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Biotransformation of inorganic arsenic: Influence of gender, arsenic dose level, and creatinine formation

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ABSTRACT

Biotransformation of inorganic arsenic (inorg-As) is not well understood. We studied 191 individuals from Lagunera area of Mexico by measuring arsenic metabolites using HPLC-ICP-MS and total arsenic in urine as well as blood using DRCe ICP-MS. We also measured creatinine in urine using the Randox Creatinine Colorimetric kit. Our results indicated that urines of females had lower % MMA and higher % DMA than males ($p<0.001$ and $p<0.01$, respectively). The ratios of % MMA to % inorg As, and the ratios of % DMA to % MMA were lower ($p<0.05$) and higher ($p<0.001$) for females than males, respectively. The concentrations of total arsenic in drinking water were positively correlated with total arsenic in urine as well as total arsenic in blood. Urinary total arsenic was negatively correlated with urinary % inorg As ($p<0.000001$) as well as % MMA ($p<0.001$), and positively correlated with % DMA ($p<0.000001$) as well as the ratios of % MMA to % inorg As ($p<0.05$) and the ratios of %DMA to % MMA ($p<0.00001$) for both females and males. Total arsenic level in blood was also negatively correlated with urinary % inorg As ($p<0.05$) and positively correlated with % DMA ($p<0.01$) for both females and males. Urinary creatinine was negatively correlated with % inorg As (females: $p<0.000001$ and males: $p<0.00001$) as well as % MMA (females: $p<0.001$ and males: $p<0.0001$), and positively correlated with % DMA (females: $p<0.000001$ and males: $p<0.0001$). In conclusions, biotransformation of inorg-As may influence by gender, arsenic doses, and creatinine formation.

Abbreviation: SAM, *S*-adenosyl-L-methionine; SAHC, *S*-adenosyl-L-homocysteine.

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Introduction

In different countries of the world including Mexico, arsenic concentration in ground water is much higher than accepted levels[1]. The Lagunera Region of north central Mexico has arsenic problem in groundwater with significant resulting chronic health problems[2].

An important limitation on the scientific understanding of arsenic toxicity is the complexity of arsenic metabolism. Differences in susceptibility to arsenic toxicity might be manifested by differences in arsenic metabolism or in the prevalence of arsenic-associated diseases among people of either gender, ages, nutritional factors, polymorphisms of the arsenic biotransformation genes in different ethnic group[3,4] and may other unknown factors. Previous studies indicated that females are less susceptible to the arsenic related skin effects than males[5-7].

Inorg-As is metabolized in the body by alternating reduction of pentavalent arsenic to trivalent form by enzymes and addition of a methyl group from S-adenosylmethionine[3,8]; it is excreted mainly in urine as DMA (V)[9]. Inorganic arsenate [Inorg-As (V)] is biotransformed to Inorg-As (III), MMA (V), MMA (III), DMA (V), and DMA (III) (Fig. 1)[3]. Therefore, the study of the toxicology of Inorg-As (V) involves at least these six chemical forms of arsenic. Studies reported the presence of 3+ oxidation state arsenic biotransformants [MMA (III) and DMA (III)] in human urine[10]and in animal tissues[11]. The MMA (III) and DMA (III) are more toxic than other arsenicals[12,13]. In particular MMA

(III) is highly toxic[12,13]. In increased % MMA in urine has been recognized in arsenic toxicity[14]. In addition, people with a small % MMA in urine show less retention of arsenic[15]. Thus, the higher prevalence of toxic effects with increased % MMA in urine could be attributed to the presence of toxic MMA (III) in the tissue. Previous studies also indicated that males are more susceptible to the As related skin effects than females[14,16]. A study in the U.S population reported that females excreted a lower % Inorg-As as well as % MMA, and a higher % DMA than did males[17].

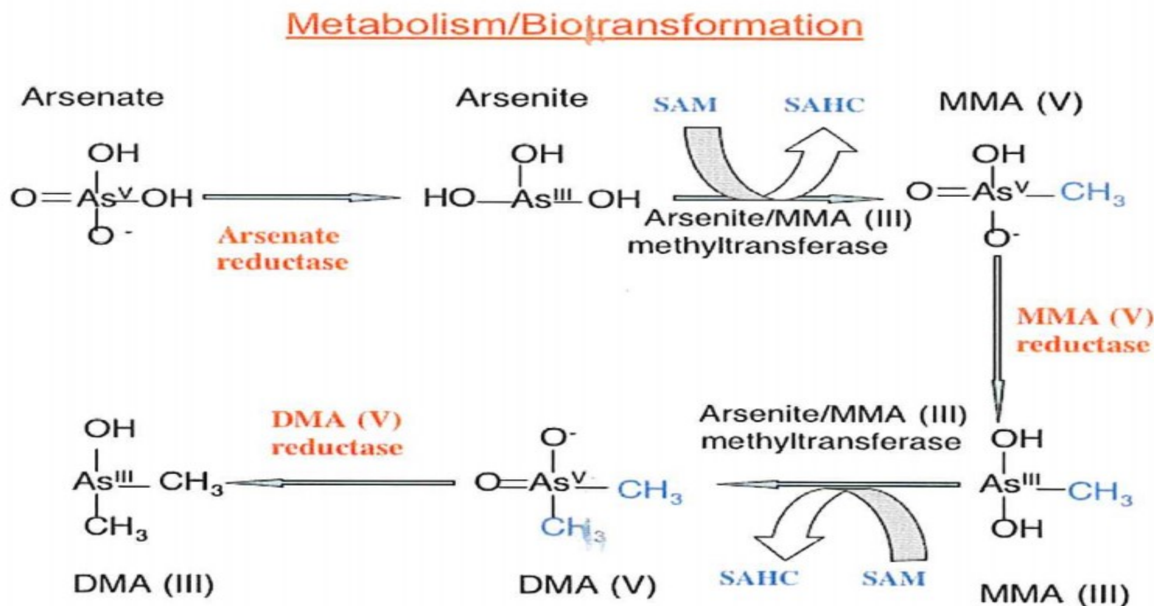


Figure 1. Metabolism of Inorg-As.

Gamble et al. (2008)[18] has found that higher urinary creatinine is associated with reduced risk for premalignant skin lesions among the arsenic exposed population in Bangladesh and folic acid supplementation significantly increased urinary creatinine.

It is well known that the concentrations of pollutants in spot urine sample are highly dependent on the dilution of the sample caused by variation in the intake of fluids, physical activity, temperature, etc[19]. Commonly applied method to control for this variation is adjustment by the creatinine concentration in urine[19-21]. However, creatinine is a waste product formed by the spontaneous, essentially irreversible dehydration of body creatine and creatine phosphate from muscle metabolism and meat intake[20-23]. Thus, urinary creatinine (U-cre) varies by gender, age, body size, race/ethnicity, diet, renal function, etc[20,24,25]. Recent studies have been reported that urinary arsenic levels (µg/L) were found significantly correlated with urinary creatinine levels[26,27]. Hindwood et al. (2002) has been suggested that creatinine adjustment of urinary inorganic arsenic (Inorg-As) concentrations may not be required in population studies investigating environmental exposure.

In this study, we investigated the influence of gender, creatinine, and total arsenic concentrations on the percentage of arsenic metabolites in urine as well as blood among the population in Lagunera area of Mexico, who drunk arsenic concentration above 10 µg/L (range: 38-116 µg/L). Our results indicate that more efficient methylation of arsenic in females compared to males. Total arsenic in urine, in blood as well as urinary creatinine concentrations were negatively associated with % inorg-As as well as % MMA, but positively associated with % DMA in urine for both females and males.

Materials and Methods

Reagents.

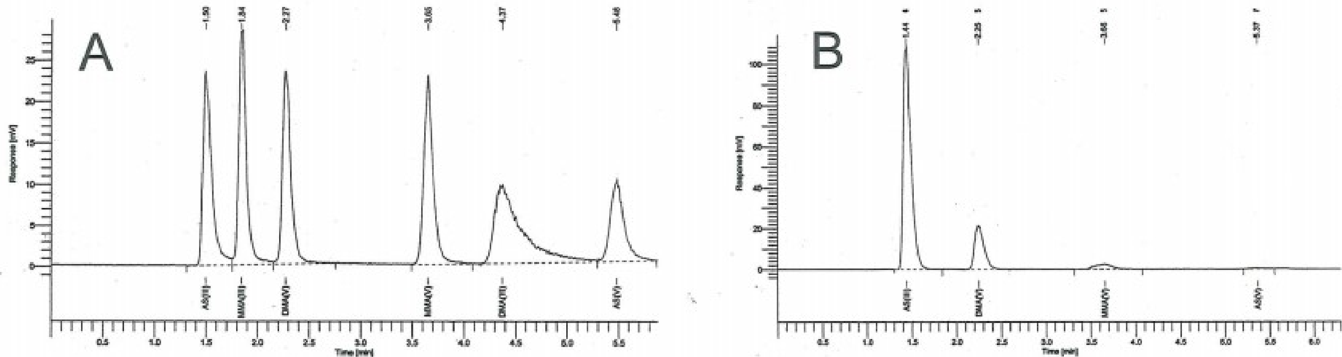
The chemicals used and there sources are as follows: Sodium arsenate (ACS reagent grade) from MCB Reagents (Cincinnati, OH); dimethylarsinicacid (sodium salt), ammonium phosphate (dibasic), and arsenobetaine from Sigma Chemical Co. (St. Louis, MO); sodium m-arsenite and ammonium nitrate from Sigma-Aldrich Co. (St. Louis, MO); disodium methyl arsenate from ChemService, Inc. (West Chester, PA). The arsenic standard solution was from SPEX Certiprep (Metuchen, NJ). Freeze-dried urine reference material for toxic elements (SRM 2670a) and frozen bovine blood reference material for toxic metals (SRM 966) from National Institute of Standards & Technology (NIST, Gaithersburg, MD 20899). Triton X-100 from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were analytical reagent grade or the highest quality obtainable. Water was doubly deionized and distilled.

Subjects. Urine and blood samples were collected from 191 subjects (98 females and 93 males), aged 18-77 years in the Lagunera area of Mexico. There were five groups, based

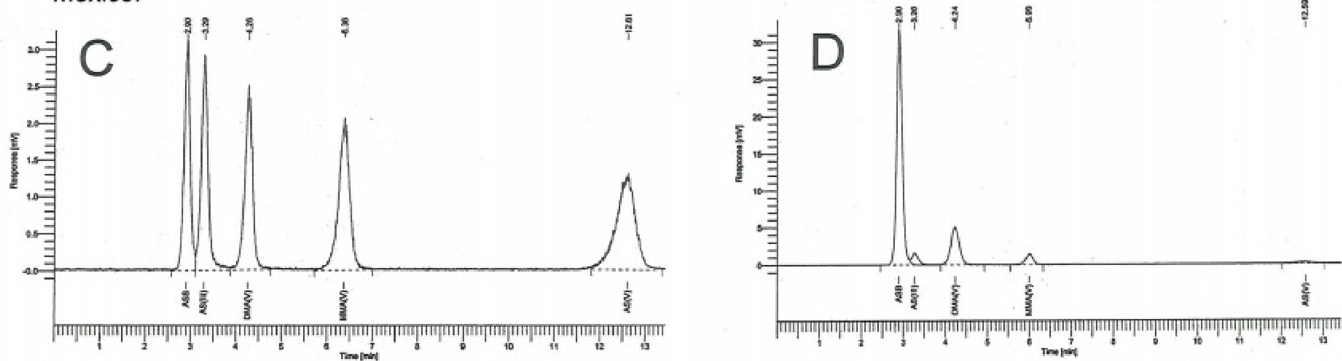
on total arsenic concentrations (38-116 µg/L) in their drinking water.

Urine and blood collection. All collecting containers were soaked overnight in 2% nitric acid (Baker analyzed for trace metal analysis) (J. T. Baker, Inc. Phillipsburg, NJ) and rinsed with double distilled and deionized water. All plastic measuring and collecting equipment were similarly washed, sealed in bags, placed in locked footlockers, and transported by air to the site of the study at the same time as the investigators. After collection, urine sample was immediately frozen in a portable icebox containing dry ice. Blood was collected by venous puncture, into Vacutainers containing EDTA, transferred to the vial, and immediately frozen. The samples were kept frozen while being transported to the University of Arizona, Tucson where they werestored at -70°C before analysis.

Separation techniques of urinary arsenic metabolites. Arsenic contamination in drinking water is the reason for the elevated levels of arsenic in urine. Even arsenic from seafood (arsenobetaine, AsB) may be responsible for the elevated levels of arsenic in urine. Thus, to know the nature of arsenic contamination and the measurement of arsenic metabolites, HPLC-ICP-MS is the most advance and reliable technique. The method of Gong et al. (2001)[28] could not separate AsB and AsB was overlapped with arsenite (Figs. 2B & 2D). In this study, an HPLC-ICP-MS method[29] was modified by author for the measurement of arsenic metabolites including AsB in urine (Figs. 2C & 2D). One of the urine sample (sample ID # 141) contained very high level of arsenic (697 µg/L urine) due to AsB (509 µg/L urine, i.e., 73% of total arsenic; Fig. 2D) and we were excluded this sample from our results.



Method 1: The HPLC system consisted of a PerkinElmer Series 200 HPLC with a reverse-phase column (Gemini 5 µm C18 110A, 150 x 4.6 mm, Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetra-butylammonium hydroxide, 2mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 mL/min. The column temperature was maintained at 50^0 C. **(A)** Arsenic standard containing a mixture of 10 ppb As(V), As (III), MMA(V), MMA(III), DMA(V), and DMA(III); and **(B)** Human urine sample (**sample ID # 141**) collected from Lagunera area of Mexico.



Method 2: The HPLC system consisted of a PerkinElmer Series 200 HPLC with an anion exchange column (Gemini PRP-X100, 10 µm, 250 x 4.6 mm, Hamilton Company, Nevada). The mobile phase (pH 8.5) contained 10 mM ammonium nitrate and 10 mM ammonium phosphate (dibasic) at a flow rate of 1 mL/min. The column temperature was maintained at 30^0 C. **(C)** Arsenic standard containing a mixture of 10 ppb AsB, As(V), As (III), MMA(V), and DMA(V); and **(D)** Human urine sample (**sample ID # 141**) collected from Lagunera area of Mexico.

Figure 2: HPLC methods for separation of urinary arsenic metabolites. Method 1**:** The method of Gong et al. (2001)[28] and Method 2**:** The modified method of Reuter et al. (2003)[29].

Arsenic species/metabolites analysis. Frozen urine samples were thawed at room temperature, filtered with a 0.45 µm filter (Nanosep MF Centrifugal Devices, Pall Life Sciences, Ann Arbor, MI), and diluted 5-fold using Milli-Q water before injection. An HPLC-ICP-MS (High Performance Liquid Chromatography Inductively Coupled Plasma-Mass Spectrometry) speciation method[29] was modified for the measurement of arsenic concentrations. The HPLC system consisted of a PerkinElmer Series 200 HPLC with an anion exchange column (Gemini PRP-X100, 10µm, 250 X 4.6mm, Hamilton Company, Nevada). The mobile phase (pH 8.5) contained 10 mM ammonium nitrate and 10 mM ammonium phosphate (dibasic) at a flow rate of 1 ml/min. The column temperature was maintained at 30° C. An ELAN DRCE ICP-MS (Perkin-Elmer) with a cyclonic quartz spray chamber and Meinhard nebulizer was used as a detector for the analysis of arsenic species [AsB, AS (V), As (Ill), MMA (V), and DMA (V)] in urine at 4° C. The operating parameters were as follows: $R_f$power, 1400 W; plasma gas flow, 15 L/min; nebulizer gas flow, 0.82 L/min; auxiliary gas flow, 1.2 L/min; oxygen flow for DRC, 0.87 mL/min; and arsenic was measured at miz 91.

The working detection limits were 0.80 - 1.75 µg/L for arsenic metabolites. Accuracy values were calculated by spiking standard compounds of all five species (5 µg/L) in urine samples. The recoveries of the added compounds were 98-103%. Standard samples (5 µg/L) containing all five arsenic species were also analyzed after analysis the urine samples each day. The values of mean ± SE for AsB, As (V), AS (III), MMA (V), and DMA (V) were found 4.86 ± 0.08, 5.09 ± 0.11, 5.16 ± 0.11, 5.02 ± 0.10, and 4.90 ± 0.05µg/L, respectively.

Total arsenic analysis in urine samples. Urine samples in acid washed polypropylene tubes were digested with nitric acid (5: 1) while a water bath for 40 min at 70° C. Freeze-dried urine reference material for toxic elements containing arsenic at a level of 220 ± 10 µg As/L was used for quality control and to validate the assay. After acid digestion, analysis of this standard by ICP-MS yielded a range of 216.0 - 236.0 µg As/L with a range of recoveries of 98.18 - 107.27%. We also analyzed the spiking standard compounds of all the arsenic species [As B, As (V),AS (Ill), MMA (V), and DMA (V)] at levels of 10 µg total As /L and 20 µg total As/L. The recoveries of the spiking samples were 104.20 % (10.42 ± 0.13 µg As/L) and 97.70 % (19.54 ± 0.24 µg As/L), respectively. After acid digestion, analyzed trace elements in urine samples collected from the subjects and NIST reference urine samples. The recoveries of Se, Zn, Co, Cu, Mn, Ni, Cd, Pb, and Hg in NIST reference urine were 92.16 %, 93.01 %, 101.00 %, 94.77 %, 106.06 %, 100.84 %, 109.70 %, 100.72 %, and 94.28 %, respectively. The multi-element standard solutions were digested and diluted using the same procedure and dilution factors (as the samples) for preparation of the calibration curve. The calibration correlation coefficients (r^2) of the elements were greater than 0.999.

Total arsenic analysis in whole blood samples. Whole blood samples were analyzed for total As concentrations using Perkin Elmer Elan DRCe ICP-MS. Inductively coupled plasma mass spectrometry method for elements in whole blood was developed (with modifications) based on published method[30]. Whole blood samples were thawed, thoroughly mixed, diluted 50 times with diluents containing 0.65% HNO_3 + 0.1% Triton X-100, and centrifuged for 10 min (3500 rpm at 4° C) with the supernatant reserved for analysis. The multi-element standard solutions were prepared from stock standard solution with 0.65% HNO_3 + 0.1% Triton X-100. The rinse solution contained 2% HNO3 + 1% Triton X-100. The calibration correlation coefficients (r^2) of the elements were greater than 0.999.

Frozen bovine blood reference material for toxic metals was used for quality control and to validate the assay. The reference sample was thawed in ice, mixed thoroughly, and diluted 50 times with diluents containing 0.65% HNO_3 + 0.1% Triton X-100, and centrifuged for 10 min (3500 rpm at 4° C) with the supernatant reserved for analysis. The recoveries of Pb, Cd, and Hg in the reference bovine blood samples were 92 %, 107 %, and 97 %, respectively. The certified values of As was not available. We also analyzed the spiking standard elements in the human blood samples and also the quality control (QC) standard samples. The spiking and QC samples were prepared and analyzed using the same procedures as the human blood samples. The recoveries of the elements in the spiking and QC samples were very close to the spiking and QC standard values.

Creatinine measurement. Creatinine (cre) concentration in urine sample was determined using the Randox Creatinine Colorimetric kit (San Diego, CA), which is based on the reaction of creatinine with picric acid in alkaline solution, forming a colored complex, and measured at 492 nm[31].

Statistical analysis: The mean and standard error (SE) were calculated. The unpaired t test (Graph Pad Software, Inc., 2005) was used to analyze the significance difference. The correlation coefficients for different variables were tested using the Spearman rank order correlation test (Richard Lowry, 1998, 2008). *P* values less than 0.05 (two-tailed) were considered significant.

Results

Study population. In this study, out of 191 participants in Lagunera area of Mexico, 98 were females (F) and 93 were males (M). The average age of females versus males was not statistically significant (Table 1). There were five groups of participants based on total arsenic concentration in their drinking water. The concentrations of As in drinking water were positively associated with urinary total As (mean values) of different groups of population (Figs. 3 and 4).

Table 1. The study population in Lagunera area of Mexico.

	Sex	Group 1	Group 2	Group 3	Group 4	Group 5	All groups
As in drinking water (µg/L)		38.2	43.5	96.0	105.1	116.3	
No. Of participants	F	17	19	19	22	21	98
	M	21	14	20	18	20	93
Ages (mean±SE, years)	F	37.94±3.1	39.32±2.9	43.17±3.1	49.00±3.5	43.92±3.5	42.69±1.5
	M	40.00±3.6	46.92±5.0	41.44±4.6	42.06±2.8	39.00±3.3	41.47±1.7

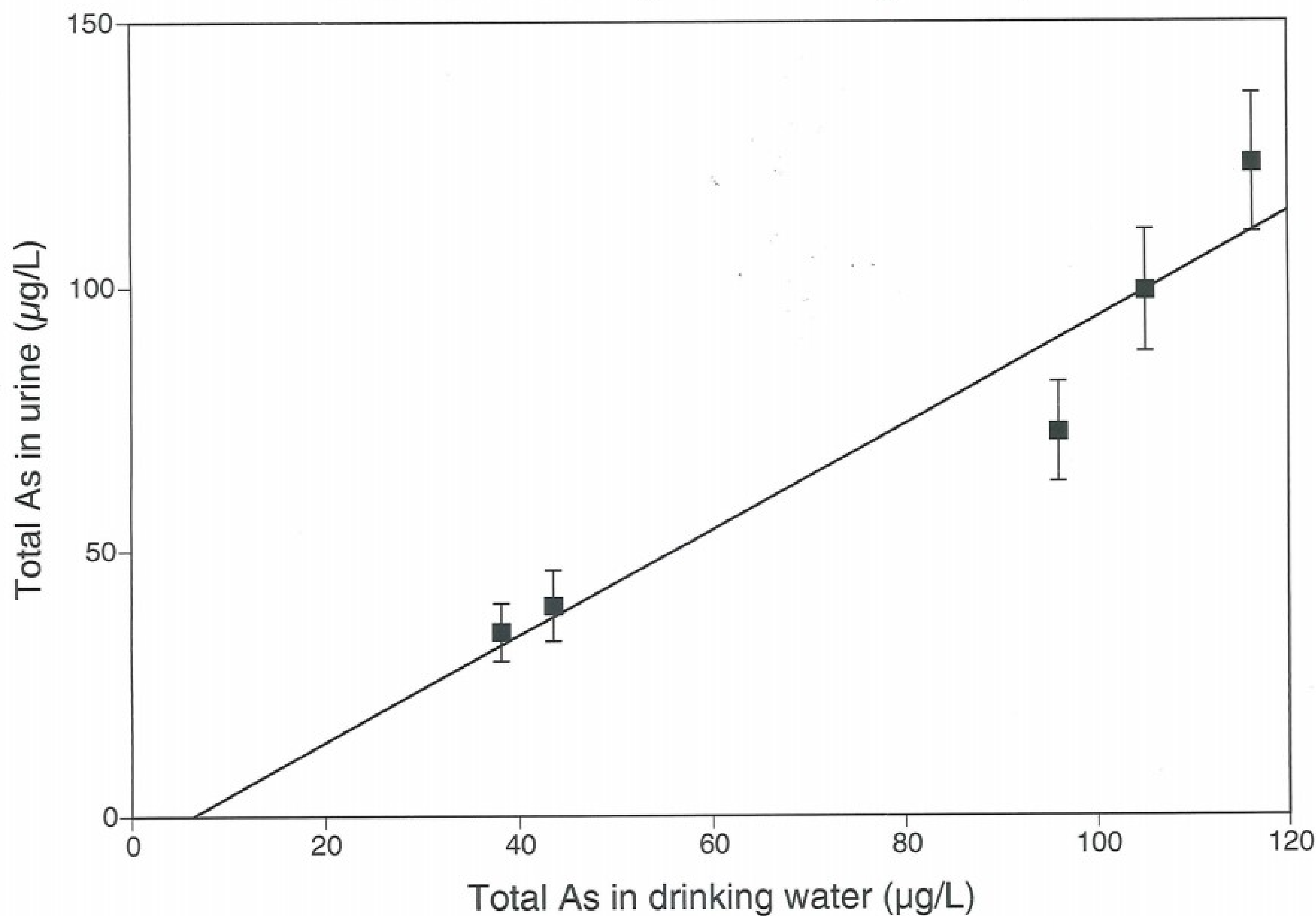


Figure 3: The correlation between total arsenic in drinking water and total arsenic in urine samples (after acid digestion) of different groups of population.

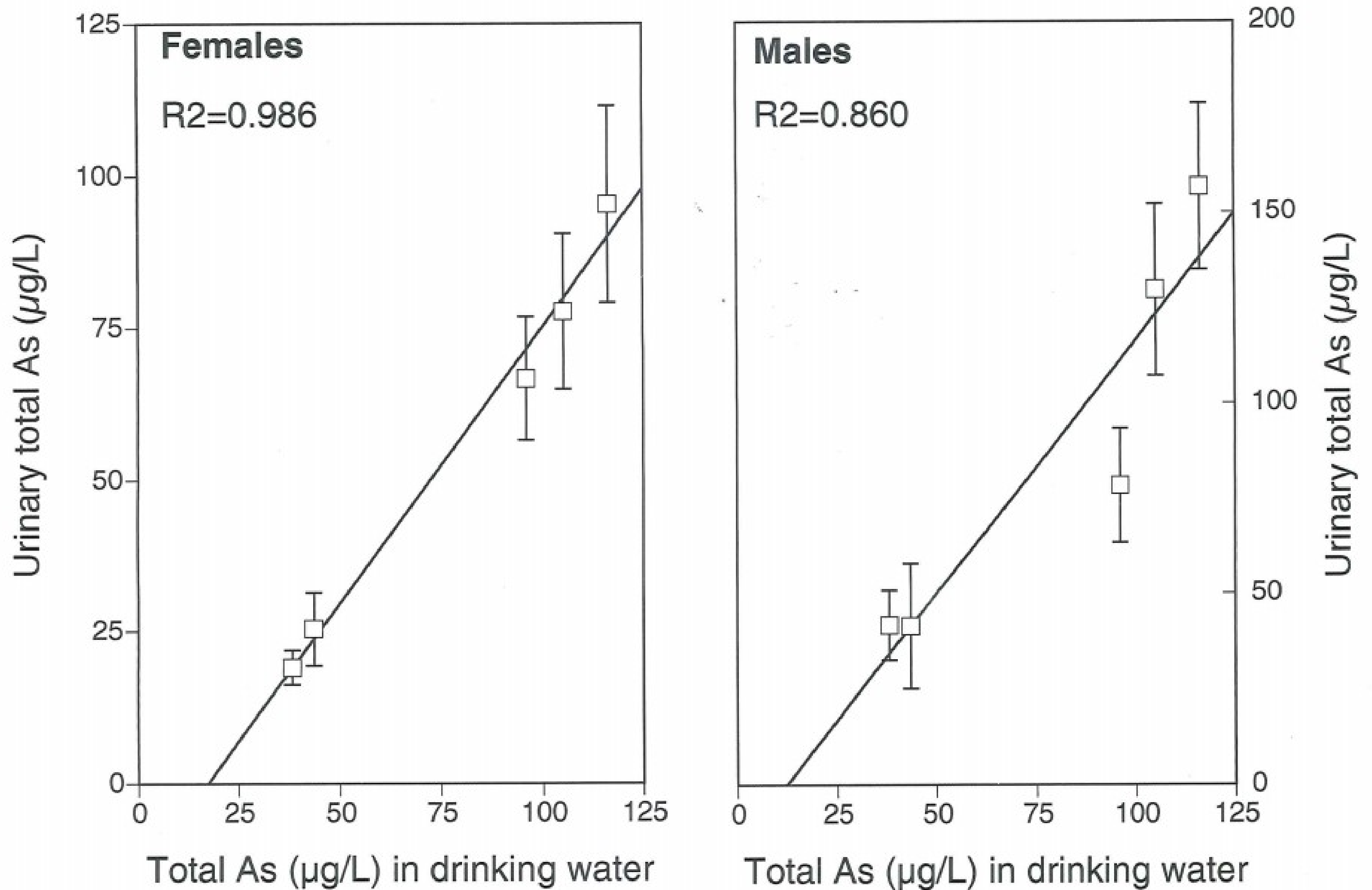


Figure 4: The correlations between total arsenic in drinking water and total arsenic in urine samples (after acid digestion) of females and males from different groups of population.

Urinary arsenic metabolites. The distribution of the percentage (%) of arsenic metabolites [AsB, As (V), AS (III), MMA (V), and DMA (V)], sum of arsenic metabolites (As Sum), and total arsenic (Total As) in urine (after acid digestion) of different groups of population were shown in the Fig. 5. The mean values of sum of arsenic metabolites and total arsenic in urine samples of individual groups of population were very close.

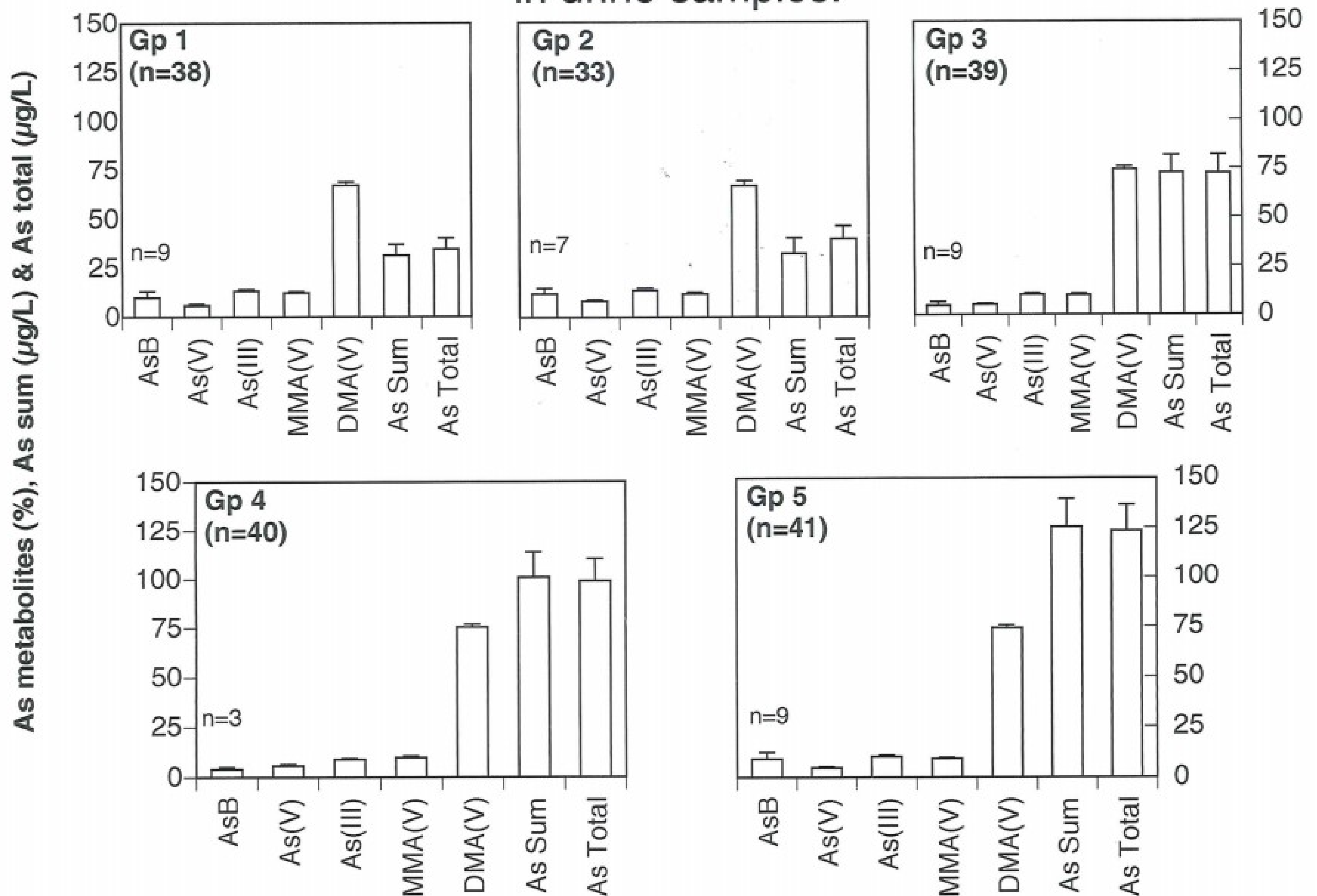


Figure 5: Distribution of the percentage of arsenic metabolites, sum of arsenic metabolites, and total arsenic in urine samples (after acid digestion) of different groups of study population.

Gender differences in the distribution of urinary arsenic metabolites. Urinary arsenic metabolites measured of 191 participants showed a wide inter-individual variability in arsenic methylation capacity. Fig. 6 shows that the highest percentage of females (33 %) had the percentage of inorganic arsenic (% Inorg-As = % As (III) + % As (V)) ranged from 10 to 15 % and males (28.72 %) had ranged from 15 to 20 %. The percentage of MMA (% MMA) ranged from 5 to 10 % had 44.9% of females population and ranged from 10 to 15 % had 44.68 % of males population. On the other hand, the percentage of DMA (% DMA) ranged from 80 to 90 % had 36.73 % of females and ranged from 70 to 80 % had 39.78 % of males population. The percent of DMA ranged from 35.46 to 94.21 %, with the majority of the participants falling ranged from 60 to 90 %.

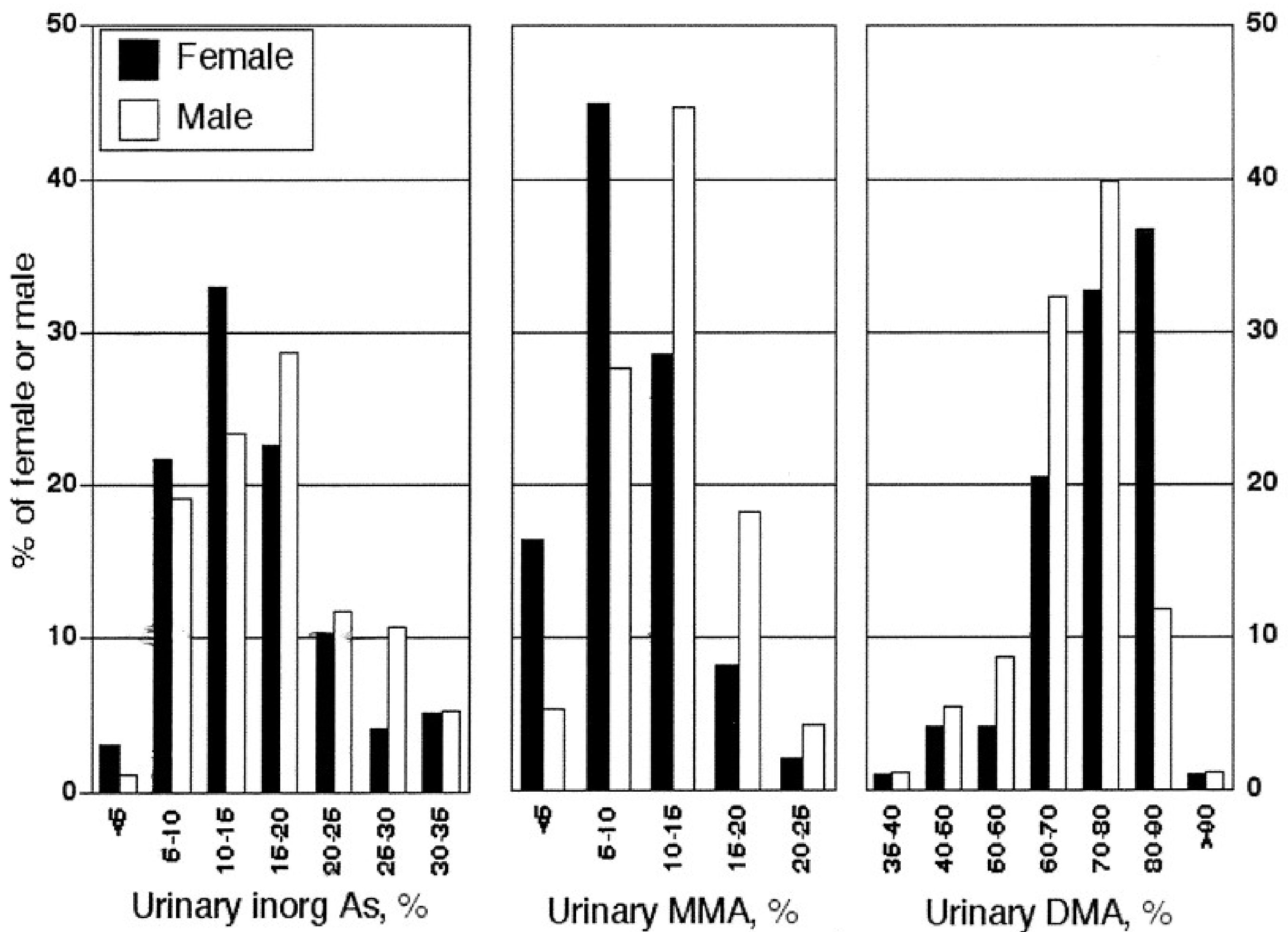


Figure 6: Frequency distribution of arsenic metabolites in urine of females and males.

Overall results show that female participants had less % inorg-As as well as % MMA, and higher % DMA in urines compared to male participants. The mean values of % inorgAs, % MMA, and % DMA in urines for females were 15.13 ± 0.76, 9.43 ± 1 0.48, and 73.97 ± 1.16 %, respectively and for males were 16.75 ± 0.76, 11.71 ± 0.45, and 69.71 ± 0.99 %, respectively. The results indicated that methylation capacity differed by sex: on average, females had a lower % MMA than males (9.43 ± 0.48 vs. 11.71± 0.45 % MMA, respectively, $p<0.01$) and a higher % DMA (73.97 ± 1.16 vs. 69.71 ± 0.99 % DMA, respectively, $p< 0.01$). The % inorg-As did not significantly differ by sex (15.13 ± 0.76 vs. 16.75 ± 1 0.76 % inorgAs, respectively, $p= 0.14$). The mean value of the ratios of % MMA to % inorg-As was significantly lower and the mean value of the ratios of % DMA to % MMA was significantly higher in urine for females compared to males (0.69 ± 0.04 vs. 0.82 ± 0.05, $p<0.05$, and 10.35 ± 0.69 vs. 7.26 ± 0.45, $p\leq0.01$, respectively). Our overall results indicate that second methylation step was more active and first methylation step was less active in females compared to males. The mean value of the ratios of % DMA to % inorgAs was also significantly higher in urines for females compared to males ($p<0.05$).

The correlation between drinking water arsenic concentrations and urinary arsenic as well as blood arsenic concentrations. Total arsenic concentrations in drinking water expressed as µg/L were positively and strongly correlated with arsenic concentrations in urine expressed as µg/L (r_s= +0.56, $p<0.01$) or µg/g cre (r_s= +0.64, $p<0.01$) as well as blood expressed as µg/L (r_s= +0.65, $p<0.01$) of this study population (Fig. 7). Blood arsenic concentrations were also positively correlated with urinary total arsenic concentrations expressed as µg/L (r_s= +0.56, $p<0.01$) or µg/g cre (r_s= +0.68, $p<0.01$).

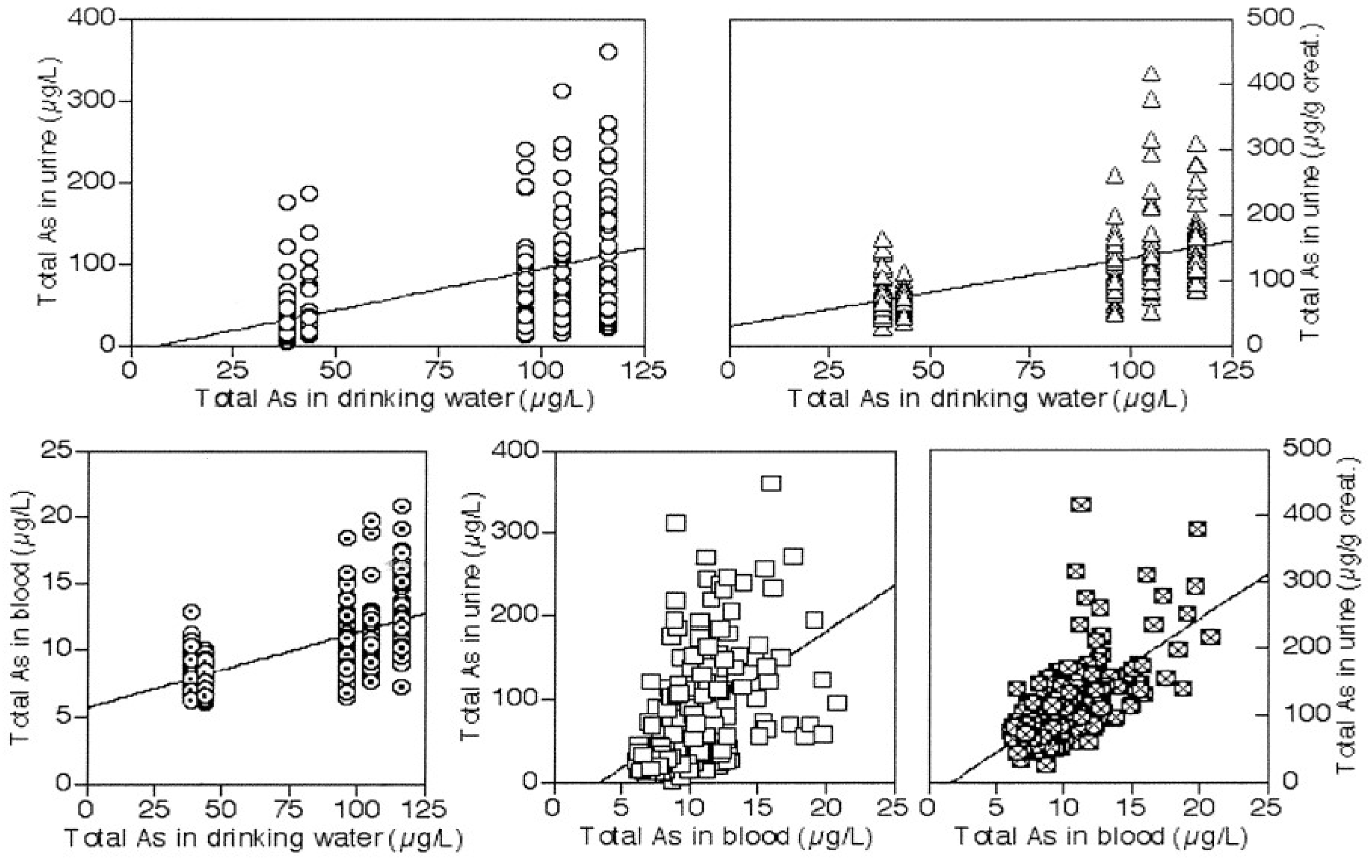


Figure 7: Correlation between As in drinking water and As in urine as well as blood of arsenic exposed people in Lagunera area of Mexico.

The correlations between arsenic concentrations in urine as well as blood and percentage of urinary arsenic metabolites.

The correlations between arsenic concentrations in urine as well as blood and percentage of urinary arsenic metabolites for females and males are shown in Table 2 and Fig. 8.

Table 2. Spearman correlation coefficients (r_s) between urinary as well as blood arsenic concentrations and percentage of urinary arsenic metabolites for females and males.

	Urinary As levels (µg/L)	Urinary As levels (µg/g cre)	Blood As levels (µ/L)
Females			
Log % Inorg As	-0.541^{e}	-0.267^{b}	-0.319^{b}
Log % MMA	-0.433^{d}	-0.276^{b}	-0.257^{a}
Log % DMA	+0.540^{e}	+0.278^{b}	+0.286^{b}
Log % MMA/% inorg As	+0.216^{a}	+0.048	+0.149
Log % DMA/%MMA	+0.474^{d}	+0.308^{b}	+0.298^{b}
Males			
Log % Inorg As	-0.494^{e}	-0.236^{a}	-0.247^{a}
Log % MMA	-0.365^{c}	-0.074	-0.075
Log % DMA	+0.502^{e}	+0.290^{b}	+0.281^{b}
Log % MMA/% inorg As	+0.211^{a}	+0.183	+0.223^{a}
Log % DMA/%MMA	+0.470^{d}	+0.153	+0.164

$^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, $^{d}p<0.00001$, $^{e}p<0.000001$

The concentrations of arsenic in urine expressed as µg/L or µg/g cre and arsenic concentrations in blood expressed as µg/L were negatively associated with % inorg As (r_s= -0.54, p<0.000001; r_s= -0.27, p<0.01, and r_s= -0.32, p<0.01, respectively) as well as % MMA (r_s= -0.43, p<0.00001; r_s= -0.28, p<0.01, and r_s= -0.26, p<0.05, respectively), and positively associated with % DMA (r_s= +0.54, p<0.000001; r_s= +0.28,

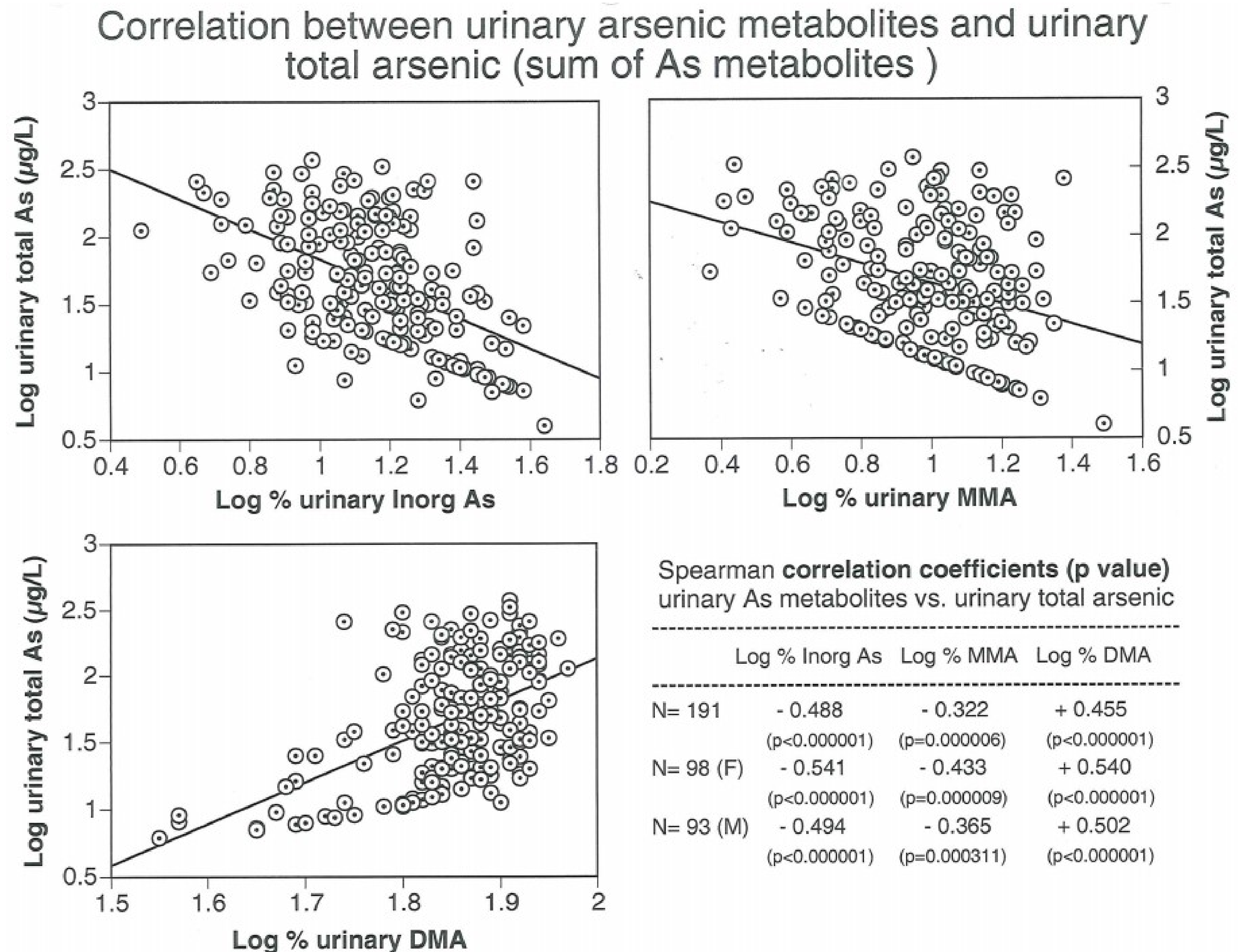


Figure 8. The correlation between urinary arsenic metabolites and urinary total arsenic (sum of arsenic metabolites).

p<0.01, and r_s= +0.29, p<0.01, respectively) in urine for females. The ratios of % DMA to % MMA in urine were also positively and significantly correlated with arsenic concentrations in urine as well as blood (r_s= +0.47, p<0.00001; r_s= +0.31, p<0.01, and r_s= +0.30, p<0.01, respectively) for females. For males, arsenic concentrations in urine expressed as µg/L or µg/g cre and arsenic concentrations in blood expressed as µg/L were also negatively correlated with % inorg As, (r_s= -0.49, p<0.000001; r_s= -0.24, p<0.05, and r_s= -0.25, p<0.05, respectively), and positively correlated with % DMA (r_s= +0.50, p<0.000001; r_s= +0.29, p<0.01, and r_s= +0.28, p<0.01, respectively). The percentage of MMA (% MMA) was not significantly correlated with arsenic concentrations in urine expressed as µg/g cre or in blood expressed as µg/L for males. The ratios of % MMA to % inorgAs in urine were positively and significantly correlated with arsenic concentration in blood (r_s= +0.22, p<0.05) and As concentrations in urine expressed as µg/L (r_s= +0.21, p<0.05) for males, but not with arsenic concentrations in urine expressed as µg/g cre. The correlations between the ratios of % DMA to % MMA and arsenic concentrations in urine expressed as ug/gcre or in blood expressed as ug/L were not statistically significant for males. We also found that the correlation coefficients between arsenic concentrations in blood expressed as µg/L and the percentage of

urinary arsenic metabolites were very close to the correlation coefficients found between urinary arsenic concentrations expressed as µg/g cre and the percentage of urinary arsenic metabolites for both females and males.

Concentrations of total arsenic in urine expressed as µg/L versus µg/g cre for females and males. Urinary total arsenic concentrations (after acid digestion) expressed as µg/L were significantly lower for females compared to males ($p<0.01$) (Table 3). But after urinary creatinine adjustment, urinary total arsenic concentration expressed as µg/g cre was not significantly difference between females and males ($p=0.14$). This was due to significant sex differences in urinary creatinine concentrations and urinary creatinine concentrations were significantly higher for males than females ($p<0.0001$). Urinary creatinine concentrations were not significantly correlated with ages for both females and males participants in the Lagunera area of Mexico.

Table 3. Urinary arsenic (U-As) and urinary creatinine (U-cre) concentrations for females (F) and males (M). Values are the Mean ± SE.

Sex	As (µg/L)	Creatinine (g/L)	As (µg/g cre)
F (N=98)	62.05±5.58	0.52±0.04	121.43±6.35
M (N=93)	90.57±8.38	0.88±0.08	108.52±6.11
p value	p<0.01	p<0.0001	p>0.05

The correlation between urinary total arsenic as well as blood total arsenic concentrations and urinary creatinine (U-Cre) concentrations. Urinary arsenic concentrations were positively associated with urinary creatinine ($r= +0.801$, $p=0.000001$) (Fig. 9). This positive correlation was more strong in males ($r= +0.823$, $p<0.000001$) compared to females ($r= +0.771$, $p=0.000001$).

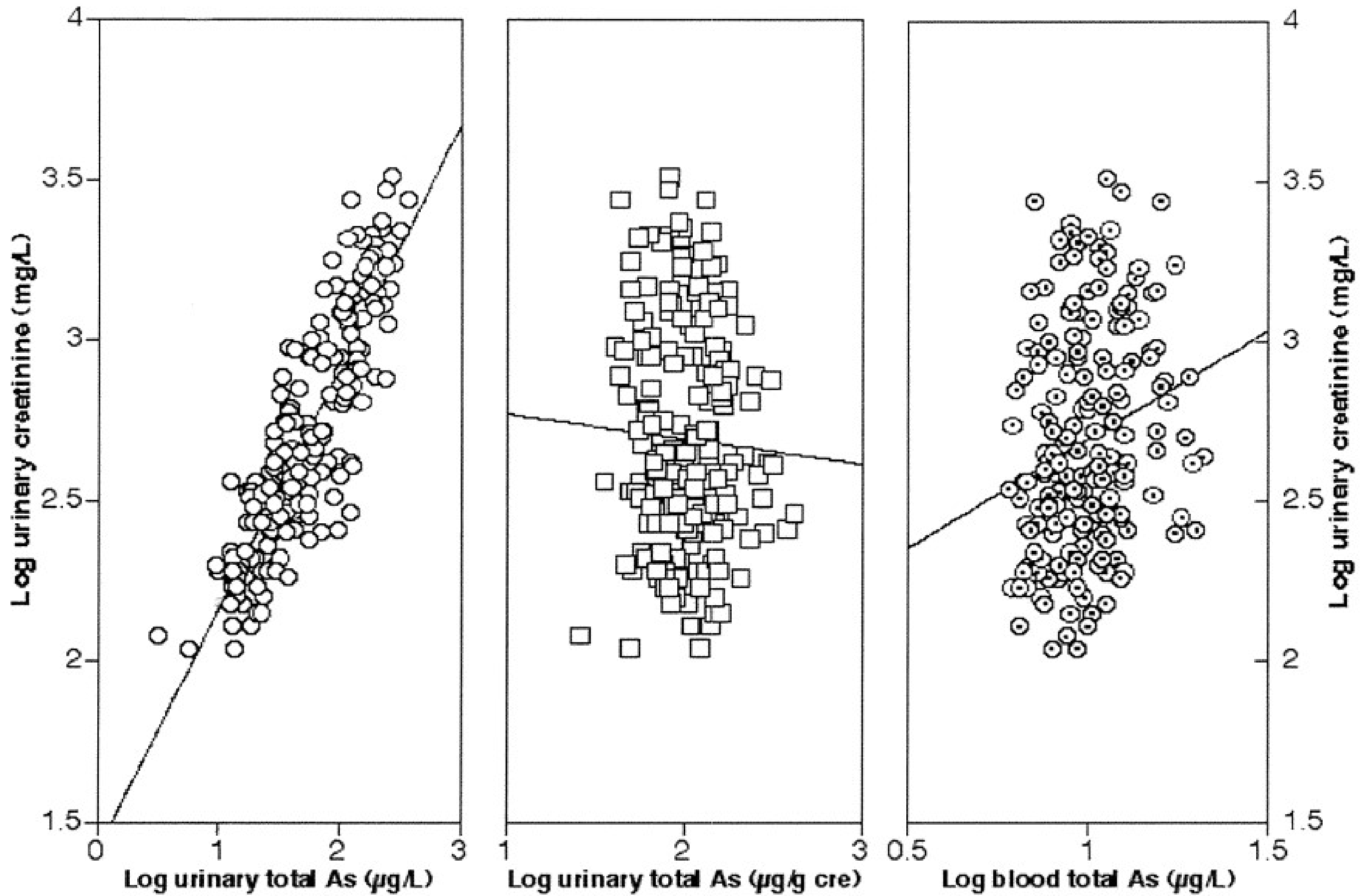


Figure 9: Correlation between Log urinary arsenic as well as blood and Log U-cre concentrations.

The correlation between urinary creatinine concentrations and percentage of urinary arsenic metabolites. The results show (Fig. 10) that urinary creatinine concentrations (g/L) were negatively associated with % inorg As as well as % MMA in urines for both females (r_s= -0.59, p<0.000001 and r_s= -0.34, p<0.01, respectively) and males (r_s= - 0.45, p<0.00001 and r_s= -0.39, p<0.0001, respectively). But urinary creatinine concentrations were more positively associated with % DMA in urines for females (rs= +0.56, p<0.000001) compared to males (r_s= +0.41, p<0.0001). The ratios of % MMA to % inorg-As and the ratios of % DMA to % MMA were positively associated with creatinine in urines for both females and males (Table 4). These positive correlations were stronger for the ratios of % DMA to % MMA (females: r_s= +0.43, p<0.01 and males: r_s= +0.46, p<0.01) than the ratios of % MMA to % inorg-As (females: r_s= +0.26, p<0.01 and males: r_s= +0.14, p=0.167).

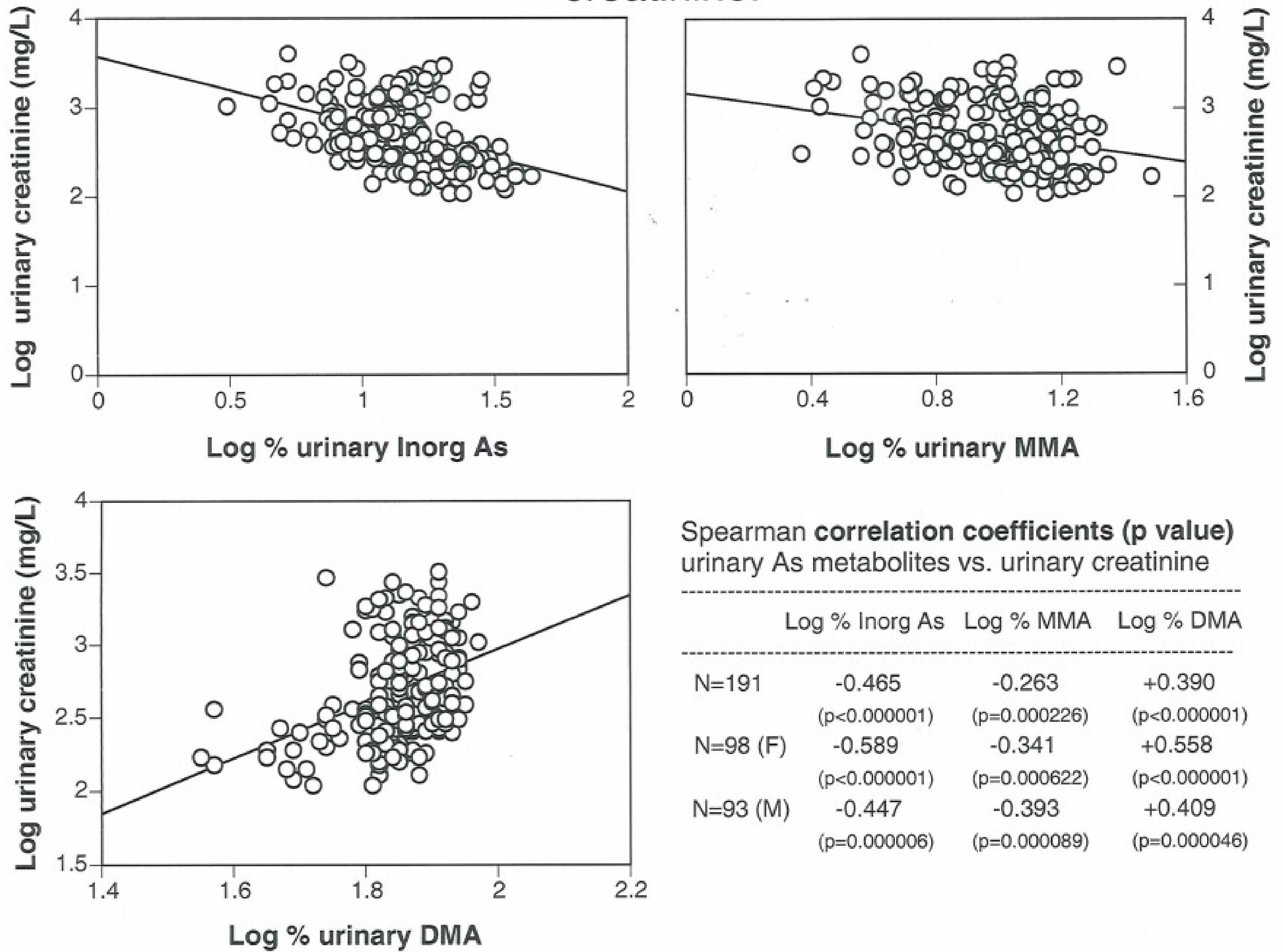


Figure 10. The correlation between percentage (%) of urinary arsenic metabolites and urinary creatinine (mg/L).

Table 4. Spearman correlation coefficients (r_s) between urinary creatinine concentrations (g/L) and percentage of urinary arsenic metabolites for females and males.

	Urinary creatinine (g/L)
Females:	
Log % Inorg As	-0.589[e]
Log % MMA	-0.341[b]
Log % DMA	+0.558[e]
Log % MMA/% inorg As	+0.260[a]
Log % DMA/%MMA	+0.426[c]
Males:	

Log % Inorg As	-0.447[d]
Log % MMA	-0.393[c]
Log % DMA	+0.409[c]
Log % MMA/% inorg As	+0.144
Log % DMA/%MMA	+0.458[d]

[a]p<0.01, [b]p<0.001, [c]p<0.0001, [d]p<0.00001, [e]p<0.000001

Discussion

The present study clearly shows that the participants in the Lagunera area of Mexico had remarkably influenced of sex, dose level, and urinary creatinine concentrations on the percentage of arsenic metabolites in urine.

Sex differences in urinary arsenic metabolites. An important finding of interest in our observation that metylation capacity differed by sex: females had a significantly lower % MMA and a higher % DMA in urine compared to males. The ratios of % MMA to % inorg-As and the ratios of % DMA to % MMA *w*ere also significantly lower and significantly higher in urines for females compared to males, respectively. The results suggest that arsenic methylation capacity was higher in females compared to males. In our knowledge, this will be the first reporting that the efficiency of arsenic methylation was significantly higher in females compared to males who drunk water contain low level of arsenic (range 38-116 µg/L*)* and not showing arsenic related skin effects in Mexican. One study of human exposure to arsenic via drinking water (up to 600 µg/L) in northeastern Taiwan also indicated that females had a higher % DMA and a lower % MMA in urines than males[32]. Another study of human exposed to high arsenic concentrations in drinking water in Bangladesh found a higher fraction of MMA and a lower fraction of DMA in urines among males as compared to females[6]. A study in the U.S. population reported that females excreted a lower % inorg-As as well as % MMA, and a higher % DMA than did males[17]. Another study in Mexican people showing skin effects due to exposure to arsenic via drinking water had a higher % MMA and a lower % DMA in urines than those without such effects[2]. However, they did not compare the % MMA and% DMA in urines for females and males, separately.

In our results, it also appears that first methylation reaction is less active and second methylation reaction is more active in females compared to males for inorganic arsenic biotransformation process. This means that inorganic arsenic converted to MMA faster in males compared to females. But, MMA converted to DMA faster in females than males, and a higher proportion of DMA and a lower proportion of MMA found in urines for females as compared to males. The results suggest and support that more than one methylase may be involved in the oxidative methylation of inorg-As[3,11,33]. A slow second methylation reaction in combination with a faster first methylation reaction seems to be most critical from a toxicological point of view. Higher proportion of MMA in urines, probably higher concentrations of the highly reactive and toxic MMA (III) in the tissues[34,35] leading to a higher retention of arsenic in the body[36-38]. Previous studies reported that females are less susceptible to the arsenic-related skin effects than males[5-7,39]. May be, due to higher methylation capacity and lower retention of arsenic (specially, MMA (III)] in the tissues, females are less susceptible to the arsenic-related skin effects compared to males. Additionally, the two steps of arsenic methylation efficiency involving different methylated metabolites with different concentrations in individuals may likely have distinct features of arsenic health effects.

S-adenosylmethionine (SAM) is the main methyl donor for arsenic menylation reactions[40]. Another important methyl donor, besides SAM, is choline, which could either be derived from the diet or from phosphatidylcholine. Experimental studies on rabbits fed diets with low amounts of choline or methionine, have shown a marked decrease in the urinary excretion of DMA[41]. Recent studies have indicated that the synthesis of phosphatidylcholine is up regulated by estrogen[42]. Possibly, explaining the better methylation of arsenic among females compared to males is related to the higher endogenous production of choline in females, which after oxidation to betaine is the sole alternate methyl group to folate for the remethylation of homocysteine to methionine[43,44]. Elevated homocysteine levels, which are indicative of a lower one carbon metabolism, are associated with less efficient methylation of arsenic[45] and elevated plasma homocysteine level was correlated with high levels of %MMA in urine[46]. S-adenosylhomocysteine (SAH) is an inhibitor of the activity of many methyltransferase[47]and it has been reported that SAH decreased methylation of arsenite, especially DMA production[48]. It was also reported that arsenic methylation is induced during pregnancy[6,49]and may be sex hormones play an important role for arsenic methylation. Another important explaining for the efficient second metylation of arsenic in females compared to males is most likely genetic polymorphisms in genes coding for enzymes involved in arsenic methylation[3]. In near future this will be cleared the mechanisms of arsenic methylation involving sex hormones.

Influence of arsenic doses on urinary arsenic metabolites. The association between arsenic concentrations in drinking water and urines had a positive linear coefficient of +0.56. The drinking water As (WAs) concentrations (µg*/*L) were positively and strongly correlated with blood arsenic *(*BAs) concentrations (µg/L) for both females and males. BAs concentrations (µg/L) were also positively and strongly correlated with urinary arsenic (UAs) concentrations expressed as µg/L as well as µg/g cre for both females and males. Similarly to our findings, Hall et al.

(2006)[50] found a strong positive correlation between BAs and UAs, and both *w*ere positively and significantly correlated with WAs. Our results show that urinary as well as blood As concentrations were negatively correlated with urinary % inorg As as well as % MMA, and positively correlated with % DMA, the ratios of % MMA to % inorg As as well as the ratios of % DMA to % MMA for both females and males. These results indicated that the methylation of arsenic was increased (specially, second methylation reaction) with increasing urinary arsenic, i.e., increasing arsenic concentration in drinking water (38- 116 µg/L) of our study population. A number of experimental studies on human subjects receiving specified doses of inorganic arsenic, indicated that the urinary excretion of total arsenic metabolites increased with decreasing % inorg-As as well as % MMA but increasing % DMA in urine[51-54].

Influence of urinary creatinine concentrations on urinary arsenic metabolites. A number of significant correlations were observed regarding urinary arsenic metabolites and urinary creatinine concentrations in this study. Urinary creatinine was negatively associated with % inorg-As as well as % MMA, and positively associated with % DMA in urine for both females and males. Urinary creatinine concentrations were also positively and strongly associated with the ratios of %DMA to %MMA, but not significantly associated with the ratios of %MMA to %inorg-As in urine for both females and males. The results indicate that there are some difference mechanisms between the first methylation reaction and the second methylation reaction of arsenic biotransformation process. Gamble et al. (2005)[26] also observed that urinary creatinine concentrations were positively and significantly associated with % DMA for both females and males, and negativel*y* associated with % inorg As as well as % MMA in urines for females only. Other researchers have not been reported significant correlation between creatinine concentrations and % MMA in urine for males with the exception of our observation in this study.

Creatinine is derived from creatine and creatine phosphate in muscle tissue. It is produced and excreted from the body in the urine via the kidney at a constant rate which is proportional to the body muscle mass[20]. In our study, the association between urinary creatinine concentrations and the percentage of arsenic metabolites were remarkable. The urinary creatinine concentration was significant predictor of arsenic methylation for both females and males. The urinary creatinine concentration is highly correlated with muscle mass[20,55] and may have some unknown impact on arsenic methylation process. Boeniger et al. (1993)[56] reported that 15-20% of the creatinine in urine could occur by active secretion from the blood through the renal tubules, i.e., urinary creatinine is influenced by renal function, which could have some unclear function for arsenic methylation process. Studies are needed to know the mechanisms of the correlation between creatinine formation and arsenic methylation process in humans.

Conclusions. The results of this study suggest to conclude the following information: (i) More efficient methylation of arsenic among females compared to males of the population in the Lagunera area of Mexico, who drunk arsenic concentration above 10 ug/L (range: 38-116 µg/L), (ii) Due to slower 'first methylation reaction' and faster 'second methylation reaction', females may less susceptible to arsenic-related skin effects compared to males[5-7], (iii) The methylation of arsenic was increased (specially, second methylation reaction) with increasing urinary arsenic, i.e., increasing arsenic concentration in drinking water, (iv) Creatinine formation may influence arsenic metabolisms with unknown mechanisms, and we need to study for understanding of these mechanisms, and (v) Data from females and males should be reported separately.

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