Is prenatal arsenic exposure associated with salivary cortisol in infants in Arica, Chile? An exploratory cohort study

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INTRODUCTION

Environmental exposure to inorganic arsenic (iAs) through drinking water or consumption of polluted food remains a public health concern for countries with natural arsenic pollution [1]. In fact, iAs leads the priority list proposed by the Agency of Toxic Substances and Disease Register (ATSDR) as a public health issue due to its ubiquity, toxicity, and potential for human exposure [2].

There is strong evidence of an association between high-level iAs exposure and different types of cancer (e.g., bladder, lung, kidney, and skin), cardiovascular diseases, skin lesions, and higher mortality rates compared to the general population [3]. Many countries have implemented water-processing plants, reducing the current exposure levels considerably. However, recent data have suggested that even low-level arsenic exposure is associated with negative health outcomes in adults and children [4, 5, 6].

Exposure during critical periods, such as gestation, may be considered as an environmental stressor under the Developmental Origins of Health and Disease (DOHaD) paradigm [7]. In mouse models, arsenic exposure causes disruption of early developmental processes with less expression of glucocorticoid receptors (GR) in the hippocampus and hypothalamic-pituitary-adrenal (HPA) axis [4]; consequently, less expression of GR is related to increased corticosterone basal concentration. Mice with higher corticosterone concentration show impairment of spatial-learning and memory functions [5, 6]. Despite the experimental validity, there is no certainty whether low-level arsenic exposure during pregnancy would have the same effects in humans. If that were the case, basal cortisol concentrations would be affected in children exposed during gestation, specifically during the neurodevelopment stage.

Cortisol is the analogous hormone of corticosterone in humans. Cortisol concentrations are influenced by natural circadian rhythms and can be modified by socio-economic status (SES), working conditions, childhood trauma, mental illness, medical treatments, and pathologies [8, 9, 10]. In children, cortisol concentrations correlate with maternal concentration [11]. For most full-term infants, the circadian
rhythm of cortisol is fully developed after the first year of life [12], implying that age must be taken into account when studying children’s cortisol.

OBJECTIVE

The aim of this study was to explore the association between prenatal iAs exposure and salivary cortisol in a cohort of infants, and compare human findings with experimental evidence studied on mice. Additionally, the modifying effect of SES on the relationship between arsenic and cortisol was examined. The infants in this study were born in Arica, a city in the north of Chile, with a history of natural and anthropogenic arsenic pollution [13]. A recent study showed a broad low-level iAs variability in this area [14], which made the exploration of the effect of arsenic exposure on cortisol feasible.

MATERIALS AND METHOD

A prospective cohort of pregnant women between their thirteenth and twenty-eighth week of gestation was recruited. The sampling frame was comprised of 591 women who accessed health services in public health centres in Arica, Chile; all women with a singleton pregnancy were invited to participate. In total, 242 women invited to be part of the study accepted, signed an informed consent, completed a socio-demographic and health history questionnaire, and provided a urine sample. The research protocol, questionnaires, and consent forms were reviewed and approved by the Ethical Committee of the Faculty of Medicine of the University of Chile in Santiago (Project Nº 069–2014).

Each woman was asked to provide a urine sample to assess iAs exposure between June – October 2013. Using pre-labeled urine containers, the samples were collected at their home, stored frozen, and sent to Trace Metals Core Laboratory of Columbia University in New York, USA. Urine iAs was determined by high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICPMS), separating and quantifying each of the following metabolites: arsenocholine, arsenobetaine, arsenenate (AsV), arsenite (AsIII), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA). Total urinary iAs was considered as the sum of AsV, AsIII, MMA, and DMA. No value was under the limit of detection.

Salivary cortisol collection and analysis in infants. Families were contacted when children were between 18–24 months old; this period was chosen since younger children have greater variability of cortisol concentration [15]. Fieldwork was conducted between July – October 2015. Trained staff took salivary samples from infants and mothers between 09:00–12:00 in a previously scheduled visit. A swab was placed in the child’s mouth for 60 seconds and then transported and frozen in a swap storage tube, following the manufacturer’s protocol. The same procedure was followed with mothers [16].

Each salivary sample was thawed and centrifuged before analysis. Cortisol concentration was measured using an expanded range high sensitivity salivary cortisol ELISA Salimetric kit. Sample analyses were performed at the Biotechnology Laboratory of Bernardo O’Higgins at the University of Chile in Santiago, Chile. All samples were analyzed in duplicates.

In a previous pilot study, a test was performed to determine salivary cortisol variability, the reproducibility of the sampling and analytical technique in the same age group. The distribution of salivary cortisol was asymmetric with a median of 0.15 μg/dL (min: 0.073; max: 0.266). Duplicate sampling showed a concordance index (rho) of 0.73, while duplicate analytical technique reached 0.96.

Covariates. A theoretical causal framework between arsenic exposure during pregnancy and salivary cortisol in infants was developed based on previous literature (Fig. 1), using the directed acyclic graph (DAG) approach. The potential pathway between prenatal arsenic exposure and salivary cortisol in infants was based on murine models proposed by Martínez and Allan [4, 5, 6]. Reduced expression of glucocorticoid receptor (GR) was demonstrated in mice exposed to arsenic during gestation. Decreased hippocampal GR levels were associated with increased basal plasma corticosterone levels in mice exposed prenatally to arsenic. The same mechanism is proposed for children, expecting a slight increase in basal concentration of cortisol during the neurodevelopment stage. As a negative feedback loop was not reported in mouse offspring, it was also assumed that this physiological mechanism would not be active in children, experiencing smooth increases in cortisol due to gestational exposure to iAs. Additionally, other variables related to cortisol control that could open back-door paths and confound the relationship between the arsenic exposure and cortisol in infants were considered: socio-economic status, having had a stressful pregnancy, mother’s depression, and cortisol in mothers.

Figure 1. Proposed DAG for the arsenic-cortisol association

Socio-demographic information, such as monthly family income (< 307 USD, 307–614 USD, >614 USD), mother’s educational level (years of education), ethnicity (mixed-race, Aymara or other) and occupation (student, homemaker, paid work) were obtained via in-person questionnaires during the first visit. Mothers reported having had a stressful pregnancy (very low or low, moderate, high or very high) and history of depression (medical diagnosis) in a second home visit the year after delivery.

For saliva collection, the time of collection, gender and age of infants, and corticoid use (both the infant and the mother) were registered. Additionally, the α-amylase activity (U/mL) was used to rule-out the autonomic nervous system activation...
due to the sample collection process as a source of stress [17]. α-amylase activity varies between 3.1–423.1 U/mL in healthy populations, according to the manufacturer (Salimetrics, LLC.).

**Statistical analyses.** Univariate analyses included central and dispersion measures for continuous variables (median and interquartile range), and frequencies for categorical variables. Bivariate comparison of urinary iAs or salivary cortisol concentration among covariates were performed using non-parametric tests (Mann-Whitney and Kruskal-Wallis test). Given the non-linear relationship between total urinary iAs and salivary cortisol, arsenic exposure was categorized into quartiles, and salivary cortisol was transformed using the natural logarithm.

Multiple linear regression was used to estimate the effect of prenatal arsenic exposure on the cortisol concentration in infants. Maternal cortisol, a stressful pregnancy, and depression were controlled in the multiple regression analyses; these were considered as potential back-door covariates (Fig. 1) [18, 19]. In addition, the DAG in this study reflected the conceptual framework of the social determinants of health inequalities: poor working conditions, work overload, poor material conditions, and social vulnerability realities [20], which have been linked to changes in the cortisol concentration [21, 22, 23]. In children, a useful proxy for socio-economic status is the monthly family income, which was considered as a potential modifier of the association between arsenic exposure and cortisol concentration.

The goodness of fit of the linear regression model was evaluated considering:
1) multi-collinearity analysis using a variance inflation factor; 2) outlier detection through Cook’s distance and leverage against residual squared plot; 3) normality residual analysis using Kernel density plot, Shapiro-Wilk test, and quantile-quantile plots.

A sensitivity analysis was performed to evaluate the robustness of the regression coefficients. Missing data on salivary cortisol (n=74) were ascribed by multiple imputation procedures, and coefficients for linear regression models with and without imputation were contrasted thereafter. Epidata Entry v3.1 was used to create the database. Data were entered in duplicate to avoid typing errors and conduct quality control checks. Stata IC v12.0 was used for all analyses.

**RESULTS**

Between 2013–2015, data collection was completed for 168 infants from the initially enrolled 242 pregnant women. Seventy-four participants dropped-out during the follow-up. Three stillbirths and 30 withdrawals (3 refused to continue, 10 moved out of town, and 17 were not available after 3 home visits) occurred during the first year; an additional 41 subjects were lost in the 2nd year. Baseline characteristics are shown in Table 1.

**Urinary iAs in pregnancy.** The distribution of total urinary iAs and each arsenical species were asymmetric with a positive bias; the median was 14.1 µg/L (Interquartile Range, IQR: 10.4–21.7). The most prevalent species was DMA. According to the occupational cut-off for arsenic exposure [13], 14 samples (8.3%) were above 35 µg/L. Infant gender (p=0.140), mother's education level (P=0.960), and cortisol in mothers (P=0.732) were independent of urinary iAs. Family income, occupation, ethnicity, stressful pregnancy, and maternal depression were not associated with urinary iAs concentration (Tab. 2).

**Salivary cortisol in infants.** Salivary cortisol distribution was skewed with a positive bias. The median was 0.17 µg/dL (IQR: 0.11–0.38) with minimum and maximum values of 0.0096 µg/dL and 1.3 µg/dL, respectively. α-amylase activity, a biomarker for acute stress in infants, and educational level in mothers were independent of cortisol concentration (P=0.196 and P=0.683, respectively). There were no differences between males and females regarding cortisol concentration (P=0.572).

Maternal cortisol concentration (used as a biomarker for stress) was linearly correlated with cortisol concentrations in infants (r=0.791; P<0.0001). Family income, ethnicity, occupation, stressful pregnancy, and diagnosed depression were not associated with salivary cortisol (Tab. 3).

**Association between urinary iAs and salivary cortisol in infants.** In multiple linear regression analyses, the relationship between urinary iAs and salivary cortisol varied according to family income (Tab. 3). In infants belonging to the < 307 USD family income stratum and between 307–614

**Table 1.** Baseline characteristics of 168 participants, Arica 2013

<table>
<thead>
<tr>
<th>Infant Age (months)</th>
<th>n (%)</th>
<th>Median (IQR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>85 (50.9)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>82 (49.1)</td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>39 (39–40)</td>
<td></td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>25 (21–30)</td>
<td></td>
</tr>
<tr>
<td>Maternal education (years of total education)</td>
<td>12 (12–14)</td>
<td></td>
</tr>
<tr>
<td>Maternal Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed-raced</td>
<td>113 (67.7)</td>
<td></td>
</tr>
<tr>
<td>Aymara</td>
<td>47 (28.1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>7 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Monthly family income</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 307 USD</td>
<td>63 (38.2)</td>
<td></td>
</tr>
<tr>
<td>307 – 614 USD</td>
<td>70 (42.4)</td>
<td></td>
</tr>
<tr>
<td>&gt; 614 USD</td>
<td>32 (19.4)</td>
<td></td>
</tr>
<tr>
<td>Maternal Occupation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Student</td>
<td>40 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Housewife</td>
<td>76 (47.5)</td>
<td></td>
</tr>
<tr>
<td>Remunerated job</td>
<td>44 (27.5)</td>
<td></td>
</tr>
<tr>
<td>Stressful pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low or low</td>
<td>54 (33.1)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>84 (51.5)</td>
<td></td>
</tr>
<tr>
<td>High or very high</td>
<td>25 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Maternal depression diagnosed by medical doctor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>37 (22.7)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>126 (77.3)</td>
<td></td>
</tr>
</tbody>
</table>

1 Interquartile Range: Centile 25 – Centile 75.
Table 2. Urinary iAs concentration in 168 pregnant women (Arica, 2013) and salivary cortisol concentration in 168 infants (Arica, 2015) by covariates

<table>
<thead>
<tr>
<th>Covariates</th>
<th>iAs µg/L Median (IQR)</th>
<th>P</th>
<th>Cortisol µg/dL Median (IQR)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monthly family Income</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 307 USD</td>
<td>14.6 (10.4–21.7)</td>
<td>0.923</td>
<td>0.174 (0.121–0.392)</td>
<td>0.515</td>
</tr>
<tr>
<td>307 – 614 USD</td>
<td>14.0 (10.5–23.0)</td>
<td>0.145</td>
<td>0.098 (0.365)</td>
<td></td>
</tr>
<tr>
<td>&gt; 614 USD</td>
<td>14.1 (11.5–20.8)</td>
<td>0.196</td>
<td>0.117–0.402</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed-race</td>
<td>13.8 (10.4–20.9)</td>
<td>0.395</td>
<td>0.179 (0.115–0.382)</td>
<td>0.240</td>
</tr>
<tr>
<td>Aymara</td>
<td>15.4 (11.1–22.6)</td>
<td>0.170</td>
<td>0.104–0.408</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>18.3 (9.3–28.3)</td>
<td>0.127</td>
<td>0.104–0.150</td>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Student</td>
<td>14.2 (10.0–21.7)</td>
<td>0.755</td>
<td>0.181 (0.111–0.439)</td>
<td>0.743</td>
</tr>
<tr>
<td>Housewife</td>
<td>13.9 (11.8–20.8)</td>
<td>0.165</td>
<td>0.092–0.426</td>
<td></td>
</tr>
<tr>
<td>Remunerated job</td>
<td>15.0 (10.5–23.0)</td>
<td>0.167</td>
<td>0.118–0.329</td>
<td></td>
</tr>
<tr>
<td><strong>Stressful pregnancy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low or low</td>
<td>14.5 (10.4–25.5)</td>
<td>0.955</td>
<td>0.165 (0.113–0.330)</td>
<td>0.219</td>
</tr>
<tr>
<td>Moderate</td>
<td>14.6 (10.5–20.2)</td>
<td>0.247</td>
<td>0.111–0.641</td>
<td></td>
</tr>
<tr>
<td>High or very high</td>
<td>12.8 (10.0–16.0)</td>
<td>0.156</td>
<td>0.102–0.248</td>
<td></td>
</tr>
<tr>
<td><strong>Depression diagnosed by medical doctor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14.7 (10.8–26.2)</td>
<td>0.452</td>
<td>0.172 (0.124–0.382)</td>
<td>0.383</td>
</tr>
<tr>
<td>No</td>
<td>14.1 (10.0–21.0)</td>
<td>0.164</td>
<td>0.110–0.337</td>
<td></td>
</tr>
</tbody>
</table>

1 Interquartile Range: Centile 25 – Centile 75.

USD family income stratum, urinary iAs was not associated with salivary cortisol. Three outliers were detected using Cook’s distance and leverage against the residual squared plot. Infants belonging to family income strata > 614 USD and exposed to a concentration between 14.08–21.70 µg/L of iAs had ~0.769 µg/dL of the natural logarithm of salivary cortisol than those exposed to 2.05–10.43 µg/L of iAs during pregnancy (P = 0.045).

In addition, the 3 stratified models showed a strong association between the natural logarithm of salivary cortisol in infants and salivary cortisol in mothers (P<0.001). The models for each family income strata did not show multi-collinearity according to the variance inflation factor; residual means were close to zero, and residual distributions did not reject normality.

Sensitivity analyses. Regarding the 74 non-participants, the median urinary iAs concentration was slightly higher than that of the participants. The distributions of covariates which included years of education, ethnicity, family income, occupation, stressful pregnancy, diagnosed depression, child’s gender, gestational age, and mother’s age, were similar between participants and non-participants (Tab. 4). The results obtained in the stratified models with imputed data were consistent with the results without imputations (Tab. 5).

**DISCUSSION**

An association was observed between the 3rd quartile of iAs concentration and the natural logarithm of salivary cortisol in infants belonging to the > 614 USD income stratum. The median concentration of urinary iAs in pregnant women was 14.1µg/L (min: 2.05; max: 69.3), while the median salivary cortisol concentration in infants was 0.17µg/dL (min: 0.0096 µg/dL; max: 1.3 µg/dL).

The median of urinary iAs reported in this study was low, which is consistent with similar studies that have explored the effects of intrauterine exposure to low concentrations of

Table 3. Association between urinary iAs concentration and natural logarithm of salivary cortisol concentration, adjusted by covariable in each stratum of family income, Arica 2015

<table>
<thead>
<tr>
<th>Coef.</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monthly family income &lt; 307 USD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAs quartile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10.43 – 14.08 µg/L</td>
<td>-0.178</td>
<td>-0.584</td>
</tr>
<tr>
<td>&gt;14.08 – 21.70 µg/L</td>
<td>-0.094</td>
<td>-0.464</td>
</tr>
<tr>
<td>&gt;21.70 – 69.3 µg/L</td>
<td>0.271</td>
<td>-0.131</td>
</tr>
<tr>
<td>Depression diagnosed by medical doctor</td>
<td>No</td>
<td>Ref.</td>
</tr>
<tr>
<td>Stressful pregnancy</td>
<td>Very low or low</td>
<td>Ref.</td>
</tr>
<tr>
<td>High or very high</td>
<td>0.010</td>
<td>-0.471</td>
</tr>
<tr>
<td>Maternal cortisol (µg/dL)</td>
<td>2.246</td>
<td>1.774</td>
</tr>
<tr>
<td>Monthly family income 307 – 614 USD</td>
<td>iAs quartile</td>
<td></td>
</tr>
<tr>
<td>&gt;10.43 – 14.08 µg/L</td>
<td>0.111</td>
<td>-0.416</td>
</tr>
<tr>
<td>&gt;14.08 – 21.70 µg/L</td>
<td>0.013</td>
<td>-0.585</td>
</tr>
<tr>
<td>&gt;21.70 – 69.3 µg/L</td>
<td>0.137</td>
<td>-0.399</td>
</tr>
<tr>
<td>Depression diagnosed by medical doctor</td>
<td>No</td>
<td>Ref.</td>
</tr>
<tr>
<td>Stressful pregnancy</td>
<td>Very low or low</td>
<td>Ref.</td>
</tr>
<tr>
<td>High or very high</td>
<td>-0.180</td>
<td>-0.762</td>
</tr>
<tr>
<td>Maternal cortisol (µg/dL)</td>
<td>1.968</td>
<td>1.363</td>
</tr>
<tr>
<td>Monthly family income &gt; 614 USD</td>
<td>iAs quartile</td>
<td></td>
</tr>
<tr>
<td>&gt;10.43 – 14.08 µg/L</td>
<td>-0.052</td>
<td>-0.809</td>
</tr>
<tr>
<td>&gt;14.08 – 21.70 µg/L</td>
<td>-0.769</td>
<td>-1.538</td>
</tr>
<tr>
<td>&gt;21.70 – 69.3 µg/L</td>
<td>-0.232</td>
<td>-0.641</td>
</tr>
<tr>
<td>Depression diagnosed by medical doctor</td>
<td>No</td>
<td>Ref.</td>
</tr>
<tr>
<td>Stressful pregnancy</td>
<td>Very low or low</td>
<td>Ref.</td>
</tr>
<tr>
<td>High or very high</td>
<td>0.090</td>
<td>-0.639</td>
</tr>
<tr>
<td>Maternal cortisol (µg/dL)</td>
<td>1.873</td>
<td>1.007</td>
</tr>
</tbody>
</table>
arsenic. Davis et al. [24] reported a median of 3.1 μg/L with a range from 0.0–22.0 μg/L, and excluded organic arsenical species from the analysis to evaluate the effect of arsenic exposure during pregnancy and foetal growth.

Murine models have shown that exposure to 10 μg/L of iAs through water consumption during and after pregnancy causes adverse health effects in mothers and their offspring, especially with respect to neurodevelopment and adaptation to stress conditions [25]. In humans, prenatal As exposure has been associated with global DNA methylation in cord blood DNA, which suggests a potential mechanism of early-life As exposure and health outcomes later in life [26, 27].

Total urinary iAs concentration was used as a proxy for exposure, based on the assumptions that this measurement is correlated with intrauterine exposure [28, 29, 30], and that the exposure is steady throughout pregnancy. The latter assumption is supported by official records of heavy metal content of drinking water which show that arsenic levels in drinking water did not vary during the 2013–2015 period [31].

The range of salivary cortisol in children (0.034–0.645 μg/dL) were wider than the values suggested by the manufacturer [32]. The sample in the current study differed from the sample used for reference values whose ages ranged from 2.5–5.5 years. According to a study by Watamura, the hypothalamus-pituitary-adrenal axis maturation develops even after the third year [33]. Age-dependency was confirmed in another study showing that younger children had higher basal cortisol concentrations [15]. In the presented study, cortisol differences by age were not significant, and were likely related to the age homogeneity of the sample. To further study the effect of total urinary iAs in pregnancy, the sample in the current study was stratified into quartiles of monthly family income. The association between the third quartile of urinary iAs (reference group: first quartile) and the natural logarithm of salivary cortisol in infants, occurred at low concentrations of exposure (>14.08–21.70 μg/L), and only in the group of infants belonging to > 614 USD income stratum. Specifically, arsenic exposure did not explain cortisol variability by itself, but depended on family income.

It is suggest that the variability of cortisol concentration in infants of lower income strata (monthly family income < 614 USD) may be explained by the cortisol level in mothers and other detrimental unmeasured variables, such as violence, psychological adversities, traumatic experiences, or adverse working conditions [34, 35, 36]. On the other hand, in infants from higher-income families (monthly family income > 614 USD), the variability of cortisol concentration was also related to mothers’ cortisol levels, but environmental factors such as the exposure to arsenic may become influential, i.e. environmental pollutants, such as arsenic, could explain the cortisol variability under special conditions when the individuals are not exposed to traumatic experiences or adverse conditions.

In the presented sample, monthly family income was neither associated with the iAs concentration nor with salivary cortisol. Interestingly, when exploring the association
of family income with other maternal outcomes, such as
gestational diabetes, preeclampsia, low birth weight, preterm
birth, postpartum depression, and mothers’ diagnosed
depression, it was noticed that those with lower income
(monthly family income < 614 USD) had a higher prevalence
of diagnosed depression (data not shown). This association
could be a potential pathway that links socio-economic status
with cortisol concentration in infants. Thus, mediators as
mother health status – especially, mental health – should be
considered in future research.

No association was found between cortisol concentrations
in children or mothers who had had a stressful pregnancy.
Nevertheless, the questions regarding stressful situations
were based on self-perception and conceivable expectations,
rather than objective facts, which may have led to an inflated
frequency, hence biasing the association.

According to the literature, social stressors seem to be
powerful factors that explain the variability of cortisol
concentrations, [37]. Even with strong evidence of the effect
of arsenic over corticosterone in experimental studies,
replicating the same results in humans might be difficult
considering unmeasured social factors. Based on mouse
models, cortisol concentration might be higher in infants
exposed to higher concentration of iAs, presumably exposed
through drinking water during gestation; however, in the
presented study it was not possible to estimate the influence
of unmeasured traumatic experiences in the sample.

Strengths and limitations of the study. Sensitivity analysis
showed the robustness of the obtained regression coefficients.
Despite missing data on salivary cortisol, the association
estimated on the stratified models were found in data
without imputation (Tab. 3) and with imputation (Tab. 5).
The characteristics of non-participants were similar to the
characteristics of the obtained sample (Tab. 4), and although
a selection bias due to losses during the follow-up was one of
the limitations in this study, our missingness seemed to
be random.

The DAG approach is one of the strengths of this study,
which considers the causal network underlying the effect
of arsenic on salivary cortisol. Using this approach, response
to two aims was achieved: to incorporate the evidence based
on mouse models, which gave biological plausibility to the
authors’ research question, and to develop an analytical
strategy that recognizes confounding and mediating
pathways, and the identification of vulnerable subgroups.
The consideration of variables, such as cortisol in mothers,
mother’s depression, stress during pregnancy, and socio-
economic status, was supported by the review of literature
[18, 19, 21, 39].

The second strength on this study was the use of a data
collection and measurement technique that is highly
reproducible, implying that the results are reliable in the pre-
analytical and analytical phases. Both exposure and outcome
variables were measured on continuous scales using high-
precision analytical techniques, avoiding misclassification
bias in the two main variables of this study.

There is no reference range for salivary cortisol
concentration for infants, and few studies have assessed
it longitudinally [12, 40]. In a cohort of Swedish infants,
serum and salivary cortisol concentrations were assessed
from birth to 12 months old. The results showed that
variability of salivary cortisol concentrations increased each
month, and achieved the higher variance at five months
old (min: 0.1015 µg/dL; max: 23.59 µg/dL). Furthermore,
in an Argentinian cohort of newborns, serum and salivary
cortisol levels were measured at birth and one month later.
The authors noted a higher variability of values at birth,
which decreased one-month later. Prior to the presented
study, a pilot study was undertaken to calibrate the analytical
technique and to explore the salivary cortisol concentration
in a similar population. However, there is no assurance that
the results obtained are conclusive regarding the cortisol
range.

As previously noted, the dropouts that occurred during
the follow-up represent a limitation of this study. Although
sensitivity analysis with imputed data did not show important
differences from the results with complete cases, the obtained
results could be more accurate with a complete data set.
With the presented sample size, the estimated error for the
multiple linear regression coefficient was 0.113, assuming
80% of confidence.

One potential bias in this study was the information bias.
Information for some variables, such as stressful pregnancies,
was collected through self-reported questionnaire, and
therefore susceptible to own perception or previous
experiences. The pilot study attempted to minimize this
research bias.

Socio-economic status was a latent variable in this study,
but family income was used as a proxy, which could have
introduced a misclassification bias. Moreover, the sample
had little variability with respect to income. Future studies
may consider taking a structural equation modeling approach [41],
considering ethnicity, education, occupation, and income as
factors behind the socio-economic status construct, and
compare results with the current study. These variables are
included in the conceptual framework for action on the social
determinants of health to challenge health inequities [20].

CONCLUSIONS

This is the first study estimating the association between
prenatal concentrations of iAs as a proxy of intrauterine
exposure and concentrations of cortisol, as a biomarker of
prenatal effect of arsenic exposure in infants aged 18–24
months. In this sample of infants, whose mothers attended
public health centres, prenatal exposure to arsenic was
associated with salivary cortisol (third quartile of iAs), only
in infants belonging the highest income strata (> 614 USD).
More studies are needed to confirm these preliminary results.

Although the level of exposure to iAs in this cohort was
low, current evidence verifies the importance of studying
the effects of exposure to low concentrations during critical
periods of development. In humans, it is necessary to
investigate the effects of low concentrations of arsenic, as well
as other pollutants, during prenatal life and early childhood,
given the impact of these exposures on medium and long-
term health outcomes. The study of biomarkers, such as
cortisol, is a fundamental area of environmental research
given its contribution to our understanding of disease
pathways, and the identification of vulnerable subgroups.
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