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ARTICLE

# Assessment of arsenic species in human hair, toenail and urine and their association with water and staple food

Hifza Rasheed[1] · Paul Kay[1] · Rebecca Slack[1] · Yun Yun Gong[2]

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## Abstract

Arsenic intake from household drinking/cooking water and food may represent a significant exposure pathway to induce cancer and non-cancer health effects. This study is based on the human biomonitoring of 395 volunteers from 223 households with private water sources located in rural Punjab, Pakistan. This work has shown the relative contribution of water and staple food to arsenic intake and accumulation by multiple biological matrix measurements of inorganic and organic arsenic species, while accounting for potential confounders such as age, gender, occupation, and exposure duration of the study population. Multi-variable linear regression showed a strong significant relationship between total arsenic (tAs) intake from water and concentrations of tAs, inorganic arsenic (iAs), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) in urine and toenail samples. tAs intake from staple food (rice and wheat) also showed a strong significant relationship with hair tAs and iAs. The sole impact of staple food intake on biomarkers was assessed and a significant correlation was found with all of the urinary arsenic metabolites. Toenail was found to be the most valuable biomarker of past exposure to inorganic and organic arsenic species of dietary and metabolic origin.

**Keywords:** Monomethylarsonic acid · Dimethylarsonic acid · Toenail arsenic · Dietary exposure · Urinary arsenic metabolites

## Introduction

Human exposure to toxic inorganic arsenic (iAs) via water is a recognised public health and scientific concern [1]. Recently detected arsenic concentrations in food have also raised the question as to the contribution from food. Based on the evidence of carcinogenicity in humans, the International Agency for Research on Cancer (IARC) classified arsenic and iAs compounds as 'carcinogenic to humans' (Group 1) and classified dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) as 'possibly carcinogenic to humans' (Group 2B) [2]. A sequence of reduction and methylation reactions in the human body metabolises iAs into MMA, which is further methylated to DMA [3, 4]. Following ingestion, iAs compounds are well absorbed by humans at an estimated rate of 50 and 95% [5].

Most of the ingested arsenic is excreted as methylated arsenic within 1–3 days following exposure, although a part of it is stored in sulphydryl-rich tissues, such as skin, nail and hair [6]. Average per day growth rates for fingernails (0.1 mm), toenails (0.1 and 0.03–0.5 mm) and hair (0.2–1.12 mm) depict exposure during the last 6, 12–18 and 3–12 months, respectively [7–9]. This makes nail and hair effective biomarkers of past exposure, however arsenic toxicokinetics depend on the forms of arsenic and variations in association with various factors such as age, sex, nutritional status and genetic polymorphisms [10]. Types and levels of excreted methylated arsenic as a useful biomarker may vary with such factors, although few studies have assessed their impact [11–14]. Arsenic speciation in hair, toenail/nail has been inadequately performed, whilst the association of arsenic intake from water and food with inorganic and organic arsenic species in hair, toenail and urine has also been insufficiently studied. For this reason, the present study aimed to assess the impact of arsenic exposure in a population of rural settings of the Punjab province, Pakistan using urine, hair and toenail biomarkers.

✉ Hifza Rasheed
gyhj@leeds.ac.uk
hifzajohar@gmail.com

[1] School of Geography, University of Leeds, Leeds LS2 9JT, UK

[2] School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK

The specific objectives of this research were set to [1] assess human exposure to As through the measurement of total arsenic (tAs) and arsenic species in hair, toenail and urine, and [2] study the impact of dietary exposure (including water) on the internal dose of arsenic species in relation to potential modifiers.

## Materials and methods

### Study area and study participants

The study villages were located within four districts of Pakistan (Kasur, Sahiwal, Bahawalpur and Rahim Yar Khan), where at least one ground water source was found to be contaminated with arsenic above 50 $\mu g\,L^{-1}$. The sampling frame consisted of 398 volunteers (223 households in villages Chak-46/12-L, Chak-48/12-I and Chak 49/12-l, Badarpur, Basti Balochan and Basti Kotla Arab) enrolled and interviewed in our previous studies which aimed to assess the household ground water arsenic concentrations [15] and dietary consumption patterns [16]. Residents of these villages were mostly dependent on the household ground water sources (wells, hand pumps) installed 8 to 44 years ago and previously found to have tAs of 0.48–3090.00 $\mu g\,L^{-1}$ [15]. The participants were non-smoking males and females who used their household ground water for drinking and food preparation, did not eat seafood, use any homoeopathic or herbal medicines and were not away from their houses for more than a week during the sampling months of August–October, 2014 for collection of urine, hair and toenail samples. Pregnant women were excluded from the study, and after all exclusions, urine ($n = 395$), toenail ($n = 20$) and hair ($n = 19$) samples were collected.

### Collection of urine, hair and toenail samples

Spot urine samples from 246 males and 149 females of six villages of the Punjab province, Pakistan were collected in labelled sterile 2-oz polyethylene urine collection containers and kept in an ice box at 4 °C prior to return to the laboratory. All urine samples were transferred to a field freezer within 2 h for storage at −20 °C and transported to the National Water Quality Laboratory, where creatinine was determined on a 1mL subsample. All samples were then shipped with dry ice to the Brooks Applied Laboratory (BAL), USA by air, stored at −70 °C, and finally measured for urinary arsenic metabolites within 4 months.

Using ethanol-rinsed stainless-steel scissors, a full strand of hair sample was obtained by the sampling team from the nape of the head as near as possible to the scalp (at a distance of 1 cm from scalp). Hair samples were stapled on cardboard, placed in sealed plastic bags and stored at room temperature until analysis. Participants were asked to remove nail polish, if any, and collect their toenail clippings from all toes using the provided stainless-steel clippers [7, 17]. These were placed in individual polyethylene bags, shipped to BAL and stored at ambient temperature (20 °C) until analysis.

### Urine samples processing and analysis

Urinary concentrations were corrected for creatinine concentrations, which were determined by the Jaffe's method as described by Bonsnes and Hertha [18]. This correction was done by dividing the concentration of arsenic metabolites ($\mu g\ L^{-1}$) by U-Cre ($g\ L^{-1}$) to express the urinary arsenical species as $\mu g\ g^{-1}$ creatinine.

Frozen urine samples were thawed to room temperature and centrifuged at 3000 rpm for 10 min, and the resultant supernatants were diluted tenfold with ultrapure water and analysed for tAs following the U.S. Environmental Protection Agency method 1638 (mod.) using inductively coupled plasma dynamic reaction cell-mass spectrometry (Model: ELAN DRC II ICPMS, Perkin Elmer SCIEX, Concord, Ontario, Canada). For measurement of urinary arsenic species i.e., arsenate (AsV), arsenite (AsIII), MMA, DMA and arsenobetaine (AsB), aqueous samples were filtered through a 0.45-µm filter. The filtered aliquot were analysed by high-performance liquid chromatography system (Dionex GP-40) coupled to an inductively coupled plasma-mass spectrometer (ICP-MS) (Agilent 7700x ICPMS, Agilent Technologies) following the method described by Hata et al. [19]. Urine samples after processing were rapidly analysed to ensure appropriate preservation of organic species. Since As(III) can oxidise to As(V) [5] during samples handling and laboratory processing, urinary iAs was presented as the sum of As(III) and As(V). The limits of detection were 0.1 $\mu g\,L^{-1}$ for tAs, As(III), DMA and AsB, and 0.3 $\mu g\,L^{-1}$ for As(V) and 0.2 $\mu g\,L^{-1}$ for MMA.

### Hair and toenail samples processing and analysis

Each hair sample was cut to a length of 0.125 inch (0.3 cm), representing approximately the last 2 months of As exposure before sampling. Past studies evaluating the external contamination of hair and nail have reported that washing procedures effectively removed the exogenous As from toenail and hair samples [20, 21]. Thus, external contamination from hair and toenail clipping samples was removed by immersing the samples three times in 5 ml of a 0.5% Triton TX-100 solution and shaking thoroughly by hand for 30 s. Samples were rinsed three times with 18.2 MΩ deionised water (DIW) and then twice with HPLC grade acetone [21]. Hair samples underwent the same

cleaning and digestion procedure as toenail samples. Polycarbonate filters (0.4 μm) and an anti-static device were used for the transfer of hair samples between vessels. Following rinsing, samples were dried overnight at room temperature and weighed. Following the USEPA method 3050b [22], an aliquot of dried toenail or hair sample was prepared by adding multiple additions of $HNO_3$ and hydrogen peroxide ($H_2O_2$) and heating at 95 °C ± 5 °C. After cooling, the volume was made up to 100 mL with DIW, centrifuged and stored at room temperature until analysed exclusively for endogenous arsenic and its species. tAs was measured using the technique of inductively coupled plasma dynamic reaction cell-mass spectrometry (Model: ELAN DRC II ICPMS, Perkin Elmer, Shelton, CT, USA). All sample extracts for arsenate (AsV), arsenite (AsIII), MMAs and DMAs quantitation were also analysed employing an Agilent 7700 CRC ICP-MS with a Dionex GP40 HPLC (IC) Systems.

For speciation, an aliquot of filtered sample was injected using a Dionex HPLC onto an anion-exchange column and mobilised isocratically using an alkaline (pH > 7) eluent. The mass-to-charge ratio (m/z) of As at mass 75 was monitored using an Agilent 7700, whilst selenium at m/z 82 was monitored as an internal standard. Retention times for eluting species were compared with NIST traceable known standards for species identification.

## Quality assurance

Species data was provided by the analysis of NIST (National Institute of Standards and Technology) traceable standard reference materials (SRMs-1640A, trace elements in natural water). Background contamination was monitored using laboratory-fortified blanks for urine analysis. Duplicate measurements were made on 10% ($n = 40$) of urine samples for total arsenic and arsenic species. The reliability of the arsenic species determination was evaluated by analysing the samples in duplicate and spiking the samples with As(III), As(V), MMA, DMA and AsB. Arsenic measured in SRMs-1640A was 7.59 ± 0.36 tAs μg $kg^{-1}$ ($n = 6$), within the certified range of 8.010 ± 0.067 μg $kg^{-1}$, yielding a mean recovery of 96%. The spike recoveries of tAs, AsIII, AsV, DMA, MMA and AsB in digests of matrix spikes ($n = 31$), matrix spike duplicates, duplicates ($n = 40$) and laboratory-fortified blank ($n = 6$) met the data quality standards in terms of relative percent difference (RPD) of < 25%, percent recovery of 75 to 125% and completeness of 80%.

For quality control of hair and nail samples, method blanks, blank spikes, standard reference materials (SRMs) and duplicates were treated in the same way as the samples and incorporated into each digestion batch and analytical run. Human hair SRM (NCS DC 73347 from China National Analysis Centre for Iron and Steel Beijing, China) was used for both hair and nail samples. Arsenic measured in SRM NCS DC 73347 was 274 ± 0.5 tAs μg $kg^{-1}$ ($n = 2$), within the certified range of 280 ± 50 μg $kg^{-1}$, yielding a mean recovery of 98%. There is no available SRM of human hair or nail containing certified concentration for arsenic species. The organic species represented a minimum fraction of tAs in SRM NCS DC 73347, whilst iAs more than 65% of the extraction indicated the main proportion of As in hair. The spike recoveries of tAs, iAs, DMA and MMA in digests of matrix spikes ($n = 2$), matrix spike duplicate ($n = 2$), duplicate ($n = 2$), blank spikes ($n = 2$) and post spikes ($n = 2$) were 83–92% for hair and 93–123% for toenail.

## Statistical analysis

The analysed tAs represents the sum of As species as well as other unidentified forms of As species, whilst the SumAs is defined as the sum of urinary iAs, MMA and DMA. Mass balance was assessed by the difference of tAs intake and tAs excreted assuming the mean 24-h urine volume of 1.5 L $day^{-1}$ (based on urine output of 2.0 L $day^{-1}$ for men and 1.6 L $day^{-1}$ for women given by EFSA, 2010). Urine, toenail and hair As concentrations had positively skewed distributions, therefore geometric transformations were applied for statistical analysis. For this analysis, concentrations below the limit of detection (LOD) of the test methods were replaced by a value equal to half of the LOD.

ANOVA and Student's *t*-test were used to test for differences in natural log-transformed values of urine, toenail and hair arsenic concentrations between different subgroups with respect to age (≤16 and >16 years), gender, ground water tAs concentration, occupation and exposure duration of the study population. Multi-variable linear regression models were constructed to assess significant predictors of biomarkers while controlling for possible confounding factors for this study population. The independent variables were log-transformed values of daily As intake from water and staple food (rice and wheat). The dependent variables were log-transformed concentrations of toenail, hair (tAs, iAs, MMA and DMA) and urine (tAs, iAs, MMA, DMA and SumAs). Considered potential confounders were age, gender, occupation and exposure duration. Before multivariable analyses, bivariate analyses (Pearson's correlation) were conducted to assess associations between potentially confounding factors and biomarkers. Factors associated with a *P*-value < 0.1 were first selected, then the factors with the weakest *P*-value were inserted in the multi-variable linear regression model using forward selection. The multivariable models were checked for multicolinearity and goodness of fit. Microsoft Excel, SPSS 24.0 (IBM, New York, NY, USA) and GraphPad Prism 7.0 were used for

**Table 1** Selected characteristics of study participants who provided urine, hair and toenail samples

| Characteristics | *n* | GM (min–max) | Data source |
|---|---|---|---|
| Study participants | 398 | | This study |
| Urine samples | 395 | | |
| Hair samples | 19 | | |
| Toenail samples | 20 | | |
| *Age* | | | Rasheed et al. [16] |
| ≤16 years | 66 | | |
| >16 years | 332 | | |
| *Gender* | | | |
| Male | 249 | | |
| Females | 149 | | |
| Body weight (Kg) | 398 | 52.19 (9–105) | |
| *Exposure duration from ground water tAs (years)* | | 14.7 (3–44) | Rasheed et al. [15] |
| 8–13 | 212 | | |
| 13–15 | 62 | | |
| 15–44 | 124 | | |
| *tAs concentration in household ground water (μg $L^{-1}$)* | | | |
| Overall | 398 | | |
| ≤10 | 50 | | |
| 10–50 | 145 | | |
| >50 | 203 | | |
| *Estimated daily tAs intake (μg $kg^{-1}$ bw $day^{-1}$)* | | | |
| Drinking/cooking water | 398 | 3.217 (0.02–236.510) | |
| Participants consumed rice only | 4 | 0.176 (0.122–0.226) | Rasheed et al. [16] |
| Participants consumed wheat only | 230 | 0.609 (0.194–2.234) | |
| Participants consumed staple food (wheat + rice) | 164 | 0.589 (0.275–2.0235) | |
| *Occupation category* | | | |
| *Labour non-Intensive* (n = *149*) | | | |
| House wives (general) | 45 | | |
| Students | 75 | | |
| Tailors | 4 | | |
| Teachers | 4 | | |
| Unemployed | 21 | | |
| *Labour intensive* (n = *249*) | | | |
| Farmers | 186 | | |
| Wives/family member of farmers (contributing in farming) | 56 | | |
| Services | 7 | | |

*GM* geometric mean

statistical analyses. The statistical significance level of $P \leq 0.05$ was set for the multi-variable analysis.

## Results and Discussion

### Study population characteristics

Data on the estimated daily tAs intake of this study population from water, rice and wheat were obtained from the previously published studies [15, 16, 34] (Table 1). The study participants living in the rural region of the Punjab Province, Pakistan had an age range of 3–80 years at the time of sampling, with 37% female participants and 10% participants above 60 years of age. The household's drinking/cooking water was found to have a GM tAs concentration of 55.33 μg $L^{-1}$ and a range of 0.48–3090 μg $L^{-1}$, with 89% of sources above the WHO provisional guideline value (10 μg $L^{-1}$) for arsenic in drinking water [23].

### Urinary biomarker levels in relation to population subgroups

The GMs for the concentrations of urinary tAs (234.43 μg $g^{-1}$ creatinine), iAs (26.98 μg $g^{-1}$ creatinine), MMA (23.32 μg $g^{-1}$ creatinine) and DMA (142.80 μg $g^{-1}$ creatinine) for all study participants and for different demographic and behavioural subsets are shown in Table 2. The DMA metabolite was the predominant form of As in urine (representing 71% of the sum of urinary arsenic metabolites), followed by iAs (13%) and MMA (12%). This conforms to the findings of Melak, Ferreccio [24] indicating As excretion as iAs (10–20%), MMA (10–15%) and DMA (60–75%) depending on inter-individual variation. AsB generated as a result of seafood ingestion, was not detected in this study population.

The significant impact ($P < 0.001$) of ground water tAs concentration (<10 μg $L^{-1}$, 10–50 μg $L^{-1}$ and >50 tAs μg $L^{-1}$) on urinary arsenic metabolites (Table 2) was found in concordance with other studies on low arsenic regions [21, 25]. There was a significant age-dependent trend for urinary tAs concentrations ($P = 0.032$), whilst males had significantly higher concentrations of urinary tAs, iAs, MMA, SumAs ($P \leq 0.05$) than females. The trend of higher MMA excretion in men than women (27.72 vs. 17.47 μg $g^{-1}$ creatinine) was consistent with previous investigations [26, 27]. This difference was reported to be linked with choline synthesis under the effect of estrogen in women of childbearing age [12, 13]. estrogen contributes to the synthesis of choline by regulating the Phosphatidylethanolamine N-methyltransferase (PMET) pathway [28]. Non-intensive labour occupations were associated with significantly increased tAs, iAs and SumAs concentrations ($P < 0.05$) compared with labour intensive occupations (Table 2). Exposure duration (≤14 and >14 years) did not have a significant impact on urinary concentrations (data not shown).

Mass balance was estimated to determine which source provided the majority of the tAs intake. Out of tAs intake (842.69 μg $day^{-1}$) from the total water (799.47 μg $day^{-1}$) and staple food (43.22 μg $day^{-1}$) intakes, the mean tAs excreted in urine was 591.18 μg $day^{-1}$. The remaining 251.51 μg $day^{-1}$ was assumed to be internally absorbed and/or excreted in faeces. The tAs intake from the consumption of food (43.22 μg $day^{-1}$) represents only 7.31% of the excreted tAs.

**Table 2** Geometric means [GM (min–max)] for creatinine-adjusted urinary arsenic metabolites (μg $g^{-1}$ creatinine)

| Characteristics | *n* | Urine creatinine | tAs | iAs | DMA | MMA | SumAs |
|---|---|---|---|---|---|---|---|
| Overall | 385 | 0.99 (0.35–2.55) | 234.43 (7.78–8743.59) | 26.98 (0.139–1411.11) | 142.80 (0.08–2353.53) | 23.32 (0.08–615.31) | 201.38 (0.30–4375.76) |
| *Age* | | | | | | | |
| ≤16 years | 62 | 0.92 (0.56–1.56) | 302.38 (27.55–8743.59) | 30.44 (0.23–1357.24) | 162.99 (0.13–1704.08) | 26.52 (0.14–615.31) | 230.81 (0.49–3676.63) |
| >16 years | 323 | 1.02 (0.35–2.55) | 223.17 (7.78–3969.70) | 26.36 (0.14–1411.11) | 139.22 (0.08–2353.54) | 22.75 (0.08–611.11) | 196.17 (0.30–4375.76) |
| *p*-values (*t* test) | | 0.03 | 0.032 | 0.424 | 0.411 | 0.415 | 0.395 |
| *Gender* | | | | | | | |
| Male | 241 | 1.03 (0.35–2.55) | 267.13 (7.78–8743.59) | 30.60 (0.14–1411.11) | 158.71 (0.08–2353.54) | 27.72 (0.08–611.11) | 226.30 (0.30–4375.76) |
| Female | 144 | 0.96 (0.54–2.01) | 188.97 (10.30–4510.20) | 21.85 (0.23–1357.24) | 119.67 (0.11–1955.22) | 17.47 (0.14–615.31) | 165.65 (0.49–3676.63) |
| *p*-values (*t* test) | | 0.02 | 0.002 | 0.013 | 0.052 | 0.001 | 0.020 |
| *tAs in water used for drinking and cooking (μg $L^{-1}$)* | | | | | | | |
| <10 | 50 | 0.98 (0.50–1.93) | 113.76 (10.297–760.60) | 14.53 (1.43–123.03) | 87.45 (9.167–488.46) | 11.81 (1.38–102.02) | 116.75 (12.12–677.58) |
| 10–50 | 140 | 0.97 (0.41–2.55) | 163.46 (18.636–1233.33) | 19.72 (1.29–229.62) | 118.15 (9.93–967.68) | 16.59 (0.76–251.28) | 159.13 (17.80–1220.64) |
| >50 | 195 | 1.02 (0.35–2.45) | 360.50 (7.778–8743.59) | 39.60 (0.14–1411.11) | 185.54 (0.08–2353.54) | 35.47 (0.08–615.31) | 274.25 (0.30–4375.76) |
| *p*-values (ANOVA) | | 0.350 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 |
| *Occupation* | | | | | | | |
| Labour intensive | 242 | 1.024 (0.53–2.55) | 213.44 (7.78–2563.64) | 24.34 (0.14–381.11) | 129.06 (0.08–1990.91) | 21.16 (0.08–415.45) | 182.22 (0.30–2767.36) |
| Labour non-Intensive | 143 | 0.96 (0.50–2.03) | 274.62 (12.62–8743.59) | 32.11 (0.23–1411.11) | 169.47 (0.13–2353.54) | 27.49 (0.14–615.31) | 238.49 (0.488–4375.76) |
| *p*-values (*t* test) | | 0.036 | 0.019 | 0.042 | 0.061 | 0.067 | 0.046 |

[a]Urine samples for tAs ($n = 395$)

**Table 3** Geometric means [GM (min–max)] for arsenic and arsenic species in toenail and hair (μg $kg^{-1}$)

| Characteristics | *n* (toenail) | *n* (hair) | Toenail, GM (min–max) (μg $kg^{-1}$) | | | | Hair, GM (min–max) (μg $kg^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | tAs | iAs | MMA | DMA | tAs | iAs | MMA | *DMA* |
| Overall | 20 | 19 | 1942.18 (586–27,500) | 1756.91 (557–22,000) | 79.44 (6–955) | 21.88 (0.8–432) | 702.16 (67.0–3100.0) | 653.25 (84–10,700) | 1.43 (0.5–55) | 2.64 (0.5–123) |
| *tAs in water used for drinking and cooking (μg $L^{-1}$)* | | | | | | | | | | |
| <10 | 5 | 5 | 593.06 (586–599.2) | 568.43 (559.2–578) | 32.82 (26–39) | 0.91 (0.8–1) | 73.82 (67–94.1) | 90.06 (84.0–95) | 2.46 (2.0–3.0) | 3.36 (2.0–7) |
| 10–50 | 4 | 3 | 1321.94 (602–4070) | 1217.91 (557–3840) | 32.72 (6–97) | 12.88 (6–57) | 1006.65 (352–2760) | 830.73 (325–2250) | 0.72 (0.6–0.9) | 10.12 (0.6–69) |
| >50 | 11 | 11 | 3830.19 (1190–27,500) | 3352.55 (1270–22,000) | 163.93 (77–955) | 112.52 (25–432) | 1771.87 (531–13,100) | 1505.83 (438–10,700) | 1.35 (0.5–55) | 1.64 (0.5–123) |
| *p*-values (ANOVA) | – | – | 0.0005 | 0.001 | 0.001 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.364 |
| *Occupation* | | | | | | | | | | |
| Labour intensive | 13 | 12 | 1766.00 (586–27,500) | 1627.03 (559–22,000) | 67.12 (6–955) | 11.81 (0.8–432.0) | 504.73 (67–13,100) | 507.16 (84–10,700) | 1.39 (0.5–17) | 1.69 (0.5–69) |
| Non-labour intensive | 7 | 7 | 2317.31 (605.0–4660) | 2026.26 (557–4070) | 108.65 (48–209) | 68.75 (9.1–310.0) | 1236.57 (352–4610) | 1008.19 (325–3590) | 1.52 (0.5–55) | 5.66 (0.5–123) |
| *p*-values (*t* test) | | | 0.53 | 0.59 | 0.25 | 0.04 | 0.190 | 0.34 | 0.89 | 0.28 |
| *Exposure duration* | | | | | | | | | | |
| ≤14 years | 13 | 12 | 1277.25 (586.0–4660) | 1163.46 (557.0–4070) | 66.54 (26.0–209) | 13.48 (0.8–310.0) | 330.06 (67.0–3770) | 329.59 (84.0–3140) | 1.57 (0.5–17.0) | 1.34 (0.5–7.0) |
| >14 years | 7 | 7 | 4229.75 (2060.0–27,500) | 3777.22 (1840–22,000) | 110.41 (6.0–955) | 53.74 (6.0–432) | 2561.18 (615.0–13,100) | 2110.63 (669.0–700) | 1.23 (0.5–55.0) | 8.50 (0.5–123 |
| *p*-values (*t* test) | – | – | 0.012 | 0.009 | 0.331 | 0.123 | 0.005 | 0.004 | 0.703 | 0.119 |

**Table 4** Multi-variable linear regression analysis of associations between log-transformed values of estimated daily intake of tAs (μg $kg^{-1}$ bw $day^{-1}$) and exposure biomarkers

| Independent variable | Biological matrix | Biomarkers | β coefficient | Std. error | *p*-value | Model adjusted R[a] |
|---|---|---|---|---|---|---|
| *tAs intake from drinking water* | Urine | tAs | 0.307 | 0.028 | 0.0005 | 0.276[a] |
| | | iAs | 0.3 | 0.038 | 0.0005 | 0.168[a] |
| | | DMA | 0.229 | 0.042 | 0.0005 | 0.069[b] |
| | | MMA | 0.284 | 0.04 | 0.0005 | 0.158[a] |
| | | SumAs | 0.259 | 0.038 | 0.0005 | 0.104[b] |
| | Toenail | tAs | 0.348 | 0.063 | 0.0005 | 0.612[c] |
| | | iAs | 0.342 | 0.056 | 0.0005 | 0.660[c] |
| | | DMA | 0.672 | 0.08 | 0.0005 | 0.606[b] |
| | | MMA | 0.24 | 0.122 | 0.008 | 0.294[b] |
| | Hair | tAs | 0.443 | 0.073 | 0.0005 | 0.792[d] |
| | | iAs | 0.386 | 0.07 | 0.0005 | 0.764[d] |
| | | DMA | −0.291 | 0.159 | 0.15 | 0.243[b] |
| | | MMA | 0.009 | 0.19 | 0.958 | −0.17[b] |
| *tAs intake from staple diet* | Urine | tAs | 0.577 | 0.106 | 0.0005 | 0.122[a] |
| | | iAs | 0.894 | 0.132 | 0.0005 | 0.105[b] |
| | | DMA | 0.773 | 0.143 | 0.0005 | 0.068[b] |
| | | MMA | 0.866 | 0.138 | 0.0005 | 0.136[a] |
| | | SumAs | 0.812 | 0.131 | 0.0005 | 0.088[b] |
| | Toenail | tAs | 1.017 | 0.291 | 0.003 | 0.547[d] |
| | | iAs | 0.995 | 0.265 | 0.002 | 0.587[d] |
| | | DMA | 2.698 | 0.598 | 0.0005 | 0.504[e] |
| | | MMA | 1.131 | 0.336 | 0.003 | 0.352[b] |
| | Hair | tAs | 1.725 | 0.357 | 0.0005 | 0.718[d] |
| | | iAs | 1.547 | 0.322 | 0.0005 | 0.718[d] |
| | | DMA | −1.139 | 0.700 | 0.128 | 0.258[d] |
| | | MMA | 0.043 | 0.591 | 0.943 | −0.169[b] |

[a]Adjusted for gender and occupation

[b]Other potential confounders that did not contribute significantly to the models were excluded by statistical programme

[c]Adjusted for gender

[d]Adjusted for exposure duration

[e]Adjusted for occupation

## Toenail and hair biomarkers levels in relation to population subgroups

A significant increase in toenail and hair concentrations of tAs and its species ($P \le 0.001$) was found with increasing drinking/cooking water tAs concentration (<10 μg $L^{-1}$ to >50 tAs μg $L^{-1}$), except for hair DMA (Table 3). The binding of iAs, dietary and/or metabolically produced DMA and MMA with sulfhydryl nails are reported to be partly dependent on the concentration available in the blood [29]. Thus, this study participants with longer exposure duration (>14 years) had significantly higher concentration of toenail and hair tAs and iAs, indicative of prolonged exposure (Table 3).

Age and gender in this study population did not show a significant impact on toenail and hair concentrations (data not shown). Type of occupation (labour intensive and non-labour professions) showed no impact. Despite the higher outdoor activities of participants engaged in labour intensive occupations (services, farmers, wives of farmers contributing in farming), significantly higher toenail DMA in this study participants engaged in non-labour intensive occupations (general house wives, students, tailors, teachers and unemployed) was unclear (Table 4).

## Intercorrelations among exposure biomarkers

The concentration of urinary iAs was significantly correlated with urinary MMA ($r = 0.905$, $P \le 0.0001$) and DMA ($r = 0.884$, $P \le 0.0001$), whilst urinary MMA was significantly associated with DMA ($r = 0.912$, $P \le 0.0001$). Urinary iAs was significantly correlated with toenail tAs

($r = 0.484$, $P = 0.036$), toenail iAs ($r = 0.494$, $P = 0.031$), hair tAs ($r = 0.513$, $P = 0.030$) and hair iAs ($r = 0.487$, $P = 0.040$). A significantly strong association between hair tAs ($r = 0.779$, $P \leq 0.0001$) and toenail (tAs) also exist.

Significant positive intercorrelations between urinary, toenail and hair arsenic species suggest that either of these may be used as biomarkers of arsenic exposure, however these biomarkers reflect the As exposure over different time periods as mentioned in section 1.

### Multi-variable linear regression analysis of relations between tAs intake and exposure biomarkers

Multi-variable linear regression analysis revealed a positive significant relationship between the tAs intake from drinking/cooking water and urinary tAs, iAs and MMA after adjusting for gender, occupation and exposure durations for this study population (Table 4). The association between urinary arsenic metabolites and drinking water arsenic concentrations in household water sources in the rural area of Punjab are in line with the results of multi-variable regression models from previous studies [30, 31], indicating a positive relation between estimated intake of tAs from drinking water and urinary As species adjusting for gender (Table 4).

A significant positive association existed between tAs intake of this study population from staple food and those of urinary arsenic metabolite concentrations when adjusted for gender and occupation. The predictor variables such as drinking/cooking water and food tAs intakes both showed significance with response variables i.e., toenail tAs, iAs, MMA, DMA and hair tAs and iAs, indicating the mean change in the response variable for one unit of change in the predictor variable while holding gender, occupation and exposure duration as constant (Table 4). The influence of gender, exposure duration and occupation subgroups on urine, hair and toenail tAs and arsenic species suggests the possible underlying reasons. These include metabolic, inter-individual, social demographic and behavioural variability, growth rate of skin appendages, health status, nutrition or exogenous contamination from dust or soil in crop field and kinetic models for peripheral tissues [29]. This study participants living in rural Punjab exposed to tAs (water) $< 1\ \mu g\ L^{-1}$ and $< 10\ \mu g\ L^{-1}$ showed a staple food tAs intake of $0.485\ \mu g\ kg^{-1}$ bw $day^{-1}$ ($n = 5$) and $0.733\ \mu g\ kg^{-1}$ bw $day^{-1}$ ($n = 50$), respectively. No significant impact of tAs intake from food was found on urinary arsenic metabolites below $1\ \mu g\ L^{-1}$. However, participants exposed to $< 10\ \mu g\ L^{-1}$ tAs concentration of drinking/cooking water ($n = 50$) showed significant Pearson correlation ($P < 0.05$: data not shown) between tAs intake from food and urinary arsenic metabolites, suggesting the sole contribution of food in human exposure to arsenic.

The regression model coefficients (Table 4) showed that for every additional unit of tAs intake from water in this study, an average increase of urinary tAs by $220.74\ \mu g\ g^{-1}$ creatinine (urine), $1944.96\ \mu g\ kg^{-1}$ (toenail) and $755\ \mu g\ kg^{-1}$ (hair) was expected. Compared to this, tAs intake from food shows increased tAs concentration by an average of 456.23 $\mu g\ g^{-1}$ creatinine (urine), $5721.58\ \mu g\ kg^{-1}$ (toenail) and $4272.70\ \mu g\ kg^{-1}$ (hair). This increase due to food tAs intake was higher by an average factor of 3.6 when compared to values derived from model coefficient of water tAs intake. These findings showed that water and food tAs intake were found as the strongest predictors of all of the urinary and toenail biomarker concentrations. When compared to food, drinking/cooking water was a relatively stronger predictor as seen by adjusted $R^2$ values (Table 4).Though the sample size of toenail and hair could constitute a limitation of this study, the degree of significant associations (Table 4) revealed that toenail arsenic speciation is a more precise biomarker of effects, a potential determinant of prolonged arsenic exposure and indicative of critical arsenic related health effects. In the same context, an elevated risk of cutaneous melanoma [32] and lung cancer [33] was reported in persons with higher toenail arsenic concentrations.

## Conclusions

The consumption of drinking/cooking water containing a range of total arsenic concentrations in household hand pumps/wells of six rural settings of Pakistan significantly increased the absorbed dose of tAs, iAs and its mono and dimethylated arsenic in urine, hair and toenail of study participants under the influence of certain biological and behavioural modifiers such as gender, exposure level, occupation and exposure duration. Levels of these species in biological matrices of rural residents of arsenic-affected region of Punjab, Pakistan can also increase significantly due to exposure through frequent consumption of staple foods such as rice and wheat. The levels of tAs, iAs and its mono- and dimethylated arsenic in urine, hair and toenail were also influenced by certain biological and behavioural modifiers such as gender, exposure level, occupation and exposure duration. Association of toenail arsenic with water and food intake of arsenic can be observed as a more favourable biomarker of arsenic exposure than urine and hair.

Given the critical role of highly reactive and genotoxic intermediate trivalent forms of MMA and DMA produced from methylation of iAs, this study underscores the need to determine these trivalent forms in association with potentially modifying effects of dietary and occupational exposure along with confounding factors such as smoking, nutrients, genetics, education on arsenic accumulation and excretion.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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