

Chronic Arsenic Toxicity: Statistical Study of the Relationships Between Urinary Arsenic, Selenium and Antimony

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Background. The groundwater of Argentina's Chaco plain presents arsenic levels above those suitable for human consumption. Studies suggest skin disorders among local populations caused by arsenic intake. The relationship between urinary arsenic and arsenic in drinking water is well known, but urinary arsenic alone is not enough for risk assessment due to modulating factors such as the intake of selenium and antimony.

Objectives. Determining the relationship between urinary arsenic, selenium and antimony could contribute to the study of arsenic metabolism in humans.

Methods. Arsenic, selenium and antimony, relative to urinary creatinine, was quantified in urine samples to assess exposure to arsenic and its toxicity modulators.

Results. Arsenic levels were higher than reference value in 89% of exposed group. Selenium concentrations were higher in 84%. Levels of antimony were also surprisingly high, not compatible with dietary intake. Urinary arsenic and selenium data showed highly significant correlations with age. Adult females presented higher urinary arsenic values than adult males.

Conclusions. Values related to urinary creatinine were much higher than values considered normal. Concentrations of arsenic and selenium diminished with age for children and increased for adults. Women showed higher concentrations than men. Positive correlation was found between arsenic and selenium. No correlation was seen between urinary antimony, arsenic nor selenium.

Competing Interests. The authors declare no competing financial interests.

Keywords. arsenicosis, urinary arsenic, urinary selenium, urinary antimony, chronic arsenic toxicity, environmental toxicity, Argentina, Salta Province, Rivadavia Banda Sur
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Introduction

Arsenic (As) is a naturally occurring element in Argentine groundwater due to Andean volcanism in the Quaternary era, which produced stratigraphic deposits of volcanic ashes on the Chaco and Pampean plains.¹ In the Chaco plains region of Salta province, northwestern Argentina, the arsenic concentration in groundwater varies from less than 0.005 mg/L up to 1.22 mg/L.² Chronic intake of arsenic via drinking water or the consumption of crops irrigated with contaminated water leads to toxic poisoning, or arsenicosis, which has been linked to an increased risk of internal and skin cancers, as well as skin pathologies, disorders of the cardiovascular and

peripheral nervous systems and even to psychiatric disturbances.³ Most epidemiological studies rely on arsenic concentrations found in drinking water to estimate the grade of exposure, but bio-monitoring is considered a superior method. Levels of arsenic can be measured in hair, fingernails, blood and urine.⁴ The linear relationship between urinary arsenic and concentrations found in drinking water is well known and so the quantity of arsenic excreted in urine can be related to manifestations of arsenicosis.⁵ However the sole appearance of arsenic in urine is not enough for risk assessment due to nutritional factors such as the intake of selenium (Se) and antimony (Sb),

trace elements that modulate the toxicity of environmental arsenic.⁶

Selenium is an essential nutrient involved in the normal functioning of the immune system and of the thyroid gland. Arsenic and selenium are considered mutual antagonists, each one inhibiting the other's toxicity, though there is some evidence as to their synergistic effects as well.⁷ Convenience of alimentary complementation with selenium is under discussion, owing to the narrow gap between its nutritional dose and toxic level.⁸ In contrast with arsenic, selenium is considered anti-carcinogenic, although its anti-oxidant properties are still under examination.

Antimony, less widely distributed in the environment than arsenic, is rarely present in biological samples and therefore is usually excluded from toxicological assays. Its role in modulating arsenic toxicity is as yet ambiguous.^{6,9}

Disagreement in arsenic content in individual urine excretions makes the 24-hour cumulative sample the optimal collection method; however, because of the associated difficulties of handling, random spot samples are of common use in most exposure studies.⁴ To reduce the influence of variations in urine output and state of hydration at the time of sampling, the concentration of toxic substances is routinely adjusted by the density of urine or its creatinine content.

In earlier studies, characterization of ground waters by major component analysis from Rivadavia Department showed that sodium sulphate, hydrogen carbonate and chloride-type waters presented high arsenic concentrations (0.13 to 1.22 mg As/L), while in hard waters arsenic concentrations were below 0.06 mg/L.¹⁰ According to the guideline for total arsenic concentration in drinking water, established at 0.010 mg As/L by the Argentine Alimentary Code and the World Health Organization (WHO), most of the wells in the region were found not suitable for human consumption.^{11,12} Research on suitable arsenic mitigation methodologies were carried out in a complementary project.¹³

Most of the 6,500 inhabitants of the district are residents of the town of Rivadavia Banda Sur, while the rural population mainly consists of widely scattered small landowners of Spanish descent, as well as Wichí families, an indigenous ethnic group. Spot urine samples of residents of eleven small locations and Rivadavia Banda Sur

Abbreviations

$\mu\text{g/gUC}$	Microgram per gram of urinary creatinine	$\text{Mg}(\text{NO}_3)_2$	Magnesium nitrate
		MgO	Magnesium oxide
APHA	American Public Health Association	<i>mL</i>	Milliliter
As	Arsenic	NaBH_4	Sodium borohydride
As/gUC	Arsenic per grams of urinary creatinine	PAHO	Pan American Health Organization
AWA	American Waterworks Association	<i>RfV</i>	Reference values
		<i>Sb</i>	Antimony
CAT	Chronic arsenic toxicity	SDR	Standard deviation ratio
$\text{Cu}(\text{NO}_3)_2$	Copper nitrate	<i>Se</i>	Selenium
DL	Detection limits	UAs_T	Total urinary arsenic content related to creatinine
ET-AAS	Electrothermal atomization – atomic absorption spectrometry	USb_T	Total urinary antimony content related to creatinine
GBC	GBC Scientific Equipment Pty Ltd	USE_T	Total urinary selenium content related to creatinine
GPS	Global positioning system	<i>UC/L</i>	Urinary creatinine per liter
H_2O_2	Hydrogen peroxide	WAs_T	Total arsenic content of water supply
<i>HCl</i>	Hydrogen chloride	WHO	World Health Organization
HG-AAS	Hydride generation – atomic absorption spectrometry	WSb_T	Total antimony content of water supply
HNO_3	Nitric acid	WSe_T	Total selenium content of water supply
<i>kg bw/day</i>	Per kilograms of body weight per day	<i>w/v</i>	Weight per volume
<i>mg/L</i>	Milligrams per liter		

Sample ID	Location	Latitude (S)	Longitude (W)	WAsT (mg As/L)	Sampled Individuals, Age and Gender		
					Children (<15 yrs)	Adults Male	Adults Female
P1	Rivadavia	24° 11' 05.4"	62° 53' 14.0"	0.26	2	3	0
P2	La Fortuna	24° 16' 27.2"	62° 35' 17.7"	0.060/0.045	7	1	1
P3	Siervo Cansado	24° 23' 49.7"	62° 21' 40.4"	0.27	3	0	4
P5	El Mirador	24° 20' 32.0"	62° 25' 34.0"	0.19	0	1	0
P6	Tres Horcones	24° 16' 38.9"	62° 29' 27.3"	0.17	3	2	1
P7	El Breal	24° 10' 01.1"	62° 51' 00.4"	0.13	5	1	1
P8	Santa Rosa	24° 01' 39.2"	63° 04' 55.8"	0.24	2	1	1
P10	El Ocultar	23° 50' 41.0"	63° 24' 14.1"	0.002	0	1	0
P11	San Felipe	24° 07' 53.0"	62° 41' 54.5"	0.006/0.002	5	1	2
P12	La Esperanza	24° 08' 00.9"	62° 36' 18.1"	0.002/0.005	0	0	2
P14	Nueva Población	24° 12' 43.0"	63° 11' 29.0"	1.22	1	1	2
P17	Aguas Muertas	24° 13' 48.4"	62° 40' 52.1"	0.10	4	1	1

Table 1 — Geographic location, well water arsenic content and demographic data

town were collected in three sampling campaigns, and each sample's total content of As, Se and Sb relative to urinary creatinine (UAs_T , USe_T and USb_T respectively) were quantified in order to assess the actual exposure to arsenic and some of its toxicity modulators. Total arsenic content of the water supply (WAs_T) was used to divide the sampled population into two groups: one exposed to chronic arsenic toxicity (CAT), and a control group.

Determining a relationship or a lack thereof between UAs_T , USe_T and USb_T for the exposed population could contribute to the study of arsenic metabolism in humans. Correlation of UAs_T with age and gender, first described for the local population, along with future nutritional and epidemiological research, should help to create strategies to counteract CAT.

Methods

Sampling Sites and Demographic Data

Sampling sites in Rivadavia Department were selected according to previous knowledge of arsenical water distribution, ground water availability and arsenicosis manifestations.¹⁴ In three consecutive sampling campaigns, groundwater from 12 sites, 22 samples of unprocessed and composite food, and 60 urine samples were collected in an attempt to find a correlation between ambient exposure and urinary excretion of arsenic. Eleven of the sampled sites were widely dispersed, with no access to standard-quality drinking water; these small settlements consisted of only four or five families so that at most eight or nine individuals could be selected for the study. All tested individuals had resided for over a year in the affected area. Inhabitants of Rivadavia Banda Sur, on the other hand, commonly consume bottled water, a fact that

could have hindered the conclusions if they were included in large numbers. Demographic data were split into the categories of "less than 15 years old" and "adults" as per WHO/Pan American Health Organization's (PAHO) guidelines, with no further distinction by age.¹⁵ Geo-referenced location of the sampling sites, arsenic water content and demographic data are shown in Table 1.

Water Sampling and Analysis

Collection, storage and chemical analysis of groundwater samples followed the recommendations of American Public Health Association (APHA) Standards Methods.¹⁶ Physicochemical characterization, as well as major and trace component total contents (WAs_T , WSe_T and WSb_T and others) were published elsewhere; WAs_T , WSe_T and WSb_T chemical analysis were performed by flow injection hydride generation atomic absorption spectrometry (FI-

HGAAS), using a GBC 904-AA atomic absorption spectrometer coupled to a GBC-HG-3000 flow injection device.¹⁰ Solutions to be analyzed for As and Sb were previously reduced with KI 15% (w/v); pretreatment for Se consisted in acidification to 50% HCl and heating at 70°C/158°F for one hour. Hydride generation was achieved by pumping concentrated HCl and NaBH₄ 0.48% (w/v) in the other two lines of the manifold.

Quantification of Urinary Analyte Total Concentration

The acquisition of urine samples followed the protocols of the Argentine National Ministry of Health and associated organisms in a collaborative study related to arsenicosis, and was executed with the consent of the Ministry of Public Health of Salta province.¹⁷ All urine samples correspond to random spot urinations; at least 10 mL of each sample were collected in sterilized polyethylene vials, kept in an iced cooler during transportation to the laboratory, and stored at -17°C/1.4°F until analysis.

For the quantification of urinary total concentration of the analytes, the liquid samples were dry-ashed in a programmed furnace using MgO/Mg(NO₃)₂/HNO₃, a digesting procedure that inhibits volatilization, improves precision and gives better recovery of inorganic substances.¹⁸ Hydrochloric acid solutions of the ashes were used to quantify UAs_T and USb_T by the same FI-HG-AAS technique used for water analysis. USE_T in the first lot of urine samples was analyzed by reflux wet digestion (HNO₃(c)/H₂O₂ 30%) in a digester block at 150°C/302°F, followed by atomic absorption by electrothermal atomization (ET-AAS) using Cu(NO₃)₂/Mg(NO₃)₂ as a modifier of the atomizing stage. For the second and third batches, the same digestion and quantification procedure used for

Technique	Recovery (%) of Laboratory Fortified Samples			
	UAs _T	USE _T	USb _T	
HG—AAS	Range	69–127	75–125	85–105
	Mean	98	97	97
	N	11	5	5
ET—AAS	Range	—	73–126	—
	Mean	—	92	—
	N	—	24	—

Table 2 — Recovery of urinary analytes in fortification assays (%)

UAs_T and USb_T was employed, spiked samples recovery assuring validation of both methods.

Urinary creatinine was tested using Wiener Lab's colorimetric assay kit for biochemical analysis, based on a modified Jaffe's alkaline picrate method that overcomes protein interference.¹⁹ Widely accepted reference values for urinary creatinine (WHO) are 0.3 to 1.4 mg UC/L, but Barr et al. recommend a higher limit of 3.0 mg/L for Hispanic ethnic groups.^{20,21} In consequence, concentrations outside this range were considered to mean that the specimen was either too dilute (UC < 0.3 mg/L) or too concentrated (UC > 3.0 mg/L), and therefore ignored.

Quality Assurance of Urine Analysis

All chemicals were analytical reagent grade. Fortification solutions and daily calibration curves for total analyte content were prepared from Merck standard solutions (Merck Titrisol® 1.09939 1000 mg As/L, Merck CertiPur 1.19796.0500 1002 ± 5 mg Se/L and Merck CertiPUR® 1.70204.0500 995 ± 2 mg Sb/L). Samples were treated by duplicates, and ashing/acid dissolution blanks were prepared by triplicate. To assess the efficiency of recovery, three to five urine samples from each batch

of around 20 under simultaneous analysis were fortified by adding suitable volumes of standard solutions of As, Se and Sb at the digestion stage; results are presented in Table 2 according to analyte and technique (HG-AAS and ET-AAS).

Up to 30% discrepancy in replicates (calculated as the relative standard deviation, %SDR) for each analyte concentration was considered acceptable; when results differed by a larger amount new replicates were prepared and analyzed starting with the ashing step. Detection limits (DL) for the three analytes were calculated as the sum of the reagents' blank concentration plus 3.3 times its standard deviation, taking into consideration the dilution factor necessary for chemical analysis. DLs were 0.003, 0.002 and 0.006 mg/L for total concentration of As, Se and Sb in urine, respectively. A default value of one-half the detection limit of each element was assigned to specimens with urinary content lower than the detection limit of the technique employed.

Statistical treatment of data was performed using XLSTAT 2007—Microsoft Excel software. Locations were geo-referenced by Garmin GPS II.

	Analyte concentration [$\mu\text{g/gUC}$]					
	Control group (N=10)			Exposed group (N=46)		
	UAs _T	USe _T	USb _T	UAs _T	USe _T	USb _T
Median	36	28	20	154	50	9
Range	8-103	13-254	5-70	9-2379	6-653	1-77
RfV	50	25	1	50	25	1

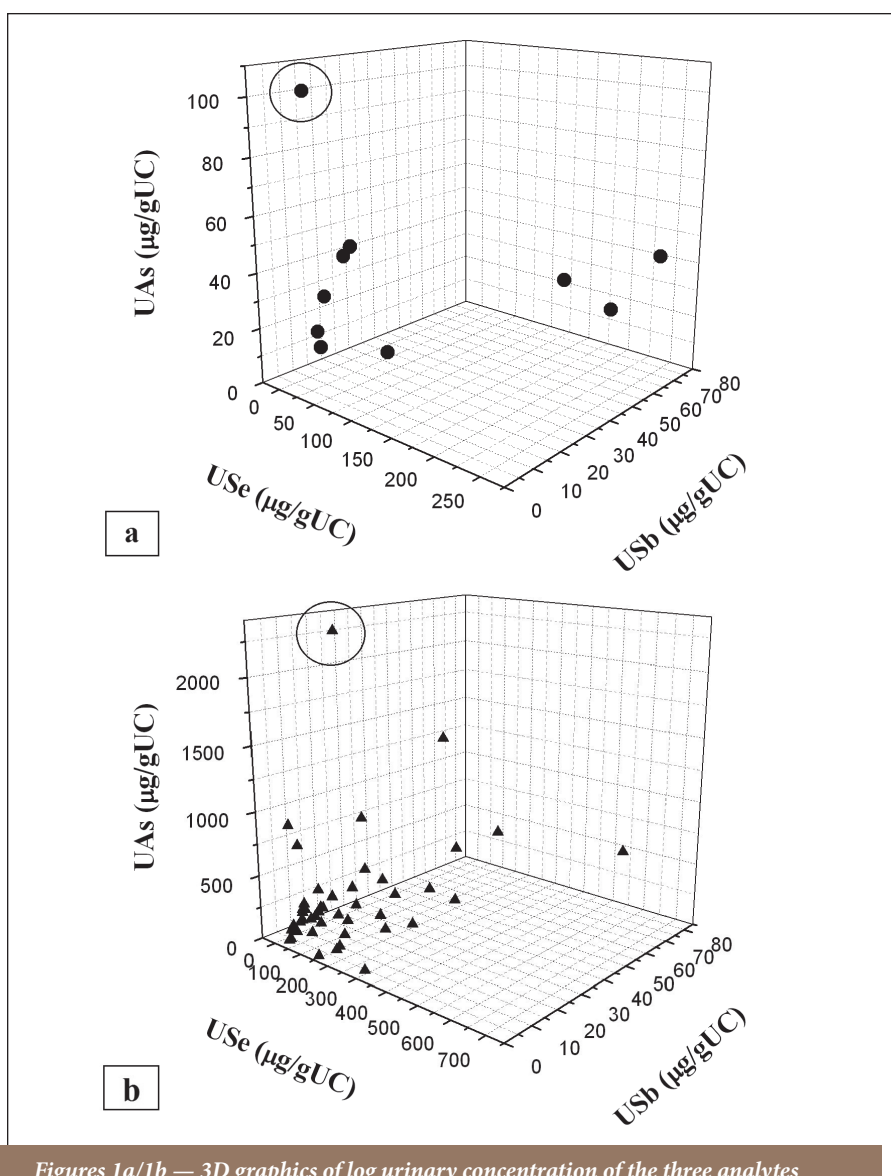
Table 3 — Urinary concentration parameters in the control and exposed groups

Results

Analyte Content in Water and Food

As shown in Table 1, only 3 of the 12 wells had arsenic levels below the WHO standard for drinking water. One of them had a particularly high value (1.22 mg As/L, Site P14) and four of the five members of the resident family manifested symptoms of arsenicosis, including a 3-year-old child. These individuals showed exceedingly high arsenic levels in urine, in conjunction with high selenium and low antimony concentrations. In all of the 12 water samples, WSe_T and Wsb_T were below AAC guideline values of 0.050 and 0.010 mg/L respectively,¹¹ with WSe_T levels below even the detection limit of the technique used, i.e. 0.001 mg/L.

Dietary arsenic contents were estimated in previous research, indicating that the major route of As intake was drinking water (4.8 $\mu\text{g/kg bw/day}$ vs. tolerable limit of 3 $\mu\text{g/kg bw/day}$) in the population under study, and that Se ingestion is lower than the recommended daily amount (1.04 $\mu\text{g/kg bw/day}$ for male adults).¹⁰ According to the evaluation of nutritional exposure, the recommended daily intake for Se could only be achieved by the ingestion of a high amount of red meat and its composite foods (12.5 $\mu\text{g/kg bw/day}$ vs. 1.04 $\mu\text{g/kg bw/day}$).²²



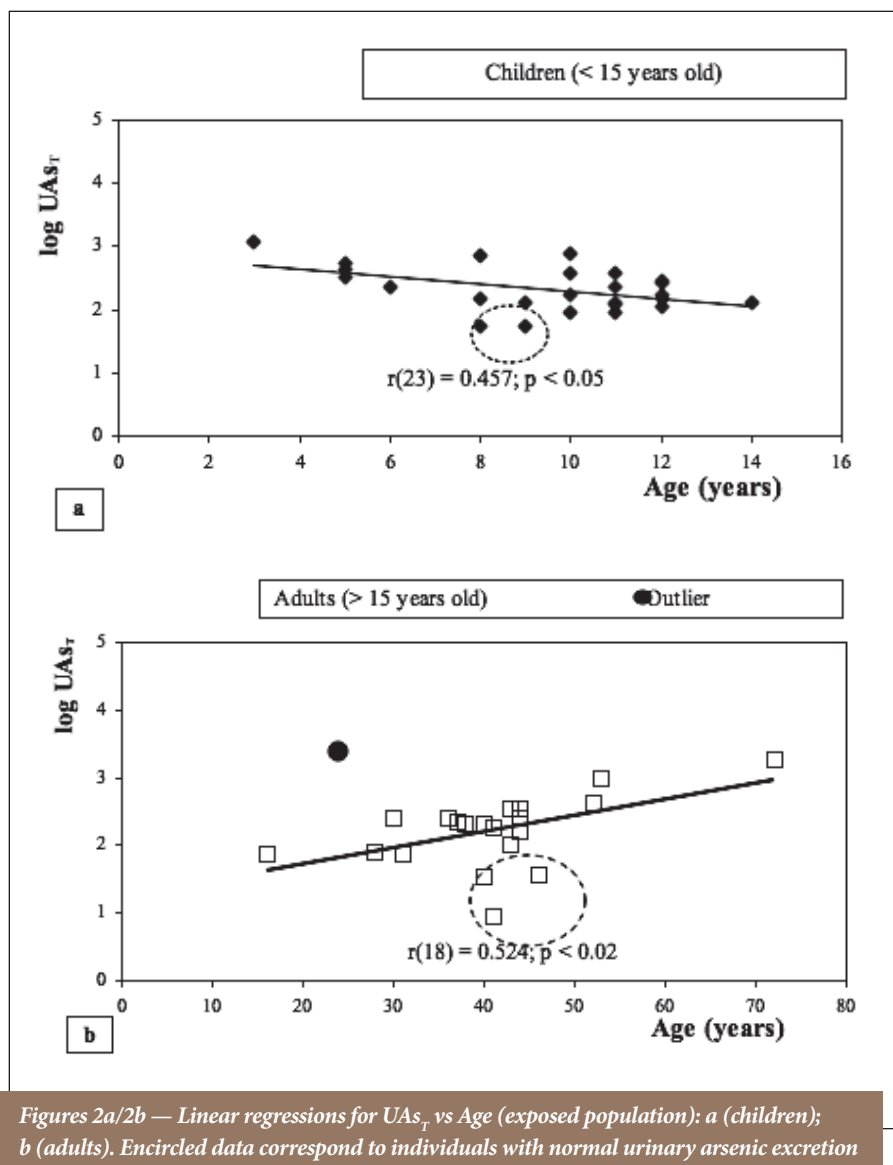
Figures 1a/1b — 3D graphics of log urinary concentration of the three analytes relative to creatinine for the a (control) and b (exposed) groups

Pre-treatment of Urinary Data

Four urine samples of the original sixty were rejected due to their extremely low content of urinary creatinine (0.04 to 0.12 g/L). Median and range of the reduced sample population together with the RfV are shown in Table 3.^{23,24}

The relationship between WAs_T content and urinary excretion, generally accepted as valid, was confirmed: log transformed water and urine concentrations, following normal distributions, showed a high correlation ($r(54) = 0.667$; $p < 0.01$). Using the WAs_T local guideline value for arsenic in drinking water of 0.010 mg As/L, individuals were separated according to the relationship (of WAs_T content and excretion) into two groups: *Control* (N=10) and *Exposed* (N=46). This criterion for the splitting of data was evaluated by application of the *t* test for mean values comparison of water arsenic content in both groups (previous evaluation of their respective standard deviations by *F* test), proving to be satisfactory since $t(\text{exp}) = 2.44 > t(p = 0.05) = 2.31$ for a degree of freedom (d.f.) of 8. In the same way, when this test was used with urinary arsenic concentration, $t(\text{exp}) = 4.50 > t(p = 0.01) = 2.68$ (d.f. = 46), confirming the validity of the grouping methodology.

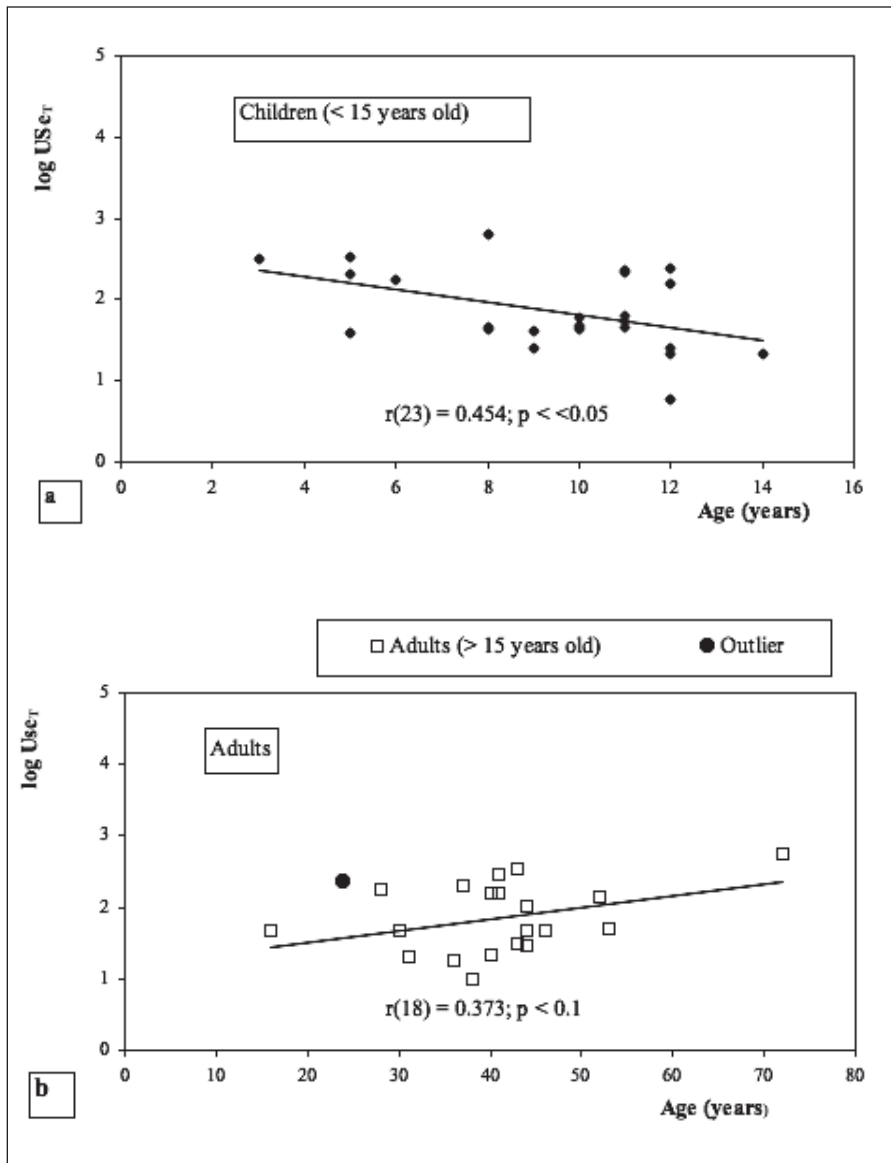
The two groups showed distinct behaviors for the three analytes: in the control group, UAs_T was below RfV for every person but one (103 $\mu\text{g/gUC}$); in the exposed group, only 11% of individuals met RfV requirement for UAs_T . USE_T median for the exposed population was significantly higher than the RfV, but not for the control group. USb_T was extremely high for both groups; these values cannot derive from the diet, its probable origin being certain medications used to mitigate the tropical diseases endemic to the area.



The percentage of the population (N=56) with urinary content below RfV was 25.0% for UAs_T , 26.8% for USE_T and 5.4% for USb_T . It seems relevant that no individual achieved RfV condition concurrently for the three analytes.

Frequency distributions of UAs_T , USE_T and USb_T data were log-normal type, so all statistical treatments were applied to the log-transformed data of the exposed group. Although the size of the control group was too small

to detect a tendency for frequency distribution, log-transformed data were used for comparison with the exposed group. Three-dimensional representations of control and exposed population (Figures 1a/1b) revealed the presence of two uncertain values, enclosed in circles: the individual with $UAs_T = 103 \mu\text{g/gUC}$ (control group) and the one with 2390 $\mu\text{g/gUC}$ (exposed group). This last individual was excluded from further statistical analysis.



Figures 3a/3b — Linear regressions for USe_T vs Age (exposed population): a (children); b (adults)

Correlation Analysis of the Exposed Population Data

1. Correlations with age

To detect the existence of correlation between urinary excretion of arsenic and age, UAs_T data were plotted against age after splitting it into two categories: children and adults, with 15 years of age as the cut-off value (Figure 2a/2b).

Correlation coefficients were calculated for the adult group with and without the $UAs_T = 2390 \mu\text{g/gUC}$ value. When the Fisher r -to- z transformation for significance assignment to disparity between two correlation coefficients ($z > p$ value, one-sided test) was applied, this urinary concentration proved to be an outlier.²⁵ The values of $\log UAs_T$ inside the dotted circles (Figures 2a/2b) represent individuals who, in spite of belonging to the exposed group, present normal values of urinary arsenic concentration. When these cases were excluded as well, significance of the correlation coefficient became even higher, despite the lower degree of freedom; figures of merit are shown in Table 4. It is remarkable that the slopes of the regression lines are of opposite signs, as it seems to evidence an increased capacity of elimination of arsenic via urine in childhood and old age. This would be contrary to the general rule that assigns a greater vulnerability to very young and aged populations.

Correlations for Log UAs_T vs Age								Data Group
Children (<15)				Adults				
N	Slope	R	P	N	Slope	R	P	
25	-0.0573	0.457	$p < 0.05$	20	0.0244	0.525	$p < 0.02$	Exposed
23	-0.0643	0.594	$p < 0.01$	17	0.0261	0.856	$p < 0.01$	Exposed (< RfV excluded)

Table 4 — Correlations between $\log UAs_T$ and Age: figures of merit

LogUSE_T data showed the same distinctive behavior with age: negative correlation for children (slope = -0.0782, r = 0.454, p < 0.05) and positive for adults (slope = 0.0162, r = 0.387, p < 0.1) (Figures 3a/3b). LogUSb_T showed no correlation with age, neither for children nor adults (p > 0.1).

2. Correlations with age according to gender

When data corresponding to the adult population was separated according to gender, two lines of different slopes were found for the logUAs_T/age graph; Figure 4 illustrates exposed adult population behavior, individuals with low urinary

arsenic contents (< standard value) being highlighted by a circle.

A stronger correlation was found for females than for males, the correlation line being shifted to upper concentration values compared to males (Table 5). This could point to a gender differentiation, women being more efficient in eliminating arsenic via urinary excretion, in agreement with former studies that showed lower prevalence of hyper-pigmentation and keratoses in women than in men in a population exposed to CAT in West Bengal, India.²⁶ The figures of merit upheld this, since the removal

of data corresponding to individuals with normal urinary arsenic content enhanced the differences in the respective slopes, improving the significance. When logUSE_T data were separated according to gender, no correlations with age were found.

3. Correlations between the analytes

A statistically significant (p < 0.02) positive correlation was found between logUAs_T and logUSE_T (once the individual with UAs_T < RfV was excluded), with a slope value of 0.30, showing a great increase in urinary Se excretion (Figure 5) related to small variations in UAs_T, which is in concordance with Spallholz et al.²⁷ When logUAs_T and logUSb_T were confronted, the correlation showed no significance (p > 0.1).

Discussion

Total urinary concentrations of arsenic, selenium and antimony related to creatinine content (UAs_T, USE_T, USb_T) for a population of 60 individuals in the Chaco region of Salta, Argentina were determined. Data was separated into “control” (normal) and “exposed” groups according to degree of exposure to arsenic in the drinking water, the main route of intake for this element; this method of dividing the population also agreed with found urinary arsenic contents. Urinary arsenic levels were discovered to be higher than the reference value (RfV = 50 µg/gUC) in

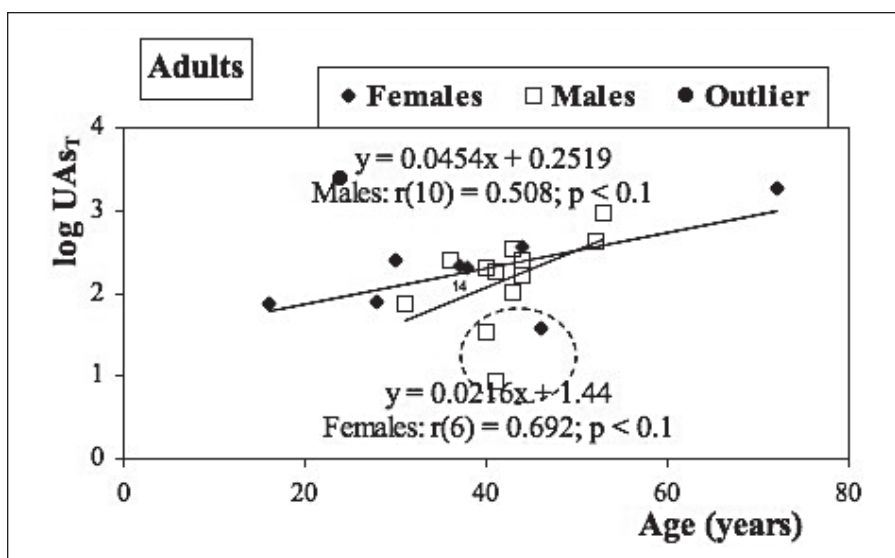


Figure 4 — Linear regressions for UAs_T vs Age according to gender (exposed adult population)

Correlations for Log UAs _T vs Age								Data Group
Males (adults)				Females (adults)				
N	Slope	R	P	N	Slope	R	P	
12	0.0454	0.509	p < 0.1	8	0.0216	0.692	p < 0.1	Exposed (total accepted)
10	0.0396	0.774	p < 0.01	7	0.0265	0.959	p < 0.01	Exposed (< Standard excluded)

Table 5 — Correlations between LogUAs_T and Age according to gender: figures of merit

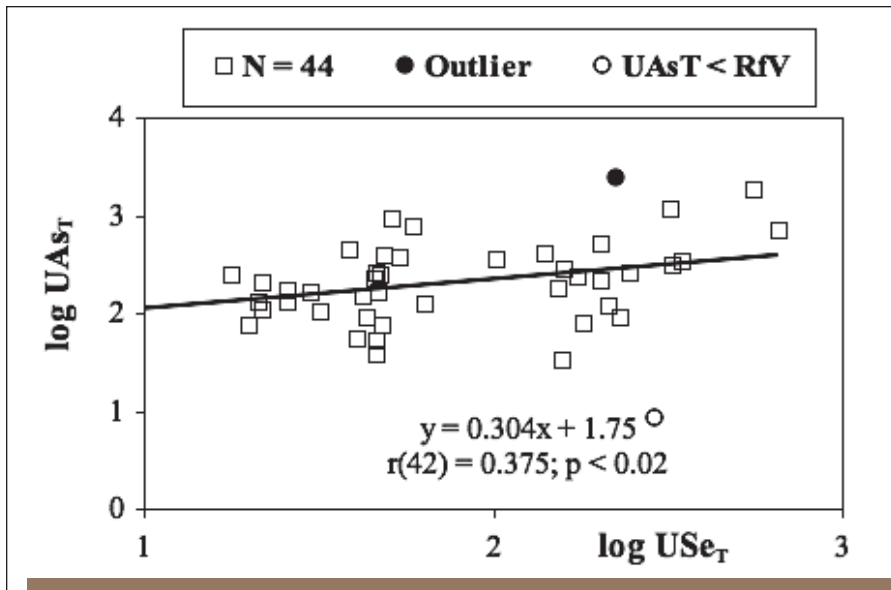


Figure 5 —Linear regression for LogUAs_T vs LogUSE_T (exposed population)

89% of the “exposed” group’s cases. At the same time, 84% of the selenium urinary concentrations found were also higher than the guideline (25 µg/gUC), in spite of the low concentration of this element in the drinking water; previous research showed that red meat is its main source of dietary intake. Levels of urinary antimony were also found to be higher than the corresponding RfV (1 µg/gUC); its origin can be presumed to be anti-parasite medications not declared in the health questionnaires, as neither the water nor the food consumed by the population contained significant amounts of this element. When data corresponding to the exposed population was log-transformed to ensure normality, both logUAs_T and logUSE_T showed highly significant correlations with age. For both elements, the slope of the regression line was negative for the sub-group of children and positive for the adult, evidencing differential patterns of detoxification.

Separate correlations for logUAs_T vs age according to gender could be established for the adult group in spite of the small sample size. The

urinary contents of arsenic in females presented higher values than the ones of the male sub-group, which could mean that the process of detoxification and/or excretion via urine is more efficient in females. Significant positive correlation was found when logUAs_T was plotted vs. logUSE_T. Median USE_T found in the “exposed” group was higher and median USb_T lower than in the control group. LogUSb_T showed no correlation with logUAs_T, logUSE_T nor age, even when adults were separated according to gender.

Conclusions

Present results will help to build a database necessary for further studies concerning population health status, arsenicosis epidemiology and medical evaluation of the convenience of selenium dietary fortification; it may also be of assistance in biological investigations concerning metabolic pathways and clinical aspects of As-Se antagonism-synergism.

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