmental pollutants.

Given the above, the aim of this work was to propose tools to control or evaluate the exposures and the effects arising from a low chronic metal co-exposure, in sub-populations living in distinct contexts of urbanization/industrialization. Therefore, we evaluated the exposure of these sub-populations to three metal pollutants relevant to urbanized/industrialized settings, namely, Pb, Mn and As, as well as changes in heme synthesis. We applied jointly BMs of exposure and/or non-specific BMs of effect, to obtain a “personalized molecular mark” which can predict the context of exposure in each subject and the effects that might ensue.

2. Material and Methods

2.1 General methodology and sample characterization

Four sub-populations non-occupationally exposed to metals, living in distinct geographical sites in Portugal (three in center/south, and the fourth in an Atlantic island) with different urbanization degrees, were studied. The origin of these populations was: Ax, a town from a Portuguese island (n=16), Sx, a non-industrialized town near a natural park (n=7), Lx, a big

city with air and car traffic (n=25), and Vx, a town near several industries (n=23). Their exposure to Pb, Mn and As was evaluated, determining urinary metal levels and urinary precursors of the heme biosynthetic pathway. The latter were assessed as indicators of biochemical changes related to metal exposure. In addition, the capability of combinations of these biological markers to predict the context of exposure of each subject was tested in order to propose a suitable combination of BMs as a tool to evaluate adverse health effects in these exposed populations.

2.2 Biological sample - urine

Biological samples (urine) from the four different urban areas previously mentioned were collected. All the participants (n=71, total) were clearly informed on the objectives of the study and provided a written consent under the guidelines of an approved IRB protocol from the University of Lisbon. They were also informed that the results were treated in an anonymous way. Urine samples were obtained from individuals that went to one chosen laboratory of clinical analysis in each of these towns, to make a periodical check-up. None of the individuals under study was occupationally exposed to metals, according with the questionnaire they filled. All the biological samples were analyzed in the laboratory of Toxicology in Faculdade de Farmácia, Universidade de Lisboa (FFUL, Portugal).

The urine samples collected in the morning, corresponded to one spot sample. After arrival to the laboratory of Toxicology FFUL, in the same day (transported at a temperature of 4°C and in the absence of light), the samples were codified, registered in a database and frozen immediately at -80 °C. All analyses were performed over the next few weeks, with priority for porphyrins quantification, due to the reduced stability of these molecules.

2.4 Chemicals:

The main chemicals were obtained from the following sources: nitric acid 65% (HNO₃), diethyl ether 99,8% (C₄H₁₀O), 5-aminolevulinic acid hydrochloride (C₅H₉NO₃.HCL) 98%, 5,5'-Dithiobis(2-nitrobenzoic acid) 98%(C₁₄H₈N₂O₈S₂), and lead, manganese and arsenic for AAS standard solution TraceCERT®, from Sigma; ethyl acetoacetate (C₆H₁₀O₃) 98% and ethanol absolute p.a.(C₂H₅OH), purity (GC) 99% from Merck; ethyl acetate 99,9 % (C₄H₈O₂), nitric acid trace metal grade (HNO₃) 67–69% assay and hydrogen peroxide 30 % W/V (H₂O₂) from Fisher Chemical; hydrochloric acid 36.5–38.0% ACS Basic (HCL) from Scharlab; glacial acetic acid 99.7 % (CH₃COOH) from Panreac.

2.5 Analytical methods:

2.5.1 Determination of metal levels in urine: Metal levels, Pb, Mn and As, were determined by Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS) in a PerkinElmer, AAnalyst-700, after sample preparation. To determine As concentrations, a hydride generator was connected to the instrument (HGAAS) (Andrade et al. 2013; Skoog et al. 2014). Daily calibration plots were performed using blanks and standards analogous to those present in the matrix of the digested solutions. After optimization, the method demonstrated to be selective and linear in the concentration range of 5–25 µg/L for Pb, 2–20 µg/L for Mn, and 1.25–12.5 µg/L for As.

Sample preparation - The analyzed samples were subjected to acid digestion to eliminate all the organic matter. After centrifugation at 2000 rpm for 3 min, 2 mL of each urine sample were digested in a water bath at 100 °C for 2 hours and 30 min, after the addition of 750 µL of HNO₃ and 750 µL of HCl. All the digested samples were transferred to volumetric flasks and diluted to the adjusted volume.

Determination of As levels in urine required a subsequent treatment, this is, the addition of 1 mL of ascorbic acid, 1 mL of concentrated HCl and 1 mL potassium iodide (KI), to 1 mL of each digested sample. A reduction reaction was performed in the HGAAS using HCl (10%) and NaBH₄ (0.2%) in NaOH (0.05%).

All the used glass material was previously decontaminated for 24 hours using a 15% HNO₃ (65%) solution in deionized water. Next, the material was rinsed twice with distilled water followed by deionized water. The material was dried and kept in an isolated place until use.

2.5.2 Determination of urinary delta-aminolevulinic acid levels: Urine samples (750 µL) were centrifuged at 2000 rpm during 3 min; Next: a) to 500 µL of supernatant, 500 µL of acetate buffer (pH 4.6) plus 67 µL of acetoacetate were added; b) the solutions were vortexed and placed in a 100 °C water bath for 10 min; c) after cooling, 1500 µL of ethyl acetate were added, followed by new mixing in the vortex and centrifugation to separate the two phases (2000 rpm, 3 min); d) 1 mL of the organic phase was transferred to new tubes, together with 1 mL of modified Ehrlich reagent and mixed; e) ten minutes later, the reading of the colored complex was performed in a Hitachi U-2001 spectrophotometer against a blank reagent (Tomokuni and Ogata 1980).

Daily calibrations curves were performed with ALA standards using the following concentrations: 0.5, 1.0, 2.0, 4.0, 5, and 8.0 mg ALA/L ($R^2=0.9978$).

2.5.3 Determination of urinary porphyrins levels—During the procedure, samples were protected from light since porphyrins are photo sensitive. Processing was carried out as follows: a) 100 µL of glacial acetic acid and 2.5 mL of diethyl ether were added to 1 mL of centrifuged urine; b) the solutions were mixed in a vortex, prior to porphyrin extraction and centrifuged (2000 rpm, 3 min) to separate the organic and aqueous phases; c) after recovering the organic phase, 2.5 mL of iodine chloride solution (ethanolic iodine solution 1% in HCl 5%) was added with subsequent vortex mixing before new extraction, and phase separation; d) the aqueous phase was placed in a water bath at 37 °C for 5 min (with shaking), before the spectrophotometric readings against HCl (5%) at 380, 430 e 401 nm (Andrade 2014; Soulsby and Smith 1974). The concentration of porphyrins was calculated according to the formula used in the studies referenced above: porphyrins (µg/L) = $[2 \times A_{401} - (A_{430} + A_{380})] \times 2.093 \times 1.064 \times 1000$.

2.5.4 Determination of creatinine levels in urine—All the determinations performed in urine samples were corrected with the levels of creatinine (creat) using a Creatinine kit, Randox Creatinine. The results were expressed as mg, or µg/g creat.

2.5.5 Statistical analysis: Statistical analysis was performed with the SPSS 16.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as means $\pm$ standard deviations (SD). All the parameters were compared by one-way analysis of variance (ANOVA) followed by post hoc Tuckey's test to assess differences between groups. Discriminant analysis was also performed to evaluate if the context of exposure (group) of each subject, could be correctly identified through linear combinations of the several BMs. The significance of all the results was considered when p values were less than 0.05 (Marôco 2014).

3. Results:

3.1 Metal levels in urine

The Vx population was the group with the highest concentrations of Pb-U, which were significantly higher ($p < 0.05$) from the Ax and Sx groups (Figure 1). The lowest levels of Pb-U were found in the Sx population, being significantly different ($p < 0.05$) from the Ax and Vx groups (Figure 1). Urinary Mn levels were also significantly higher in the Vx group ($p < 0.05$) compared with the Sx and Lx groups (Figure 2). In contrast, the Lx population had the lowest concentrations of Mn-U, with significant differences ($p < 0.05$) compared with the Ax and Vx groups (Figure 2). With respect to As, the urinary levels were highest in the Lx population, and significantly different ($p < 0.05$) from all the other groups, while the lowest levels were found in the Sx group (Fig.3).

Concentrations of heme precursors in urine—The highest levels of ALA-U were observed in the Lx group (Figure 4a), being significantly different ($p < 0.05$) from all the other groups. The Sx population had the lowest ALA-U values, when compared with all the other groups ($p < 0.05$) (Figure 4a). With respect to urinary porphyrins concentrations (Figure 4b), the highest levels were observed in the Vx population, showing statistically significant ($p < 0.05$) differences from the Ax and Sx groups. The second highest concentrations of urinary porphyrins were observed in the Lx group, which were significantly different ($p < 0.05$) from the Ax and Sx groups. The Sx group had the lowest urinary porphyrin levels in urine, and they were significantly different ($p < 0.05$) from all the other groups (Figure 4b).

3.3 Combination of biomarkers

Table 1 shows that the integration of Pb, As, ALA and porphyrins urinary levels resulted in a correct classification of 89.8% of all the subjects concerning their origin. The highest correct classifications were achieved in the Sx group, with 100% of the cases correctly classified, while the lowest correct classification was observed in the Vx group, corresponding to 84.6% (Table 1). Thus, Figure 5 illustrates the trend for a clear discrimination among all the groups, except between the groups Ax and Sx, which overlapped.

A slight decrease in the number of correct classifications (88%) was observed when urinary Pb and As levels were removed from the model (Tables 1 and 2). In fact, it was in the Ax and Vx groups that the number of correct identifications declined to 77.8% and 79.1%, respectively (Tables 1 and 2). Figure 6 shows the overlapping trend between these groups.

4 Discussion

Environmental pollution is a major cause of disease, death and disability in countries around the world. The health and well-being of human societies is intrinsically linked to the quality of the environment in we live. Over the past century, increased levels of pollution in air, water, soil and food has been associated with negative health outcomes in local communities. (Tchounwou et al. 2012). This has led to increased ecological and global public health concerns secondary to environmental metal pollution. Heavy metals are naturally found throughout the earth's crust in multiple environmental compartments. Most environmental contamination and human exposure result from anthropogenic activities such as mining and smelting operations, industrial production, and domestic and agricultural use of metals and metal-containing compounds (Casarett and Doull's 2013; He et al. 2005; US.EPA 2007). Although stringent measures and controls have been put into place during the last decades, high levels of metals persist in soils and aquatic sediments, thus entering the food chain, leading to potential health adverse consequences due to chronic environmental exposure of people living in contaminated areas (de Burbure et al. 2006). Human biomonitoring is a key instrument for health-related environmental protection. It focuses on measuring the internal exposure of people to environmental chemicals and on assessing potential health effects (2nd International Conference HBN 2016 Berlin). In this type of risk assessment, the total exposure to a toxic agent in the environment is based on the amount taken up into the body, reflecting all routes of exposure (US.EPA 2007).

In a multiple chemical context, evaluating the diverse chemicals to which each person is exposed to, is unrealistic. Thus, it is crucial to estimate the effects of both identified and non-identified chemicals on the human populations. Consequently, in addition to the identification of chemical exposure, we mean metals with high toxicological relevance through BMs of exposure, we posit the use of non-specific BMs of effect as to evaluate the effects caused by a whole mixture of chemical pollutants in exposed populations.

Given the above, our objective was to analyze human co-exposure to three metals, Pb, As and Mn, using a biological matrix, namely, urine, as well as BMs of effect in distinct non-occupationally exposed settings with distinct environmental quality. The reason for the choice of these three metals, Pb, As and Mn reflects their priority as toxic agents (Pb and As), as well as their validated combined neurotoxic effects (ATSDR 2007a, b and 2012; EEA 2013).

4.1 Levels of the three metals, Pb, Mn and As in urine:

As noted before, biological samples were collected from four sub-populations belonging to four different zones, three in the middle/south of Portugal, and the fourth in a Portuguese Atlantic Island. In the population living in the industrialized zone, Vx, levels of Pb-U and Mn-U were the highest (Figure 1 and Figure 2). With respect to As-U, the Lx group had the highest values (Figure 3).

Liang and Mao (2015) reported that the management of waste containing Pb as well as the manufacture of leaded products and their recycling, are the sources that contribute the most to the increase in environmental Pb levels thus posing the greatest threat to human health.

This may in part reflect the highest average Pb-U concentrations in populations living in the industrialized urban zone, Vx, (Figure 1). However, as Pb-U is not an optimal BM of chronic exposures, namely because of the accumulation of Pb in bones, these samples may not exhibit increased Pb body deposition over time (ATSDR 2007b; Barbosa et al. 2005; Sanders et al. 2009). Nonetheless, it is reasonable to suggest that individuals living near this industrialized zone (Vx) are exposed to greater levels of this metal in comparison to the other populations. Thus, industries in the Vx zone seem to have an impact on the emission of Pb, when Pb-U levels are compared with the those found in other urban zones, one with a great automobile and air traffic (Lx) and the other near a natural park (Sx). Our study indicates that it is necessary to consider the Pb results in this population (Vx) and to further validate them in a larger sample.

Concerning Mn levels, the Vx population showed the highest average values, which were \ significantly differences ($p<0.05$) compared to the Lx and Sx populations (Figure 2). Recognizing that one of the main anthropogenic sources of Mn is the metallurgical industry associated with the manufacture of metal alloys and iron products (Hagelstein 2009), this observation is in agreement with the values observed in Figure 2; accordingly, the population of the industrial urban zone, Vx, is the one exhibiting the highest Mn-U levels ($4.95 \mu\text{g/g}$ creatinine), showing values higher than the recommended levels ($<2 \mu\text{g/g}$ creatinine) (ATSDR 2012; Félix 2007) and $<3 \mu\text{g/g}$ creatinine (Waldron, 2013). Regarding the Ax population, the one belonging to an urban zone in a Portuguese Island, high Mn-U levels were also observed ($4.54 \mu\text{g/g}$ creatinine) above the recommended values. It is well known that the main route of excretion for Mn is through the feces, with Mn-U considered not to be an ideal BM of exposure (Aschner et al. 2007; Zheng et al. 2010). Accordingly, we suggest that the potential reasons for these elevated values in the population living in the Ax town reflect the fact that this zone is volcanic, thus likely increasing natural exposure to metals, including Mn (Weinhold 2013), as well as potentially exposure secondary to the use of fertilizers in this area.

We also hypothesize that the presence of MMT, a gasoline additive that contains Mn (marketed in Portugal with 2mg/L according to the Decree Law n° 142/2010) may have an impact on the exposure and accumulation of this metal in individuals residing in areas with heavy traffic. As a result of MMT use in unleaded gasoline, and according to EPA's statement (US.EPA 2017) certain portions of the population may be exposed to levels in the same range as the Reference Concentration (US.EPA 2017). Additionally, in Bolté et al. study (2004), a correlation was found between Mn concentration in the atmosphere and traffic density.

With regard to As-U concentrations, we point to the Lx population, which showed higher average values with significant differences ($p<0.05$) comparing to other populations (Figure 3), with average values of $6.46 \mu\text{g/g}$ creatinine. As is mainly excreted in urine; however, it needs to be considered that average urinary As values in the Lx population ($6.5 \mu\text{g/g}$) were lower than those observed in a French study (11.9 mg/g creat) with a sample of 1500 non-occupationally exposed individuals (Saoudi et al. 2012), in a population from Montana ($8.47 \mu\text{g/g creat}$), (ATSDR 2011) as well as also lower than BLV, $30 \mu\text{g/L}$ (Rodriguez et al. 2003). We must however consider that the observed concentrations for these three metals, Pb, Mn

and As, are influenced by the existence in the environment of other metals as well as other pollutants, that will interact with the analyzed metals, altering their toxicokinetics and toxicodynamics (ATSDR 2004).

4.1 Levels of porphyrins and delta-aminolevulinic acid in urine

Several studies showed that porphyrins and other components of the heme biosynthetic pathway, may be used as sensitive BMs of exposure to toxic metals (Andrade et al. 2015; Chiba et al. 1996; Ng et al. 2005). Considering that most of the concentrations of metals in urine are below the reference values, there is an interest in observing whether there are alterations on the porphyrins and ALA concentrations in urine, used here as BMs of effect.

When analyzing Figure 4a where the values of ALA-U can be observed, we verified that all the populations had values significantly different ($p<0.05$) among them, except Sx from Vx; average higher values (1.98 mg/g creatinine) were presented in the Lx population. Ogata and Taguchi (1987) noticed that average concentrations of ALA-U in non-occupationally exposed populations were 1.18 mg/g creatinine, values that are close to those observed in our study. However, we expected that in the Vx population, the one with the highest Pb values, also had the highest ALA-U values. Indeed, there are several studies that indicate that Pb exposure induces higher ALA-U levels (Higashikawa et al. 2000; Makino et al. 2000; Prista and Sousa Uva 2003; Tong et al. 2000). Nevertheless, in Makino et al.'s study (2000), which had a large number of workers exposed to low Pb concentrations (average 52 $\mu\text{g/L}$ in blood), average ALA-U value appeared to decrease with increasing blood Pb in the range of 10.4 to 178 $\mu\text{g/L}$. However, the authors did not find an explanation for the trend observed for low levels of Pb exposure. In addition, Chiba and Kikuchi (1984) observed an interference of Mn with the heme biosynthetic pathway, inhibiting ALAD activity *in vitro*. Accordingly, the perturbation of heme biosynthetic pathways depends on each individual metal, on the interaction among metals, and on their concentrations, especially in non-occupational environments in which there is no predominant metal present.

Concerning porphyrin levels in urine (Figure 4b) we observed that the four populations presented significant differences among them ($p<0.05$), highlighting Vx and Lx populations, that displayed higher average values (117.3 $\mu\text{g/g creat}$ and 67.7 $\mu\text{g/g creat}$, respectively). When considering metals' values in the analyzed biological samples, it was observed that the Vx population had the highest concentrations (Figures 1, 2 and 3), underlining the case of Pb-U (Figure 1); it is also noteworthy that Lx was the population that stood out in relation to As-U values (Figure 3). This may explain the noted urinary porphyrins in this population, since it is well known that Pb and As, may affect heme biosynthetic pathways in mammals leading to changes in porphyrins profiles, and to increased urinary porphyrins (Chiba et al. 1996; Higashikawa et al. 2000; Makino et al. 2000; Marchiset-Ferlay et al. 2011). We also suggest that mercury environmental or food exposures, which were not determined herein, may also affect heme biosynthetic pathways (Woods et al. 1993; Khaled et al. 2016).

Woods et al. (2009) addressed that porphyrins average values in a population non-occupationally exposed was 35.2 $\mu\text{g/g creat}$; herein, we verified that only the Ax and Sx populations are below this value; this may be related to the fact that these populations are

exposed to lower metal levels. In fact, Mn as well as copper, zinc, cobalt, and cadmium, due to their similarity in physiochemical characteristics to iron, are able to interfere with normal iron absorption and metabolism and consequently with heme and hemoglobin synthesis. An increase in heme oxygenase activity resulting in depletion of cellular hemoproteins can also be induced by these metals (Garnica 1981).

4.3 Combination of biomarkers:

Figure 5 represents discriminant analysis of the populations based on some biomarkers determined in urine, Pb, As, porphyrins and ALA levels (Mn was removed from discriminant analysis due to its low diagnostic performance in this model), and it was verified a percentage of 89.8 of the cases identified correctly concerning their environmental context provenance (Table 1). Thus, Mn-U has little representativeness as a BM of exposure, corroborating that its main excretion route is feces, not urine (Casarett and Doull's 2013). The selected group of urinary BMs defines well the populations, as it can be seen in Figure 5, although the separation between Ax and Sx it is not very evident, probably because they are the populations that had lower metal levels and may reflect most similar environments.

In Figure 6, urine BMs of effect (porphyrins and ALA) evidence well the populations, with 88% of the cases grouped correctly (Table 2), being also unclear the separation of Ax and Sx populations.

In agreement with Bocca et al. 2010 and Saravanabhavan et al. 2017, we observed that the background levels of metals in human biological samples varied according to the environmental contexts provenance; in addition, it is impossible, owing to the multiplicity of chemicals present in the environment, to identify and measure all the metals or other pollutants that occur in a given environmental context. That is why it is so important to propose and use non-specific BMs of effect that may reflect the total environmental contamination. As a result of industrialization, current environmental levels of metals may be elevated relative to naturally occurring levels. Due to individual, spatial and temporal variability, this may result in a wide variability in the intake or absorption of some metals in food, drinking water, or air (US.EPA 2007; Goyer 2004; Prather 2009).

Concluding Remarks: We addressed whether it is appropriate to consider that there is what is called a "control population". Even in the same country and in nearby zones, metal exposure and the consequent levels of BMs of effect are quite different, depending on the environmental context where populations live. In fact, our study suggests that it is difficult to establish baseline values for metals.

Concerning the use of combinations of BMs as a tool to control individually adverse effects in populations co-exposed to the metals under study in these environmental contexts, we show that the use of only two urinary BMs of effect (porphyrins and ALA) resulted in a percentage of cases correctly grouped just 1% lower compared with approaches investigating additional BMs. We suggest that these two BMs can be to adequately assess environmental exposure to Pb, Mn and As, saving human and financial resources.

Finally, we propose that using combined non-specific BMs of effect to identify contexts of exposure in an individual basis may better reflect each individual's exposure than the search for one specific BM of exposure to one chemical in a group of subjects. We emphasize that the evaluation of changes in non-specific BMs may show not only the exposure to Pb, Mn and As, but also the effects on health induced by the total environmental mixture, including unknown components (that are impossible to characterize) that may interfere with heme biosynthetic pathways. In conclusion, we suggest that the subjective perception that we have in considering the environment pollution of the different areas under observation was reflected in the chosen BMs.

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Highlights

- Non-occupationally exposed human populations in distinct areas are exposed to lead, manganese and arsenic, approaching regulatory limits.
- The values found reflect the quality of the environment where these populations live;
- Porphyrin levels in urine may be used as a non-specific biomarker to control and distinguish the different study populations.
- The results show the difficulty in establishing a reference population to be compared with occupational populations exposed to metals.

Figure 1.

Urinary levels of Pb in Ax, Sx, Lx and Vx groups; N=16, 7, 25 and 23, respectively. Data represent the mean $\pm$ SD. The groups were compared by one-way ANOVA and *post hoc* Tukey's tests: * and ^ are $p < 0.05$ versus Ax and Sx.

Figure 2. Urinary levels of Mn in Ax, Sx, Lx and Vx groups; N=16, 7, 25 and 23, respectively. Data represent the mean $\pm$ SD. The groups were compared by one-way ANOVA and *post hoc* Tukey's tests: *, ^ and # are $p < 0.05$ versus Ax, Sx and Lx.

Figure 3.

Urinary levels of As in Ax, Sx, Lx and Vx groups; N=16, 7, 25 and 23, respectively. Data represent the mean $\pm$ SD. The groups were compared by one-way ANOVA and *post hoc* Tukey's tests: *, ^ and # are $p < 0.05$ versus Ax, Sx and Lx.

Figures 4a and b.

Urinary levels of ALA (a) and porphyrins (b) in the Ax, Sx, Lx and Vx groups; N=16, 7, 25 and 23, respectively. Data represent the mean $\pm$ SD. The groups were compared by one-way ANOVA and post hoc Tukey's tests: *, ^ and # are $p < 0.05$ versus Ax, Sx and Lx, respectively.

Figure 5.

Graphical representation of each subject classification taking into account their levels of urinary Pb, As, ALA and porphyrins (Predicted group). A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions. N=16, 7, 25 and 23 in Ax, Sx, Lx and Vx groups, respectively. Each symbol represents a single individual.

Group identification of each subject through their levels of the urinary biomarkers of effect ALA and porphyrins

Figure 6.

Graphical representation of each subject classification taking into account their levels of the urinary biomarkers of effect, ALA and porphyrins (predicted group). A centroid value was calculated for each group and the results were plotted by the canonical discriminant functions. N=16, 7, 25 and 23 in Ax, Sx, Lx and Vx groups, respectively. Each symbol represents an individual.

Table 1:

Individuals (%) belonging to Ax, Sx, Lx and Vx groups, were classified by discriminant analysis using the linear combination of their levels of urinary Pb, As, ALA and porphyrins (Predicted group), *vs* their true origin (Group). N=16, 7, 25 and 23 in Ax, Sx, Lx and Vx groups, respectively.

Group	Predicted group				Total
	Ax	Sx	Lx	Vx	
Ax	88.9	11.1	0.0	0.0	100.0
Sx	0.0	100.0	0.0	0.0	100.0
Lx	9.5	0.0	90.5	0.0	100.0
Vx	7.7	7.7	0.0	84.6	100.0

89.8% of the subjects are classified correctly

Table 2:

Individuals (%) belonging to Ax, Sx, Lx and Vx groups classified by discriminant analysis taking into account the linear combination of their levels of effect biomarkers, urinary ALA and porphyrins (predicted group) vs their real (true) home group. N=16, 7, 25 and 23 in Ax, Sx, Lx and Vx groups, respectively.

Group	Predicted group				Total
	Ax	Sx	Lx	Vx	
Ax	77.8	22.2	0.0	0.0	100.0
Sx	0.0	100.0	0.0	0.0	100.0
Lx	9.5	0.0	90.5	0.0	100.0
Vx	7.7	14.3	0.0	79.1	100.0

88.0% of the subjects are classified correctly.