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Original Article

Ethnic characterization of a population of children exposed to high doses of arsenic via drinking water and a possible correlation with metabolic processes

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Abstract: Because the ratio between the two major arsenic metabolites is related to the adverse health effects of arsenic, numerous studies have been performed to establish a relationship between the ability to metabolically detoxify arsenic and other variables, including exposure level, gender, age and ethnicity. Because ethnicity may play a key role and provide relevant information for heterogeneous populations, we characterized a group of 70 children from rural schools in the Argentinean provinces of Chaco and Santiago del Estero who were exposed to high levels of arsenic. We used genetic markers for maternal, paternal and bi-parental ancestry to achieve this goal. Our results demonstrate that the Amerindian maternal linages are present in 100% of the samples, whereas the Amerindian component transmitted through the paternal line is less than 10%. Informative markers for autosomal ancestry show a predominantly European ancestry, in which 37% of the samples contained between 90 and 99% European ancestry. The native American component ranged from 50 to 80% in 15.7% of the samples, and in all but four samples, the African component was less than 10%. Correlation analysis demonstrated that the ethnicity and the ratio of the excreted arsenic metabolites monomethyl arsenic and dimethyl arsenic are not associated, dismissing a relationship between ethnic origin and differential metabolism.

Keywords: Arsenic, children, ethnic characterization, ancestral informative markers

Introduction

Complex human diseases and differential biological responses have been linked to multiple genetic polymorphisms through association studies and candidate genes. In these studies, the cohort selection represents a key aspect that may be determined, in part, by the genetic structure of the studied population and its ethnic background. Therefore, evidence based on genetic marker analysis reveals differences between populations that may bias the results in population-based case-control studies [1, 2].

Ancestry informative markers (*AIMs*), including maternal, paternal and bi-parentally transmitted markers are tools for investigating genetically heterogeneous populations [3, 4]. On the one hand, maternal inheritance can be established by analyzing the entire mitochondrial control region (HVI, II and III) as well as the coding region for single nucleotide polymorphisms (SNPs) [5-8], whereas paternal linages can be characterized using SNPs located in the non-recombinant region of the Y chromosome [9-12]. On the other hand, autosomal SNPs provide information about admixture between different groups with bi-parental inheritance [13, 14]. By combining these markers, it is possible to obtain reliable information about the ethnicity of each individual investigated, allowing an association between ethnicity and the physiological response to different compounds, such as arsenic (As), to be established.

Arsenic is a widely distributed metalloid that is present in surface water, ground water, land and food. In many Argentinean regions, such as the Chaco-Pampean plain, Cuyo and Puna, high levels of arsenic have been found in water sources, including rivers, lakes and groundwa-

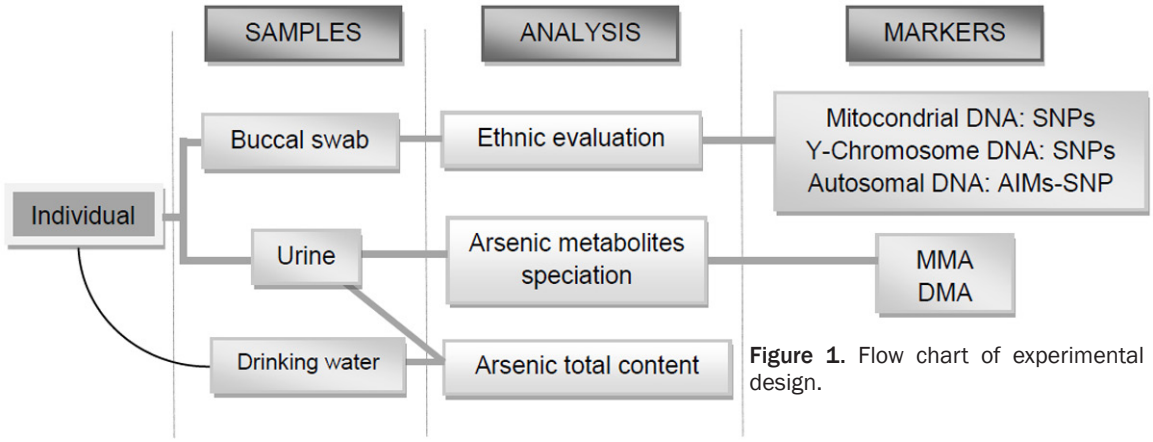

Figure 1. Flow chart of experimental design.

ter, primarily in rural areas [15-17]. In the Chaco-Pampean region, high levels of As were detected in ground water, whereas in the Puna region, As was also found in the surface water [18]. Anthropogenic activity has a minimal influence on these high As levels because the occurrence of natural arsenic as a result of various geological processes is the main contributor to the observed levels. Over 80% of the analyzed water samples from endemic regions showed As levels that exceeded the WHO recommendations (10 μg/l) by over 200-fold [19-21].

Thus, more than 4 million people in Argentina are exposed to high levels of As in their drinking water. Acute exposure to As-contaminated drinking water causes anemia, neuropathies, hyper-pigmentation and irritation of the skin, mucous membranes and gastrointestinal tract. Chronic exposure has been associated with hyperkeratosis, loss of pigmentation and skin, bladder, and lung cancers [22-24]. Among the exposed populations, children represent the most vulnerable segment, showing low cognitive development, detrimental effects in long-term memory, changes in DNA and immunodepression as a consequence of chronic exposure [25].

However, the appearance of these symptoms is related to the differential susceptibility to arsenic toxicity and may be associated with differences in arsenic metabolism. Biomethylation is the main metabolic pathway for inorganic arsenic, and arsenic-3-methyltransferase (As3MT) catalyzes the synthesis of the two major methylated metabolites, monomethyl arsenic (MMA) and dimethyl arsenic (DMA) acids [26, 27]. MMA is primarily related to the adverse health effects of As because of its low elimination rate compared to DMA. The MMA:DMA excretion ratio provides information regarding the level of harmful metabolites retained in the body, and it is hypothesized that this ratio is determined primarily by genetic variations. However, during the last few years, several factors have also been related to metabolic capacity, such as exposure level, gender, age, smoking habits, nutritional state and ethnicity [28-31]. Differences in arsenic methylation capacity were observed in populations from different regions, and several studies suggested a link between methylation capacity and ethnicity [32-34]. Hopenhayn-Rich et al. [35] assessed individuals from two towns located in the high Atacama Desert of northern Chile, who were descended from a population that lived in the region for 11,000 years. Their findings suggest that Atacameños have a lower MMA:DMA ratio compared to subjects with European ancestry, which may be related to an evolutionary adaptation over thousands of years that rendered the Atacameños less susceptible to arsenic. However, ethnic classification of the samples used in the previous studies has not performed with an AIMs analysis, which makes it difficult to accurately establish whether they belong to their declared ethnicity.

Because an AIMs analysis was not available when the previous studies were performed, the objective of this study was to analyze a group of children exposed to high levels of arsenic using ancestry informative genetic markers to determine ethnic origin and evaluate the relationship between ethnicity and arsenic methylation capability.

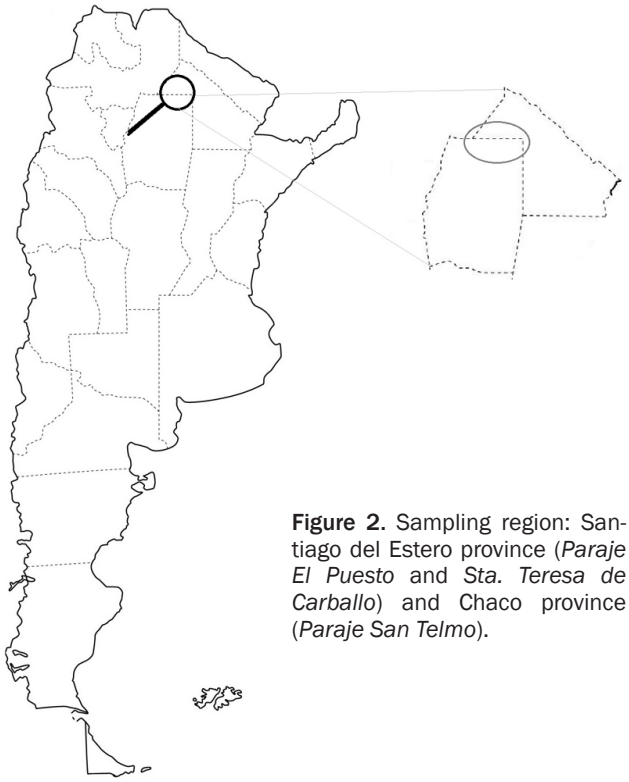

Figure 2. Sampling region: Santiago del Estero province (*Paraje El Puesto* and *Sta. Teresa de Carballo*) and Chaco province (*Paraje San Telmo*).

Material and methods

Experimental design was summarized as in **Figure 1**.

Samples

Samples were collected from 70 unrelated children, 40 girls and 30 boys, ranging from 2 to 16 years old, from 3 rural schools. Informed consent was signed by the children's parents, and the study was approved by the Ethical Committee of the Hospital de Clínicas "José de San Martín" (Buenos Aires, Argentina). The schools are located on the border zone between the north area of Santiago del Estero province (Paraje el Puesto *26,1275200 S – 63,7013300 W* and Sta. Teresa de Carballo *25,5800000 S – 63,1987400 W*) and Chaco province (Paraje San Telmo *26,0827500 S – 62,5614000 W*) (**Figure 2**). According to INDEC 2010 [36], economic activities are scarce in this area, and the socioeconomic level is very low, with unsatisfied basic needs, such as the tap water supply. Each donor provided a urine sample and buccal swab to measure the arsenic levels and perform a genetic analysis, respectively. Water samples from the declared sites of residence were also analyzed to determine the arsenic exposition level. The majority of individuals are native of this region, at least for the two previous generations.

DNA extraction and quantitation

DNA extraction was performed using the Nexttec Genomic DNA Isolation Kit for Tissue and Cells according to manufacturer's protocol. DNA quantitation was performed by real time PCR using a commercial kit (Plexor1 HY, Promega, USA) and a Rotor-Gene 6000 (Corbett, Australia) Real Time PCR machine.

Ethnic markers

Autosomal DNA: Twenty-four autosomal SNPs were analyzed according to the method of Lao et al. SNP analysis was used to determine three continental ancestry components, Sub-Saharan Africa, Eurasia, and Native America. The SNPs used in the study were as follows: rs1876482, rs2179967, rs1048610, rs1371048, rs1478785, rs1369290, rs952718, rs1405467, rs1344870, rs1391681, rs1461227, rs1907702, rs2052760, rs714857, rs721352, rs722869, rs926774, rs1448484, rs1667751, rs1858465, rs1465648, rs16891982, rs1808089, and rs3843776. SNP analysis was performed in two multiplex SNaPshot reactions. The genotype methodology was based on the principle of primer extension (Applied Biosystems, Foster City, USA). Capillary electrophoresis was performed with an ABI-3100 *Avant* (Applied Biosystems, Foster City, USA) and the results were analyzed using GeneMapper ID 3.2 (Applied Biosystems, Foster City, USA).

Mitochondrial DNA: All samples were typed for Native American linages by analyzing the SNPs at positions 8027 and 12007 (HgA2), 3547 (HgB2), 14318 (HgC) and 2092 (HgD1). The most frequent European haplogroup (HgH) was investigated by analyzing the SNP at position 7028, whereas HgI and HgL1/2 were evaluated by analyzing positions 10398 and 3594, respectively. These polymorphisms were amplified in two multiplex reactions and analyzed by real-time PCR (RotorGene). The reaction mix was prepared in a volume of 25 µl, and 20 pmol of each pair of primers was added to 5 X buffer

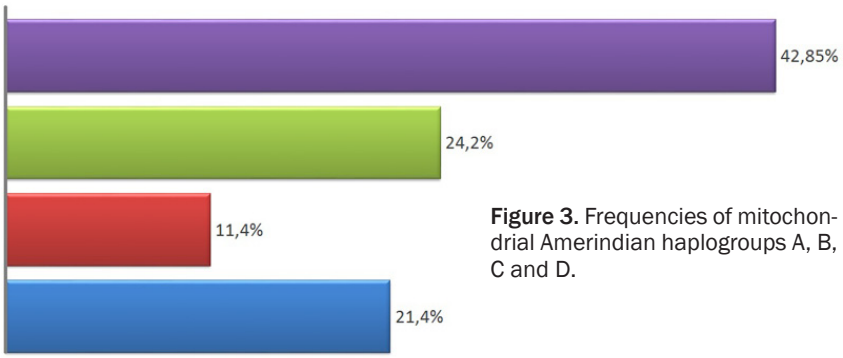

Figure 3. Frequencies of mitochondrial Amerindian haplogroups A, B, C and D.

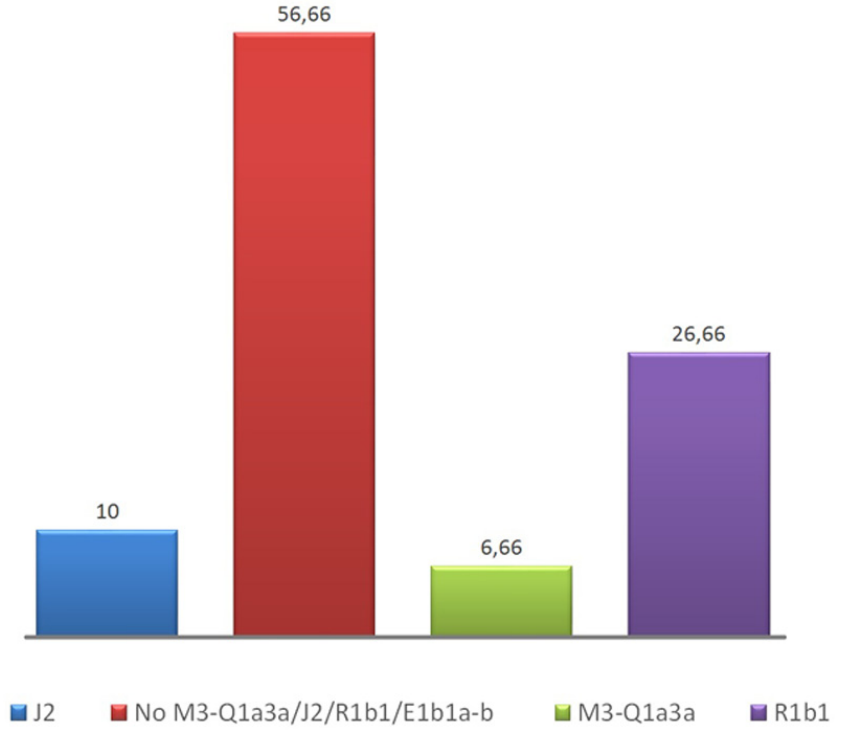

Figure 4. Frequencies of Y-chromosome haplogroups European (J2, R1b1), Amerindian (M3Q1a3a), non M3Q13a-J2.R1b1-E1b1a/b.

(MgCl: 1,5 mM), dNTPs (25 mM), Syto 9 (25 mM) and Go-Taq Polymerase (5000 U/ml, Promega, Madison, USA). The SNPs analyzed in multiplex I were 8027, 12007, 3547, 14318 and 2092, whereas the SNPs analyzed in multiplex II were 7028, 10398 and 3594. The cycling conditions were as follows: hold 95°C for 2 min followed by 30 cycles of 95°C 20 s, 56°C 30 s, 72°C 40 s and a final extension a 72°C for 2 min. High resolution melting was analyzed between 68°C and 85°C.

Y chromosome DNA: For the Y-chromosome haplogroup assessment, the most prevalent haplogroups in the Argentinean population were analyzed. R1b1 (SNP 269), I (SNP 179) and J2 (SNP M172) are the European haplogroups. The Native American M3-Q1a3a haplogroup (DsYS199) and African haplogroup E1-b1a/b (SNPs M35 and M2) were also selected. The reaction mixes for multiplex I (M3-Q1a3a, I and E1b1b) and multiplex II (R1b1, J2 and E1b1a) were prepared in a volume of 25 µl, with 10 pmol of each primer pair except M35, 269 and M172 (20 pmol primer forward and 40 pmol primer reverse). This was added to 5x buffer (MgCl: 1,5 mM), dNTPs (25 mM), Syto 9 (25 mM) and Go-Taq Polymerase (5000 U/ml, Promega, Madison, USA). The cycling conditions were as follows: hold 95°C for 2 min followed by 30 cycles of 95°C 20 s, 56°C 30 s, 72°C 40 s and a final extension at 72°C for 2 min. High resolution melting analysis was performed between 68 °C and 85°C.

Arsenic and metabolite analysis in water and urine

The total As content in water and urine samples was determined by flow injection-hydride generation-atomic absorption spectrophotometry (FI-HG-AAS) (Varian® VGA77, AA475) according to Navoni et al. [37, 38]. The urinary concentration of DMA (III+V) and MMA (III+V) was measured using high performance liquid chromatography (Thermo Separation Products®, P4000, AS3000) coupled to hydride generation atomic absorption spectrophotometry (Varian® VGA77, AA475) [39].

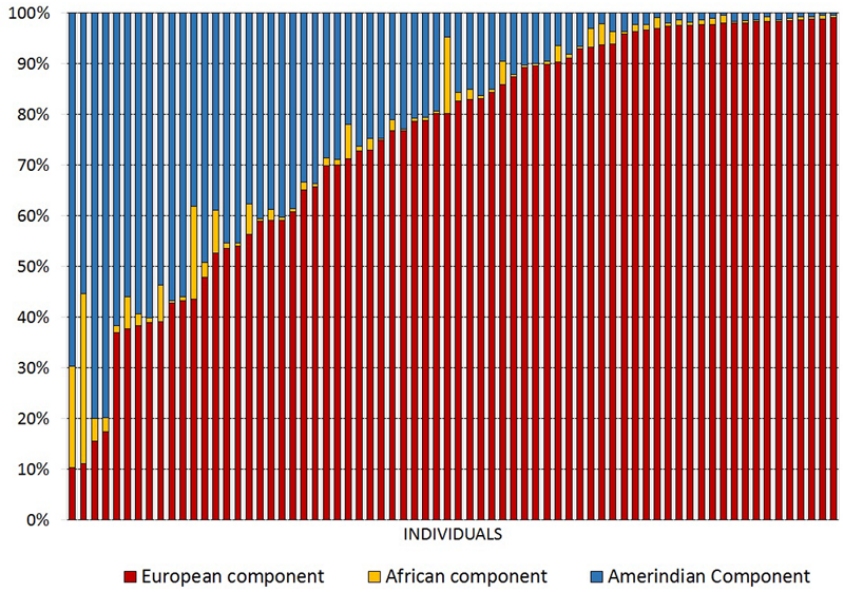

Figure 5. Bar plot in which each bar represents an individual. Ancestry estimation based on STRUCTURE 2.0 of the individual exposed to arsenic, assuming three parental populations, Amerindian, European and African.

Statistical analysis

The autosomal AIMs results were analyzed using STRUCTURE 2.0 [40] with 10.000 burnings. Three parental populations were assumed and compared to samples of individuals (HGDP-CEPH panel) that represent the closest geographic relatives of the most likely true parental populations for Argentineans (158 European, 44 native South Americans and 49 West Sub-Saharan Africans). Multi-dimensional-scaling (MDS) plots were obtained using SPSS 17.0 (SPSS for Windows, Rel. 17.0 2008. SPSS Inc., Chicago, Illinois, USA). The plot was performed with an IBS (Identity-by-state) distance matrix calculated using the R software [41]. Correlation analysis between ethnicity and arsenical metabolites was evaluated with non-parametric (Kendall's Tau_b and Spearman's Rho) statistics tools using SPSS 17.0 (SPSS for Windows, Rel. 17.0 2008. SPSS Inc., Chicago, Illinois, USA).

Results & discussion

Uniparental markers

The complete set of 70 analyzed samples showed Amerindian maternal lineages when SNPs located in mitochondrial DNA were studied, and the four Pan-American haplogroups A, B, C and D were detected. The most frequent haplogroups were C and D1, representing over 50%, whereas the B2 haplogroup was observed in only eight samples (**Figure 3**). The observed proportions are inconsistent with results obtained in a previous study in which a higher percentage of the haplogroup B2 was detected in Amerindian groups (Toba and Wichis) inhabiting a nearby area [42-45]. These differences might be related to sampling because all the samples were collected from children of different schools, whereas the samples in the previous studies were collected from well-defined Aboriginal communities. Moreover, there are no previous data for the ethnic composition of Aboriginal populations located in the border area between Chaco and Santiago del Estero where the sampling was performed. Therefore, these results could indicate either an aboriginal community with a different proportion of mitochondrial haplogroups or most likely, an admixed population sample because these three schools host children from different areas.

The analysis of Y-chromosome SNPs identified less than 10% (2 boys) of the Amerindian paternal linage (haplogroup M3-Q1a3a), and 36% of the samples belonged to the European haplogroups R1b1 and J2, whereas the remaining samples were classified as non R1b1, J2, M3-Q1a3a or E1b1a/b (African origin) (**Figure 4**). The samples that could not be classified most likely belong to other European haplogroups not typed in this work. The low percentage of Amerindian paternal linages observed is consistent with previous studies in Argentina and other Latin American countries [46-49] and may be the result of a severe reduction in the Native American male population caused by the conquest, as well as an augmented proportion of offspring between

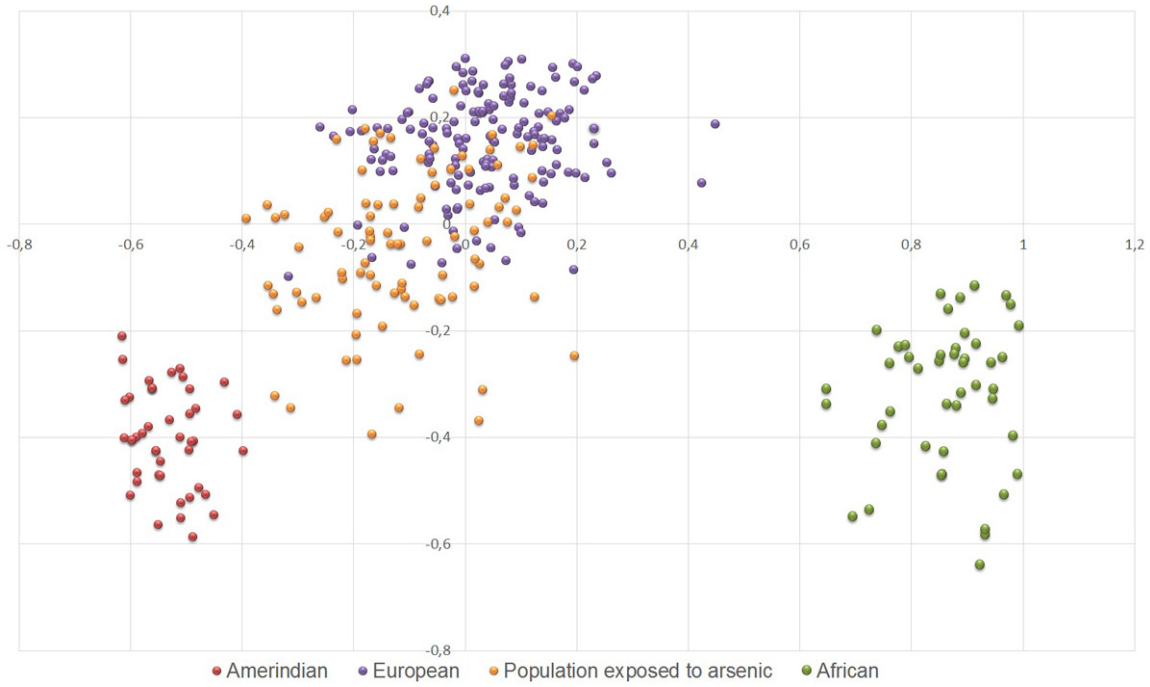

Figure 6. Multi-dimensional-scaling plot of a population exposed to arsenic together with three assumed parental populations, Amerindian, European and African.

Table 1. Correlation analysis based on *Kendall's tau_b* and *Spearman's Rho* tests

	MMA:DMA	
Kendall's Tau B	0.164	AIMs
Significance	0.078	
Spearman's Rho	0.216	AIMs
Significance	0.075	

European males and Native American women, which increases the population in the occupied territories.

Bi-parental markers

Individual clustering analyses were performed using information from 24 autosomal ancestry-sensitive SNPs. The samples were compared with three groups, incorporated as the parental population in the STRUCTURE analysis (European, Native Americans and Sub-Saharan Africans). The results show a predominantly European ancestry, in which 37% of the samples contained between 90 and 99% European component. The Native American component ranged from 50 to 80% in 15.7% of the observed samples, and in all but four samples, the African component was less than 10% (**Figure 5**). Although there was a predominantly European component, most likely related to recent immigrations, over 50% of the samples showed high intra-individual heterogeneity, with a strong Amerindian component, which may be associated with the admixture that occurred in the early days of the conquest. The multi-dimensional-analysis was performed with IBS matrix distances and revealed similar results as shown in **Figure 6**. The majority of samples clustered closest to Europeans, and some appeared between two parental groups, Europeans and Native Americans, indicating some degree of admixture. Only two samples clustered close to Native Americans and no sampled appeared close to African.

The high proportion of Amerindian maternal lineages observed in the mitochondrial DNA analysis showed no correlation with the results of the AIMs, most likely due to recombination processes that occurred in the autosomal chromosomes through the 20 generations of contact, in which the genetic information provided by the ancestral maternal lineages would have disappeared and the European contribution increased in the recombinant segment. The major European component obtained after the analysis of Y-chromosome SNPs and AIMs is

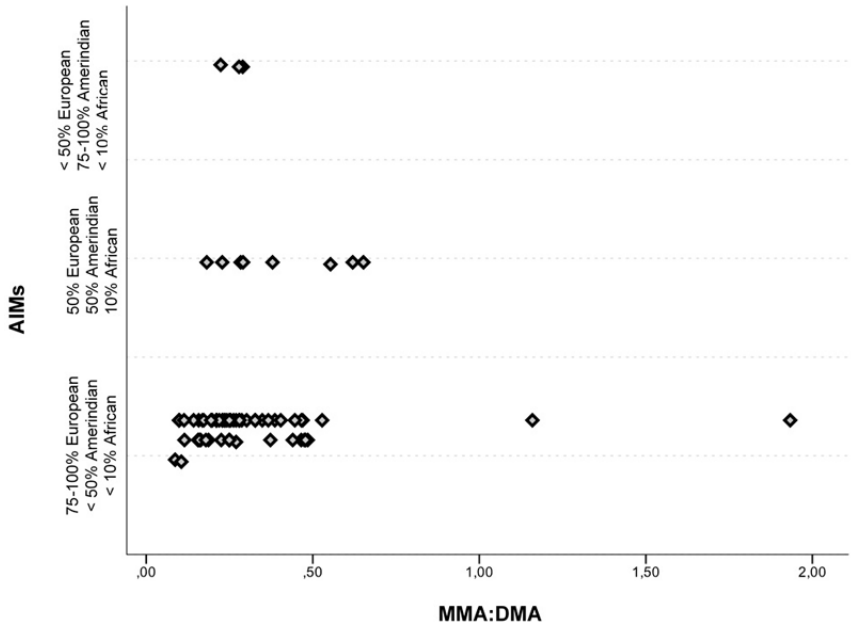

Figure 7. Correlation analysis between the MMM-DMA ratio and ethnical composition based on the autosomal AIMs results.

consistent with previous studies for the Argentinean population [50, 51]. Because of the degree of admixture in the current population, the use of AIMs for ethnic characterization emerges as a necessary step when ethnicity is intended to be incorporated as a variable of analysis, even in isolated self-declared Amerindian groups in which a strong European influence has been detected.

Arsenic metabolism and ethnicity correlation analysis

The urinary arsenic content ranged from 18 to 5000 μg/g creatinine, and only two samples were within the acceptable range below 100 μg/g creatinine. The total arsenic in the water samples was more than one hundred times the level reported by the WHO (10 μg/l) as the safe level. To perform the correlation analysis, the samples were divided into three groups according to the autosomal AIMs results based on the prevalent ethnic composition. The results of the correlation analysis (AIMs vs. MMA:DMA) showed no-correlation between variants (significance level 0.01 and 0.05) when *Kendall's tau_b* and *Spearman's Rho* tests were used (**Table 1**); therefore, there was no association between ethnicity and the excreted MMA:DMA. Evaluation of the three individuals with the highest Amerindian component (70-79%) showed a MMA:DMA ratio between 0.15 and 0.27, and similar results were obtained when individuals with a greater than 95% European component were analyzed, as shown in **Figure 7**.

Conclusions

Our analysis demonstrates that similar to other populations of Argentina, the Amerindian heritage is high in maternal lineages. The proportion of Amerindian haplogroups was higher than in other urban Argentinian populations, possibly because the sampling area is in a region with the highest concentration of Aboriginal groups.

By contrast, the low proportion of Amerindian paternal lineages and the prevalence of European paternal lineages are consistent with historical events when native men abruptly disappeared during the conquest process. In addition, the low number of European women present in the past favored the admixture between European men and native women, which originates this biased gene flow.

It was not possible to correlate the ethnic background with the ability to metabolize arsenic, excluding metabolic differences as part of an adaptive process to the environmental conditions.

To the best of our knowledge, this is the first study in which ethnic characteristics were evaluated using molecular tools in a population exposed to high doses of arsenic. In this context, it would be beneficial to extend this study to other exposed groups by considering different characteristics, such as age and geographic location, to gather enough evidence to generalize our conclusions.

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Disclosure of conflict of interest

None.

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