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RESEARCH

Micro-distribution of arsenic in toenail clippings using laser ablation inductively coupled plasma mass spectrometry: implications for biomonitoring

Jennie R. Christensen · Geriene O. LaBine · Janet S. Cheung · Renata Rosol · Asish K. Mohapatra · Brian Laird · Hing Man Chan

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Abstract Toenails are a common monitoring tool for arsenic exposure, but the risk of external contamination of toenails has cast doubt on its usefulness. The main objective of this study is to investigate the micro-distribution of arsenic through the dorsoventral plane of nail clippings to understand endogenous vs exogenous sources. We used laser-ablation inductively coupled plasma mass spectrometry to measure arsenic through a dorsoventral cross-section of the nail plate collected from reference ($N=17$) and exposed individuals ($N=35$). Our main results showed (1) bulk toenail concentrations measured using ICP-MS in this study ranged from 0.54 to 4.35 µg/g; (2) there was a double-hump pattern in arsenic concentrations, i.e., dorsal and ventral layers had higher arsenic than the inner layer; (3) the double-hump was more pronounced in the exposed group (ventral: 6.25 µg/g; inner: 0.75 µg/g; dorsal: 0.95 µg/g) than the reference group (ventral: 0.58 µg/g; inner: 0.15 µg/g; dorsal: 0.29 µg/g) on average; (4) the distribution was, in part, associated with different binding affinity of nail layers (i.e., ventral > dorsal > inner); (5) most individuals in the higher exposure group showed > 25% contamination in ventral and dorsal nail layers; and (6) there were no statistically significant correlations between LA-ICP-MS arsenic with either bulk toenail arsenic or urine arsenic from the same individuals. Our results on micro-distribution and binding affinity provide insight into the impact of external contamination on arsenic concentrations and show how LA-ICP-MS can access the protected inner nail layer to provide a more accurate result.

Keywords Biomarker · Exposure · Arsenic · LA-ICP-MS · Microanalysis · Nails

J. R. Christensen (✉) · G. O. LaBine
TrichAnalytics Inc., 207-1753 Sean Heights, Saanichton, BC V8M 0B3, Canada
e-mail: jennie.christensen@trichanalytics.com

J. S. Cheung · R. Rosol · H. M. Chan
Department of Biology, University of Ottawa, Ottawa, ON K1N 6N5, Canada
e-mail: janet.cheung@wsp.com

A. K. Mohapatra
Environmental Health Program, Health Canada, Calgary, AB, Canada

B. Laird
School of Public Health and Health Systems, Faculty of Applied Health Sciences, University of Waterloo, Waterloo, ON, Canada

Introduction

Chemical pollution is the world's largest environmental risk factor for diseases and was responsible for 9 million premature deaths in 2019 (Fuller et al., 2022). Many human activities drive accumulations of chemicals in soil, water and/or air, to which humans are exposed through inhalation, dermal absorption, or ingestion of contaminated soil, water, or food

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(Masindi & Muedi, 2018). These chemicals are often naturally occurring, but exposures to above-natural concentrations can cause, or exacerbate, myriad acute and chronic health effects. Such effects include increased mortality rates (US EPA, 2017); disease and declines in physical condition (e.g., cancer; neurological, respiratory, and cardiopulmonary disease; and birth defects) (US EPA, 2017); and psychological conditions (Schmitt et al., 2021), which all accumulate into public health concerns (US EPA, 2017).

Arsenic is a metalloid with a long history of known toxicity in humans, following both intentional and unintentional exposure (Hughes et al., 2011). Speciation of arsenic is an important factor determining its toxicity. Dietary forms of arsenic, e.g., arsenosugars that are commonly found in seaweed or arsenobetaine that is commonly found in fish, do not accumulate in the body and are not considered toxic (Cullen & Reimer, 1989; Hughes et al., 2011; Kaise et al., 1996). However, arsenic in water sources is principally comprised of the more toxic inorganic arsenic forms, such as trivalent (III) or pentavalent (V) arsenic (Saxe et al., 2006; ATSDR, 2007, 2016; Gault et al., 2008; Karagas et al., 2000). Ingestion of inorganic arsenic has been associated with various cancers, including bladder, skin, kidney, and liver (Celik et al., 2008; Chiang et al., 1993; Ferreccio et al., 2000; Liu & Waalkes, 2008; Speer et al., 2023; Tseng et al., 1968; Yuan et al., 2010), as well as skin lesions, cardiovascular disease, kidney disease, respiratory problems, diabetes, and neurodegeneration (Parvez et al., 2010; Tolins et al., 2014; Zheng et al., 2015; Abdul et al., 2015; Olabode Fatoki & Abiodun Badmus, 2022). Many population studies have been conducted in arsenic-polluted sites worldwide including Europe (Buekers et al., 2023), Africa (Nyanza et al., 2019), Taiwan (Lan et al., 2011), India (Maity et al., 2012; Mazumder & Dasgupta, 2011), and Bangladesh (Ali et al., 2010; Smith et al., 2000). In the Americas, high arsenic levels were also reported in specific areas within Canada (McIver et al., 2015), USA (Beamer et al., 2016; Calderon et al., 2013; Wasserman et al., 2014), and Mexico (Gonzalez-Cortes et al., 2017).

The ability to readily identify exposure to, absorption of, and net accumulation of arsenic in humans is critical for early intervention and prevention of health effects. Consequently, sensitive and accurate biomonitoring of body burden of arsenic in individuals who are potentially exposed to arsenic is fundamental to the successful interventions. However, invasive methods, such as blood drawing, may introduce unnecessary risks and cause undue stress to patients. Additionally, arsenic does not accumulate in blood and is quickly distributed in the body and cleared within a few hours in the urine (ATSDR, 2007; Järup & Åkesson, 2009; Tehrani et al., 2020). Therefore, blood is not a good candidate for a biomarker as it only provides information on relatively recent exposure (Laohaudomchok et al., 2011). Urine is currently the most used biomarker of recent arsenic exposure, reflecting exposure of 4–5 days (ATSDR, 2007). However, urinary arsenic only represents exposure for a small window of time and does not necessarily reflect past exposure or more long-term exposure.

There has long been interest in non-invasive monitoring methods that leverage the biochemistry of readily accessible tissues within which arsenic accumulates over time (Smolders et al., 2009). Keratin-based tissues, such as nails, are prime examples: the root portions are exposed to blood as they grow and accumulate contaminants within their keratin matrix (wherein they become metabolically inert), and clippings can be painlessly sampled without medical expertise and readily can be handled without biohazard concerns. Nails are a suitable matrix for arsenic due to their affinity to sulfhydryl groups, which are present in keratin-rich tissues such as nails (Shen et al., 2013). A recent systematic review of 129 papers covering populations from 29 different countries has used toenail arsenic to measure toxic inorganic arsenic exposure in chronic disease research. However, there is a high heterogeneity between studies (Signes-Pastor et al., 2021).

The viability of non-invasive methods that use keratinized tissues, such as toenails, as biomarkers depends on their capacity to distinguish endogenous exposure (i.e., arsenic that has been absorbed into the body) from external contamination (Bainter, 2014; Slotnick & Nriagu, 2006). The heterogeneity of the studies above (Signes-Pastor et al., 2021) may be linked with the confounding influence of external contamination. Monitoring methods must provide accurate inferences about endogenous sources of exposure since it is these concentrations (versus external concentrations unassimilated into the body) that affect health. Methods prone to contamination from external sources may be confounded and

therefore ultimately provide overestimated levels of exposure and hence health risk.

Other than arsenic, nail clippings have also been used as biomonitoring matrix in studies of drug, nicotine, and alcohol use (Cappelle et al., 2015; Palmeri et al., 2000), disease (Jaramillo Ortiz et al., 2021), and occupational exposure for other metals (Salcedo-Bellido et al., 2021). Toenails tend to be preferred as matrices to fingernails, since they are thought to be less exposed to external sources of contaminants (Bakri et al., 2017; Gherase et al., 2013; Signes-Pastor et al., 2021; Slotnick & Nriagu, 2006). However, the accumulation of dirt and dust in shoes, socks, and (ultimately) nail margins means that toenails remain susceptible to external influence. Most analytical methods involve manual, chemical, and/or sonic cleaning of samples prior to chemical analysis (Button et al., 2009; Signes-Pastor et al., 2021; Slotnick & Nriagu, 2006); nevertheless, sample cleaning does not always completely remove external contamination (Bainter, 2014) and does not remove contamination that has adsorbed through the nail surface and has bound to the nail matrix.

Most methods used to quantify arsenic concentrations in nail tissue require clippings to be digested, pulverized and/or homogenized (Signes-Pastor et al., 2021). These methodological steps facilitate adequately large sample sizes and presume homogeneity within the sample matrix. Unfortunately, these steps also mean that information on meaningful variability in concentrations throughout the nail plate ("micro-distribution") is lost. Such within-sample micro-distributions could provide a more accurate understanding of arsenic exposure or could be used to disentangle endogenous and exogenous arsenic sources (Ponomarenko et al., 2014). Analyzing nails for arsenic using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) may provide a solution, as smaller volumes can be analyzed, and therefore focused analysis on nail subportions and layers can be accomplished (Chan et al., 2023; Christensen et al., 2017; Rodushkin & Axelsson, 2003).

The main objective of this study is to use LA-ICP-MS to investigate the micro-distribution of arsenic through the dorsoventral plane of nail clippings to understand the relative contributions from endogenous vs exogenous sources. The goal is to provide practical insights into the utility of such micro-distributions to monitor arsenic exposure and disentangle confounding influence of external contamination. We also explore how the micro-distribution of arsenic compares to bulk toenail analysis, to urine arsenic, and the influence of age on arsenic exposure in urine and toenails.

Material and methods

We used a six-step approach to investigate endogenous and exogenous arsenic in toenails, with a focus on microdistribution among nail layers determined by LA-ICP-MS. First, we explored the conventional arsenic biomonitoring matrices (i.e., urine and bulk toenail) collected from individuals with higher potential arsenic exposure ("exposed group") and the potential influence of age. Second, we used the big toenails from these same exposed individuals to test the hypothesis that arsenic concentrations are non-uniform through the nail matrix, specifically with higher concentrations in ventral and dorsal layers, creating a "double-hump" dorsoventral profile. Third, we examined nail clippings of individuals with low background exposures (i.e., no occupational sources, not living in arsenic-contaminated areas, not drinking contaminated water), "reference group," and then compared the micro-distribution and concentrations of arsenic with the exposed group. Fourth, we artificially spiked nail clippings (all layers exposed equally) from individuals in the reference group with arsenic to test the hypothesis that nail layers have different binding affinity for arsenic, which contributes to a double-hump micro-distribution. Fifth, we built a model of nail layer ratios using reference group nails, both unspiked and spiked, to quantify the contamination that may be contributing to the exposure group's arsenic nail clipping concentrations. Finally, we examined the relationship between conventional bulk analysis of toenails and urine analysis with the LA-ICP-MS results from the big toenail using samples from the same individuals.

Ethics

The research was conducted following the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and, in particular, Chapter 9, research involving the First Nations, Inuit and Métis Peoples of Canada (Canadian Institutes of Health

Research, Natural Sciences and Engineering Research Council of Canada, Social Sciences and Humanities Research Council of Canada, 2010), and the document entitled: Indigenous Peoples & Participatory Health Research: Planning & Management, Preparing Research Agreements published by the World Health Organization (Fediuk & Kuhnheim, 2003). The study also follows the First Nations principles of Ownership, Control, Access, and Possession (OCAP®) of data (Schnarch, 2004).

The study was approved by the Health Sciences and Sciences Research Ethics Board of the University of Ottawa (http://research.uottawa.ca/ethics/reb) and the Aurora College Research Ethics Committee. In addition, the study has been granted a Scientific Research License from the Aurora Research Institute in Northwest Territories. Individual participation in the project was voluntary and based on informed written consent following an oral and written explanation of each project component.

Study area and population

The "exposed group" participants are located in Yellowknife and the First Nations communities of Dettah and Ndilo in Northwest Territories, Canada. The presented research is part of the Health Effects Monitoring Program, a prospective cohort study established to monitor levels of arsenic and other chemicals of potential concern (COPCs) in the human population of Yellowknife, Dettah, and Ndilo as remediation of Giant Mine progresses (Chan et al., 2020). A total of 2037 individuals aged 3 to 79 participated in the baseline study. Recruitment and data collection of the baseline cohort was conducted in two waves; the first wave occurred from September to December 2017, and the second wave occurred from April to June 2018. A detailed description of the Health Effects Monitoring Program and the methodology of the study is described previously in the reported cohort profile (Chan et al., 2020).

A subset of thirty-five ($n=35$) samples from the "exposed group" was randomly selected from the cohort for this study, where urine and toenail samples were again collected from June to December 2019. They included 17 adults (aged 18 to 62), 17 children (aged 5–17), and 1 unknown-aged individual. Seventeen ($n=17$) volunteers from British Columbia, with no known arsenic exposure, were recruited as the "reference group," where only a big toenail was provided.

Urine analysis

Sample kits were distributed to all participants by trained research assistants to collect urine at their own time. A toilet hat was provided for the collection of urine for young children (3–6 years). Participants were instructed to abstain from eating seafood 3 days before urine sampling and to provide the first-morning urine void. Samples were kept at at − 20 °C until analysis within 30 days. On the day of analysis, urine samples were thawed then kept on ice. The sample was diluted 10 times in 1% nitric acid before analysis using inductively coupled plasma mass spectrometry (ICP-MS) (7700 × ICP-MS, Agilent Technologies, Japan). The system is equipped with low flow borosilicate glass MicroMist concentric nebulizer and quartz, Scott-type double pass spray chamber. Stock solutions were diluted in 1% nitric acid and used to provide a working calibration curve of at least five points. For analytical quality controls, an element quality control standard stock (High Purity Standards, Cat# QCS-19) and urine multi-element stock (High-Purity Standards, Healt3 solution A) were used as check standard after calibration and then every 10 to 20 samples. Also different reference materials (NIST reference materials: NIST 2669 level 1 and level 2 arsenic species in toxic human urine, 2668 level 1 and level 2 toxic elements in frozen human urine, IAEA 407 and IAEA 085 references from the International Atomic Energy Agency and DOLT-4 from the National Research Council Canada) and pooled samples for a spike recovery test were included in the analysis. The results for both check standard, spike recovery, and reference materials were within 80–120% of expected values. The detection limit in the urine samples was 0.012 µg/L for total arsenic and 0.005 µg/L for the arsenic species.

Bulk analysis in toenails (for arsenic speciation)

Up to 10 toenail samples (one from each toe) were collected from each individual in the "exposed group" and sent to the University of Ottawa. One big toenail from each individual from the exposed group was sent to and ISO 17025 CALA accredited laboratory, TrichAnalytics Inc. (Saanichton, British Columbia),

for the microchemistry analysis. The remaining nine toenails were retained at University of Ottawa for bulk analysis and for arsenic speciation. Bulk toenail analysis was not conducted on the "reference group."

Toenail samples were washed according to the protocol adapted from Button et al. (2009). First, visible exogenous material (dirt, clothing fiber, etc.) was removed using forceps; then, samples were placed in 15-mL centrifuge tubes and sonicated in 3-mL acetone for 5 min. Following the sonication, the samples were rinsed again with 2 mL ultrapure water followed by 3 mL acetone and final sonication in 3 mL ultrapure water for 10 min. For the final step, the samples were rinsed 2 times with Milli-Q® water before drying at 60 °C for 1–2 days before being ground and weighed on an analytical balance before digestion in nitric acid for total metal analysis. A block digestion system comprised of 48-place, 50 mL (SCP Science model DigiPrep MS, cat. # 01–500-205) with temperature control module (SCP Science, cat. # 010–500-225) and Digi Probe (SCP Science, Cat. #010–505-115) was used for sample digestion. Briefly, 1 ml of deionized, distilled water and 2.5 ml of concentrated nitric acid were added to each digestion tube and then placed in the DigiPREP. The temperature was raised at the ramp rate of 1 degree/minute to reach 100 °C and was left for 3 to 3.5 h or until the volume was low but not completely evaporated. The samples were cooled for 20 min, and 1.5 ml of hydrogen peroxide was added to each digestion tube. The DigiPREP is turned down to 95 °C and left at the temperature for another 40–60 min. The samples were then removed and diluted with deionized water to a final volume of 10 ml then capped and vortexed. After acid digestion, total arsenic or bulk analyses were performed using inductively coupled plasma mass spectrometry (ICP-MS) (7700×ICP-MS, Agilent Technologies, Japan). QA/QC method is the same as those described above for urine analysis.

For As speciation analysis, the digestion method was modified to using a low concentration of nitric acid (0.28 M) and no hydrogen peroxide added to the samples for species preservation. The chromatographic separation of arsenite (As^{3+}), arsenate (As^{5+}), methylarsonate (MMA), dimethylarsonic acid (DMA), and arsenobetaine was performed using a 10 mM ammonium phosphate dibasic buffer with pH adjusted to 8.25 on Agilent 1200 Infinity LC system consisting of a 1260 Isocratic pump and 1260 Autosampler. The LC system was connected to the Agilent 7700×ICP-MS via Peek tubing and equipped with a low flow Micro Mist Nebulizer and quartz, low-volume Scott-type double-pass spray chamber. The mobile phase was delivered at 1 mL/min, and the injection volume was fixed at 100µL. To check the stability of the samples, fortified blanks and reference samples were prepared and preserved the same way as the sample and checked at regular basis.

Toenail microchemistry analysis (for arsenic) using LA-ICP-MS

All toenails from both the reference and exposed groups were cleaned by sonication in Eppendorf tubes filled with distilled water. Following sonication, the toenails were allowed to air dry at ambient temperature on the bench. Then, each dried toenail was placed in a base mold (15×5×5 mm, ThermoFisher Scientific), filled with EpoxiCure 2 and hardener (Buehler Canada), and allowed to cure overnight. The toenail embedded in epoxy was revealed after sanding using Silicon carbide 600 and 1200 grit adhesive backed lapping papers (Allied High Tech Products Inc.). The orientation of toenail required the cross-section of the clipping where the dorsal and ventral regions were visible and available for laser ablation (Fig. 1). Sub-samples of an in-house reference material were analyzed alongside all toenail samples to facilitate the conversion of arsenic concentrations

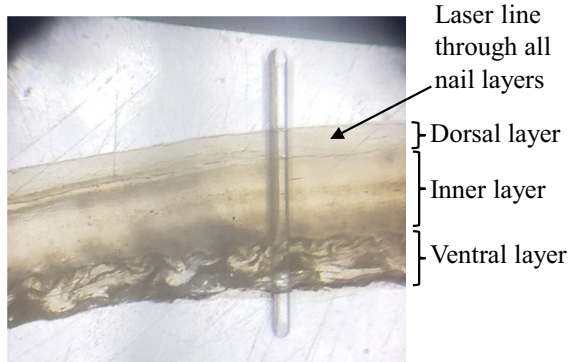

Fig. 1 A photo of a toenail clipping cross-section from one of the individuals in the exposure group. From top to bottom is dorsal to ventral. The laser scan line is visible going through the epoxy first, followed by the nail, and then the epoxy again to ensure all nail layers are captured in the scan. Note how distinct the different nail layers are

from signal intensities, calculate detection limits, and determine accuracy and precision prior to accepting data for analysis. The in-house reference material is a keratin-based matrix (hair standard) with arsenic concentration of 1.14 µg/g with an overall measurement uncertainty of 100 ± 15% (using adjusted CALA-accredited method, MET-003 scope of testing). Accuracy and precision was determined by analyzing the reference material as an unknown three times at the beginning of each run. Accuracy for sulfur and arsenic ranged from 92–98% and 87–117%, respectively, while precision ranged from 6 to 18% for sulfur and 11–30% for arsenic.

Arsenic in the reference material and toenail clippings were quantified using an iCAP RQ series ICP-MS (Thermo Scientific, Canada) connected to an NWR-213 (Elemental Scientific Lasers, USA) laser ablation system. LA-ICP-MS settings were as follows: 20% power, 20 Hz, 60 µm spot size, and 20 µm/s laser speed. Following analysis, analyte signals were screened using Qtegra (Thermo Scientific) and exported for data processing using R (Version 4.0.0) and statistical analysis using XLSTAT (Version 2023.2.0).

Artificial Dosing

Following initial quantification of arsenic concentrations in the nail samples (considered "unspiked"), the reference group toenail samples, embedded and prepared in epoxy, were then submerged in a 50 µg/mL As^{5+} solution for 1 min, rinsed with deionized water, and air-dried on the bench. As^{5+} was chosen for spiking due to the predominance of this arsenic species in the bulk toenail analysis in the exposed group (see "Results": Hypothesis 1). "Spiked" samples were then reanalyzed by LA-ICP-MS to procure spiked-treatment arsenic concentrations. Artificial dosing of the nails was conducted to test hypotheses 4 and 5.

Quantification of arsenic in nails

Arsenic concentrations (µg/g) were calculated in toenail clippings by developing set-specific signal intensity-concentration curves using in-house reference material from a sample run. In the reference material sample, the background region was defined as the initial 9 s, which always fell before laser ablation was initiated. Sample regions were identified using sulfur (34S) signal intensities, which were pronounced and distinct from background intensities in all samples. Arsenic intensities were first background-adjusted before linear models were fitted for each sample set, forced through the origin, between arsenic intensities (as the dependent variable) and the theoretical concentration of arsenic in our reference material (1.14 µg/g).

Best-fit calibration curve slopes were used to estimate arsenic concentrations along the line scan in all toenail samples. Detection limits (DLs) were calculated and used to delimit the lowest concentration arsenic that could be confidently detected. A signal-to-noise ratio of three in the blank (i.e., background gas signal intensity) was used to calculate DL, which was 0.03 µg/g for all samples. Sulfur was used as an internal standard. Although arsenic concentrations were not adjusted based on the sulfur content, as there is natural variability in sulfur content of nails of 2.38–4.25% (Dittmar et al., 2008), the calculated sulfur content was assessed to ensure the ablated nail tissue fell within that range for each sample. The sulfur content of the nails in this study averaged 3.15 ± 0.6%, which is similar to the averages for males (3.20 ± 0.3%) and females (3.36 ± 0.32%) reported elsewhere in fingernails (Dittmar et al., 2008).

Data preparation and analysis

In every sample, individual estimates of arsenic concentrations were categorized into layers based on their position in the ablation line scan: dorsal, inner, and ventral. This nominal layer delimitation is valid because dorsal and ventral layers are usually thinner than the inner layer and, hence, will fall within the outer thirds of the line scan. The internal layer will straddle the middle third. If, as predicted, arsenic peaks occur in the (actual) dorsal and ventral layers, these layers will also fall in the outer thirds—our nominal dorsal and ventral layers.

We tested six hypotheses, relating to endogenous versus exogenous arsenic and the micro-distribution of arsenic through a dorsoventral cross-section of the nail plate.

Hypothesis 1: Relationship between urine and bulk toenail arsenic

At the initial stage, an exploration of conventional matrices, specifically urine analysis and bulk toenail analysis, was conducted with the "exposed group." This included an examination of arsenic speciation within the toenails and the relationship between urine arsenic and bulk toenail arsenic within individuals and the influence of age. It was hypothesized that there would be no relationship between urine and toenail arsenic, irrespective of age. Although collected at the same time, urine arsenic reflects recent exposure and the toenail arsenic would reflect past or chronic exposure (up to 10 months previous). Additionally, toenails are likely to reflect at least some contamination on the exposed surfaces confounding any relationship with urine. Percentages for each arsenic species was calculated in the toenail samples. Linear regression analysis was used to characterize the relationship between urine and bulk toenail arsenic. *T*-test (with alpha set at 0.05) was used to determine potential differences in urine and bulk toenail arsenic between age groups.

Hypothesis 2: The double-hump micro-distribution

This hypothesis focuses on the LA-ICP-MS results from the "exposed group" big toenail samples. It is hypothesized that arsenic will not be uniformly distributed through the dorsoventral plane of a toenail clipping; rather, accumulations of arsenic will be more significant in the dorsal and ventral layers of the nail than in the internal layer following existing anecdotal evidence (Christensen et al., 2017; Forslind et al., 1976; Gherase et al., 2013; Pearce et al., 2010; Rodushkin & Axelsson, 2003). First, we compared children and adult toenail arsenic using repeated measures two-way analysis of variance (ANOVA) on log-transformed data to test for age and layer (i.e., double-hump) differences, including an age × layer interaction. Then, using all 35 toenails, we used a repeated measures one-way ANOVA on log-transformed data to test for a general double-hump distribution (layer affect).

Hypothesis 3: Double-hump micro-distribution is more pronounced in exposed group

We hypothesized that there would also be a double-hump distribution in the reference group ($n = 17$), which was tested using repeated measures ANOVA followed by post hoc paired *t*-tests. Differences between reference-group and exposed-group ($n = 35$) nail samples in arsenic concentration within each nail-plate layer was also assessed using repeated measures two-way ANOVA. Data were log-transformed prior to analysis.

Hypothesis 4: Double-hump micro-distribution associated with differences in binding affinity

We hypothesize that the double-hump micro-distribution is driven by differences in binding affinity. We assess this hypothesis by testing the prediction that despite all nail layers experiencing similar arsenic exposure when artificially spiked with an arsenic solution, a double-hump distribution will occur and be enhanced, due to differences in binding affinity. We used data pertaining to all 17 reference group nail clippings from both unspiked and spiked treatments and tested the relationship between arsenic and layers with repeated measures two-way ANOVA. Data were log10-transformed prior to analysis.

Hypothesis 5: Differentiating endogenous and exogenous arsenic exposure in the nail

Here, we assume that the patterns of arsenic among layers measured in the reference unspiked and spiked nails reflect the pattern that would occur without external contamination. In this way, the unspiked ratios reflect individuals with no to very low exposure to arsenic, and spiked ratios reflect what would be expected in a high exposure associated solely with accumulation from endogenous sources. We compare the relationships between the inner layer and the (a) dorsal and (b) ventral layers in the reference nails using linear regression and compare the exposed individual nail layer arsenic to those relationships. If the dorsal and ventral concentrations are higher than expected relative to the inner layer, it is presumed to be due to their contact with the external environment and potential contamination, while the inner layer is protected.

Percent contamination of exposed group nails was based on the relationship between nail layers, as described above, and was calculated using Eq. (1):

$$\text{Contamination}(\%) = (\text{OBSx} - \text{PREDx})/\text{PREDx} \times 100 \quad (1)$$

where OBSx is the arsenic concentration in the dorsal or ventral layer and the PREDx is the

predicted concentration in the dorsal or ventral layer based on the regression equation that uses the inner layer to predict the dorsal or ventral arsenic concentration.

Hypothesis 6: Comparing bulk ICP-MS and urine analysis with LA-ICP-MS toenail arsenic

Finally, we compare the arsenic concentrations obtained through LA-ICP-MS analysis of the big toenail with the bulk ICP-MS analysis following acid digestion of the remaining nails (up to 9 clippings) and urine arsenic from 33 individuals in the exposed group (two individuals did not have a sample available for bulk analysis). As bulk analysis would include all three layers (dorsal, inner, and ventral) in the digestion, with the potential for external contamination to be a factor, it is hypothesized that concentrations through bulk analysis will be similar to the arsenic concentrations that include all nail layers (Rodushkin & Axelsson, 2003). Linear regression was used to determine relationships between (1) bulk toenail and all-layers (LA-ICP-MS) arsenic; (2) urine and all-layers (LA-ICP-MS) arsenic; and (3) urine and inner layer (LA-ICP-MS) arsenic. Data were first log-transformed.

Results

Hypothesis 1: Relationship between urine and bulk toenail arsenic

Urine arsenic concentrations ranged from 2.3 to 57.0 µg/L, where there was no significant difference ($p = 0.948$) between children (12.5 ± 12.9 µg/L) and adults (12.2 ± 9.4 µg/L). For the same individuals, bulk nail arsenic concentrations ranged from 0.34 to 6.60 µg/g (Table 1), where children had significantly higher arsenic concentrations ($p = 0.000$; 3.45 ± 1.41 µg/g) than adults (1.40 ± 0.90 µg/g). The arsenic species, As^{5+}, dominated in the toenails at 77.6 ± 10.4%, followed by As^{3+} at 15.8 ± 11.3% (data not shown). Arsenic species AsB, DMA, and MMA made up 8.6 ± 5.9% combined. Despite the children having higher toenail arsenic concentrations, they did not have more elevated urine arsenic; as such, there was no significant relationship between urine and bulk toenail arsenic (Fig. 2).

Hypothesis 2: The double-hump micro-distribution

The predicted double-hump distributions were discernible in both adults and children (Fig. 3). There were no significant differences between age groups in any of the nail layers, and no age × layer interaction

Table 1 Summary (arithmetic mean, geometric mean, and range) of arsenic concentrations in urine, bulk toenails (acid digestion ICP-MS), and in all/each nail layer as determined by LA-ICP-MS in different treatment groups

Group	Urine (µg/L)	Bulk toenail (µg/g)	All Layers (µg/g)	Dorsal (µg/g)	Inner (µg/g)	Ventral (µg/g)
Potentially exposed: child (5–17 years)	12.5	3.45	2.18	1.14	1.21	8.78
	(9.3)	(3.16)	(0.74)	(0.80)	(0.27)	(2.58)
	3.1–57.0	1.39 – 6.60	0.08–12.83	0.14–3.44	0.06–12.35	0.17–53.54
Potentially exposed: adult (18–62 years)	12.2	1.40	0.77	0.75	0.27	3.56
	(9.3)	(1.18)	(0.45)	(0.61)	(0.17)	(1.33)
	2.3–34.2	0.34–3.38	0.12–3.31	0.06–1.04	0.06–1.04	0.11–17.41
Potentially exposed: all ages (5–62 years)	12.3	2.46	1.50	0.95	0.75	6.25
	(9.3)	(1.95)	(0.58)	(0.71)	(0.22)	(1.87)
	2.3–57.0	0.34–6.60	0.08–12.83	0.14–3.44	0.06–12.35	0.11–53.54
Reference (Unspiked)	-	-	0.11	0.29	0.15	0.58
			(0.07)	(0.23)	(0.14)	(0.33)
			0.02–0.56	0.08–0.81	0.08–0.21	0.08–4.33
Reference (Spiked)	-	-	5.03	10.78	2.08	22.06
			(4.47)	(10.72)	(2.09)	(21.88)
			2.22–10.86	2.03–39.16	0.81–9.99	8.34–54.40

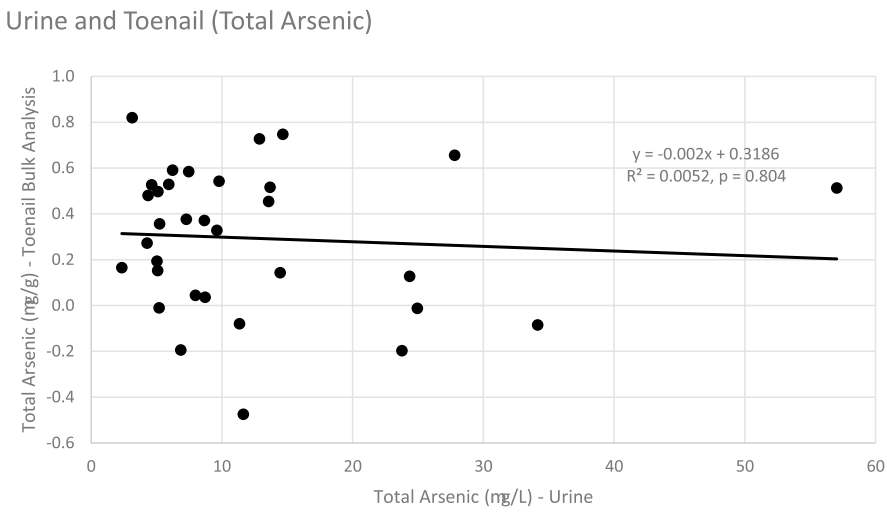

Fig. 2 Lack of relationship between total arsenic in the urine and total arsenic in the toenails (determined by acid digestion bulk analysis ICP-MS) in the exposed group

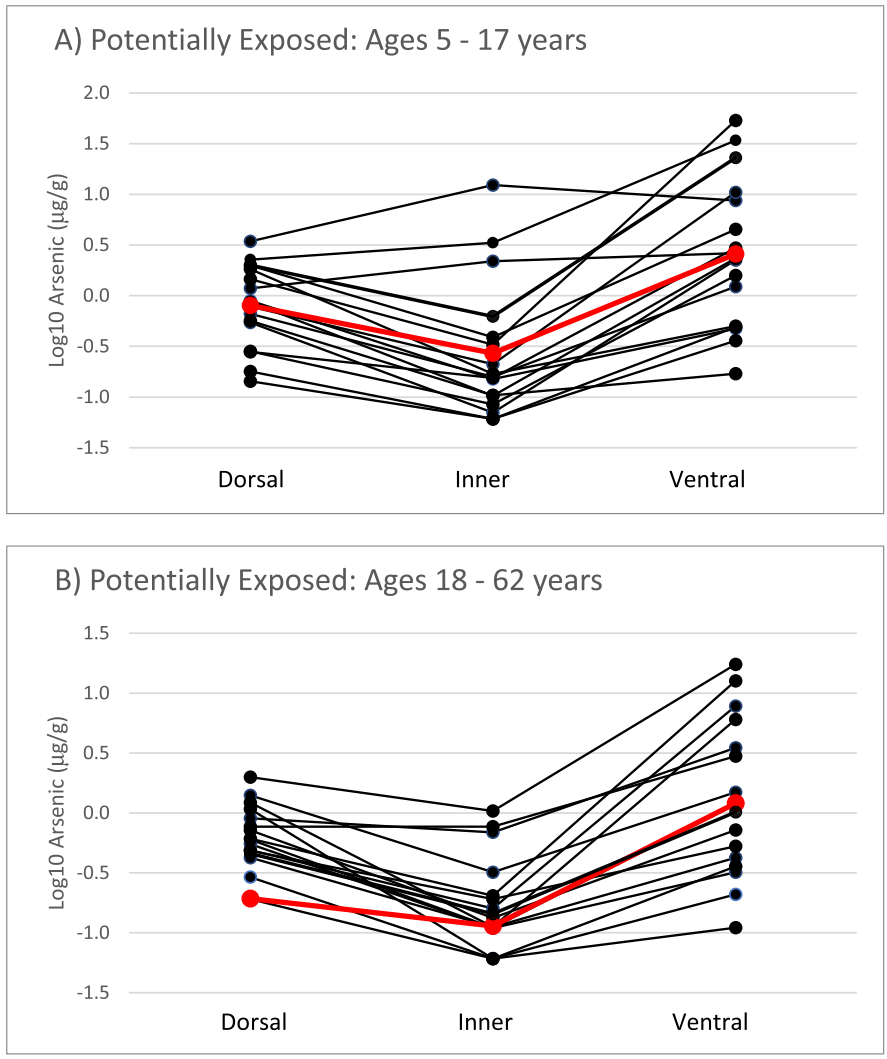

Fig. 3 Dorsal, inner and ventral nail layer arsenic concentrations (log10 transformed) in children (**A**) and adults (**B**). Black circles denote individual datapoints connected by lines for each participant in the exposed group. Red circles denote the average of all datapoints for each age group connected by lines. Note the double-hump, where the dorsal and ventral layers are almost always higher than the inner layer

(Table 2, part A). There were, however, significant differences in arsenic concentrations among nail layers, exhibiting a double-hump distribution (Table 2, parts A and B), where the ventral layer had the highest arsenic concentrations on average (6.25 µg/g), followed by the dorsal layer (0.95 µg/g), with the inner layer having the lowest arsenic concentrations (0.75 µg/g; Table 1). Arsenic concentrations in the dorsal and ventral layers of the exposed group were, on average, 1.26- and 8.32-fold higher than in the inner layer, respectively.

Hypothesis 3: Double-hump micro-distribution is more pronounced in exposed group

The reference group also had a significant double-hump pattern (Fig. 4A), where all three layers were significantly different from each other (Table 2, part C). As with the exposed group, the ventral nail layer had the highest arsenic concentrations on average (0.58 µg/g), followed by the dorsal layer (0.29 µg/g), with the lowest concentrations in the inner layer (0.15 µg/g; Table 1). The exposed group had significantly higher concentrations in all layers compared to the reference group (Table 2, part D). Overall, however, the ventral layer to inner layer ratio was less pronounced in the reference group at 3.84-fold, while the dorsal layer was slightly more elevated relative to the inner layer at 1.97-fold.

Hypothesis 4: Double-hump micro-distribution associated with differences in binding affinity

Using the unspiked and spiked reference nails to highlight theoretical arsenic binding in the absence of external contamination, there were significantly higher concentrations of arsenic in all layers following spiking (Table 2, part E). The layer × treatment interaction was significant ($p < 0.0001$), meaning the concentration increase differed depending on the nail layer. The ventral layer increased the most at 60-fold, on average (highest binding affinity for arsenic), followed by the dorsal layer at 33-fold (Fig. 4B). The lowest increase in concentrations observed was in the inner layer at 25-fold (lowest binding affinity for arsenic). Before spiking, peak arsenic concentrations in the dorsal and ventral layers were, respectively, approximately 1.97- and 3.84-fold that of the internal layer; following spiking, these differences increased 5.18- and 10.59-fold, respectively.

Hypothesis 5: Differentiating endogenous and exogenous arsenic exposure in the nail

Consistent with higher binding affinity in the ventral layer compared to other nail layers, the ventral/inner slope (1.43) was higher by 1.12-fold compared to the dorsal/inner layer slope (1.28; Fig. 5). When the exposed group nail arsenic concentrations (red points

Table 2 Results of repeated measures analysis of variance (one-way and two-way) to characterize significant differences between test groups and nail layers

Test		Dorsal	Inner	Ventral	Layers	Interaction
A	Child vs Adult	Child = Adult ($p = 0.347$)	Child = Adult ($p = 0.577$)	Child = Adult ($p = 0.246$)	Ventral > Dorsal > Inner ($p < 0.0001$)	Age * Layer ($p = 0.569$)
B	Exposed Group: Double Hump	-	-	-	Ventral > Dorsal > Inner ($p < 0.0001$)	-
C	Reference Group: Double Hump	-	-	-	Ventral > Dorsal > Inner ($p < 0.0001$)	-
D	Exposed vs Reference	Exposed > Reference ($p < 0.0001$)	Exposed > Reference ($p < 0.0001$)	Exposed > Reference ($p < 0.0001$)	Ventral > Dorsal > Inner ($p < 0.0001$)	Exposure Group * Layer ($p = 0.121$)
E	Spiked vs Unspiked (Binding Affinity Among Layers)	Spiked > Unspiked ($p < 0.0001$)	Spiked > Unspiked ($p < 0.0001$)	Spiked > Unspiked ($p < 0.0001$)	Ventral > Dorsal > Inner ($p < 0.0001$)	Spiked Group * Layer ($p = -0.002$); Ventral > Dorsal > Inner

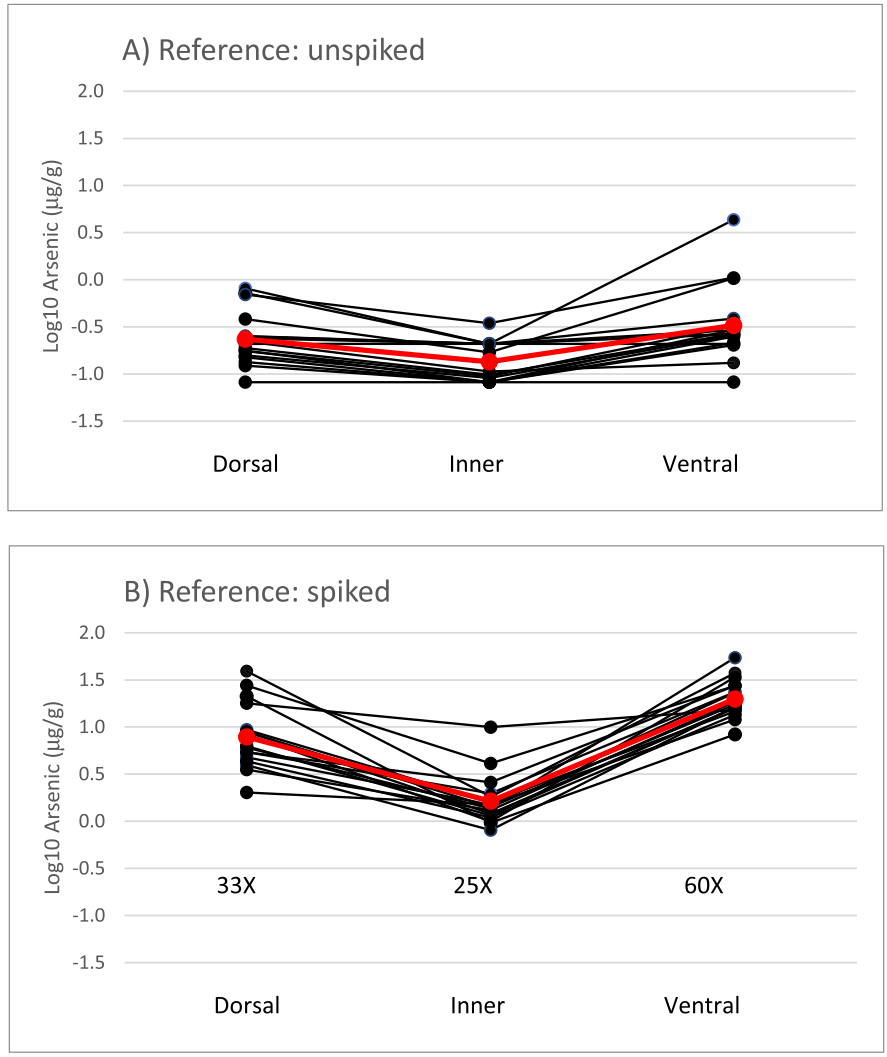

Fig. 4 Dorsal, inner, and ventral nail layer arsenic concentrations (log10 transformed) in the reference group, unspiked (**A**) and in the reference group, spiked (**B**). Black circles denote individual datapoints connected by lines for each participant in the reference group. Red circles denote the average of all datapoints for each treatment group connected by lines. Note the double-hump, where the dorsal and ventral layers are almost always higher than the inner layer, which is more pronounced in the spiked nail layers (noted by values representing the increase in arsenic concentrations compared to the reference nail layers)

in Fig. 5) were compared to the reference nails, most of the data points at lower inner arsenic concentrations (<0.32 µg/g or $<10^{-0.5}$ µg/g) fell above the linear relationship, both for adults and children, suggesting both dorsal and ventral layers were abnormally higher than they should be if no contamination of those layers had occurred. The ventral layer showed a similar occurrence of contamination compared to the dorsal layer. Specifically, the ventral layer had 63% of the individuals (22/35) with >25% estimated contamination compared to 71% (24/35) in the dorsal layer. However, the extent of contamination was, on average, lower in the dorsal (concentrations 1.13-fold higher on average than predicted in dorsal and 3.75-fold higher in the ventral). Additionally, 51% (18/35) of the individuals had greater than 100% contamination (i.e., double the concentration predicted) in the ventral layer, while 37% (13/35) had contaminated dorsal layers above that level. The dorsal and ventral layers of toenails from the children and adults were similarly contaminated.

Hypothesis 6: Comparing bulk ICP-MS and urine analysis with LA-ICP-MS toenail arsenic

Bulk analysis of the nails from potentially exposed individuals ranged from 0.34 to 6.60 µg/g, while the all-layers combined LA-ICP-MS arsenic concentrations for the same individuals ranged from 0.08 to 12.83 µg/g (Table 1). There was no significant

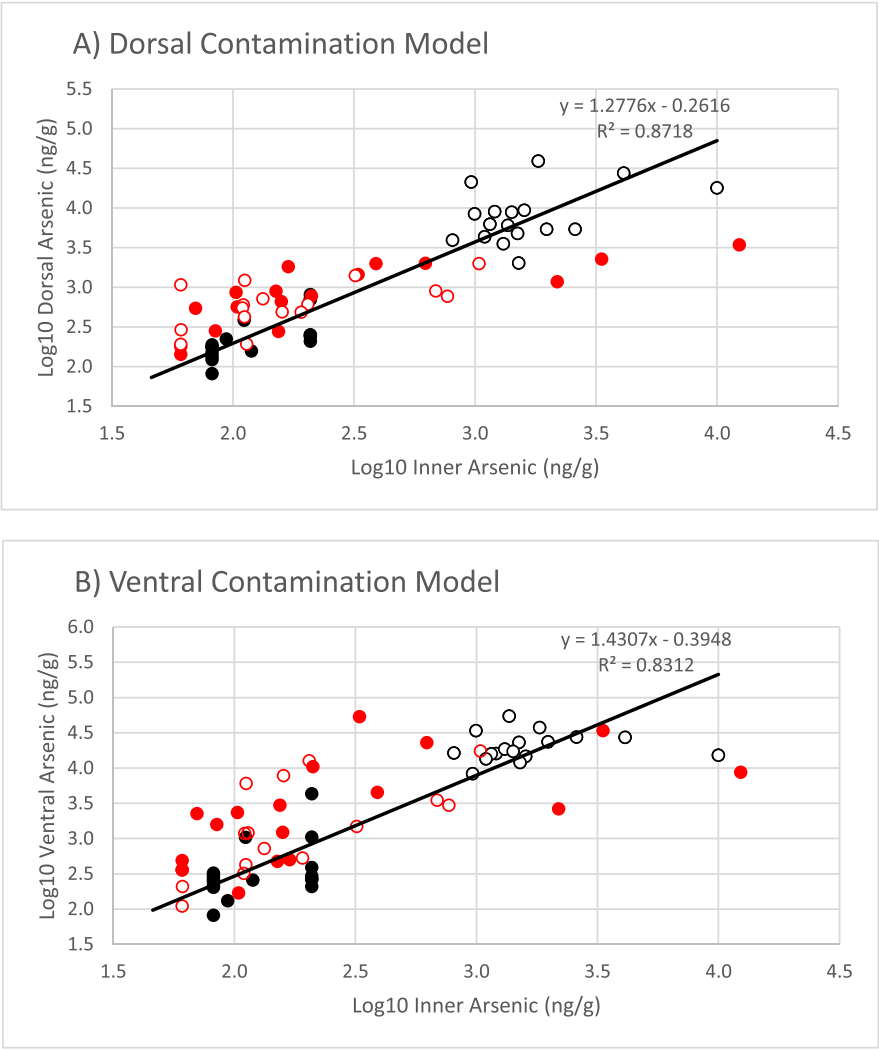

Fig. 5 Relationship between nail layers (arsenic concentrations are $\log_{10}$ transformed): **A** inner and dorsal layers; **B** inner and ventral layers. Linear relationship based on spiked and unspiked reference nails (black circles), assuming this relationship represents pure arsenic exposure with no external contamination. Exposed group nail arsenic concentrations (red circles) are compared to the relationship to determine extent of contamination (datapoints above regression line) of dorsal and ventral nail layers. Solid red circles denote children and open red circles denote adults

correlation between the two analyses (Fig. 6). There was also no significant correlation between the all-layers combined LA-ICP-MS arsenic concentrations and urine arsenic concentrations (Fig. 6B) or between the inner nail layer arsenic and urine arsenic (Fig. 6C).

Discussion

Urine arsenic concentrations ranged from 2.3 to 57.0 µg/L in the exposed group. Health Canada has established a screening level of total arsenic in urine at 21 µg/L based on the 95th percentile values of Canadians participated in the Canadian Health Measure Survey (Health Canada, 2021; St-Amand et al., 2014). This result confirms that some individuals in the exposed group have recently been exposed to some arsenic above background levels.

These same individuals had bulk toenail arsenic concentrations ranging from 0.34 to 6.60 µg/g (dominated by the inorganic As^{5+} and As^{3+} species), which were significantly higher than the reference group (i.e., whole nail arsenic concentrations averaging 0.11 µg/g, and up to 0.56 µg/g). These dissimilarities between exposure groups are mirrored in the literature. Gault et al. (2008) reported reference arsenic at 0.72 ± 0.11 µg/g, compared with people living near contaminated well water at 1.96 ± 0.33 µg/g. Nails collected from a population in Bangladesh where

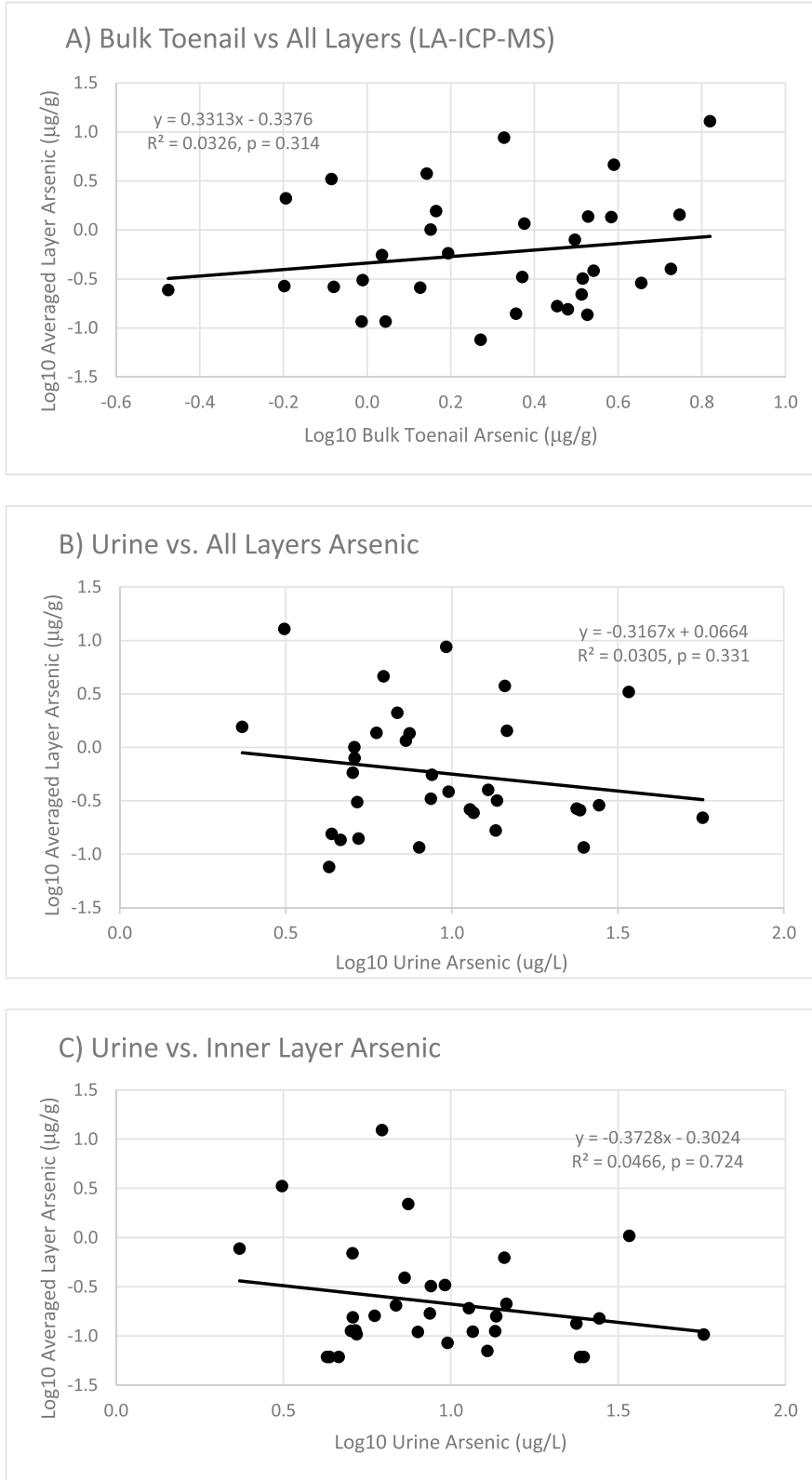

Fig. 6 Relationship between **A** bulk toenail arsenic and all-layers LA-ICP-MS arsenic concentrations; **B** urine arsenic and all-layers LA-ICP-MS arsenic concentrations; and **C** urine arsenic and inner layer arsenic concentrations. All data is log10 transformed

groundwater is highly contaminated with arsenic were 1.40 ± 0.09 µg/g for males and 2.03 ± 0.190 µg/g for females (Rakib et al., 2013). In Cambodia, in an area also having elevated arsenic groundwater, fingernail arsenic concentrations ranged from 0.20 to 6.50 µg/g (1.90 ± 0.20 µg/g) (Gault et al., 2008), an almost identical range to those in the exposed group of the present study.

Nail clippings have been used for decades to monitor exposure to toxic metals, such as arsenic (Signes-Pastor et al., 2021; Gault et al., 2008; Goullé et al., 2009; Fleming et al., 2021; Michaud et al., 2004). The bulk nail arsenic concentrations in our exposed group definitely suggest potentially elevated exposure to arsenic, as does the urine analysis. However, the two matrices were not correlated, where elevated urine arsenic did not equate to elevated toenail arsenic. There are a number of reasons for a lack of correlation, at least in this study, including that while the urine and nails were collected at the same time, they reflected different exposure windows. The urine reflects recent exposure (ATSDR, 2007; Järup & Åkesson, 2009; Tehrani et al., 2020), and the nail clippings reflect exposure months previous (Yaemsiri et al. 2010). Additionally, nine different nails were combined for a single arsenic concentration, with each nail reflecting a different time frame for exposure (Yaemsiri et al. 2010), e.g., big toenail reflecting the furthest past, up to 10 months previous to the sampling event. If exposure to arsenic is not consistent and/or chronic, each clipping would reflect different arsenic exposure events, with none of the nail clippings reflecting the time frame reflected in the urine sample. There is the added confounding influence of external contamination of the nail clippings (Bainter, 2014; Christensen et al., 2017). For people living within an area with arsenic-contaminated soils and water, there is a higher probability of those soil particles and water sources coming in contact with exposed tissues, such as toenails. As such, the main objective of this study was to focus on this latter confounding influence, by examining the micro-distribution of arsenic in toenails, with the aim to differentiate between endogenous and exogenous arsenic.

Our study verified that there are at least two possible synergistic phenomena driving non-uniform micro-distributions of trace elements across the nail plate: external contamination (Bainter, 2014; Christensen et al., 2017); and differences across nail layers in binding affinity associated with the availability of unbonded sulfhydryl (-SH) groups (Cui et al., 2013; Pearce et al., 2010; Slotnick & Nriagu, 2006; Wilhelm et al., 2005). The dorsal and ventral surfaces of nail plates, particularly those of fingernails, are exposed to external sources of contaminants (Bakri et al., 2017; Christensen et al., 2017; Signes-Pastor et al., 2021; Slotnick & Nriagu, 2006). With prolonged exposure, external contaminants could also be absorbed through the nail surfaces into the matrix (Walters & Flynn, 1983), thereby increasing concentrations near dorsal and ventral margins. While washing and/or sonicating nails may remove some arsenic contamination (Signes-Pastor et al., 2021; Slotnick & Nriagu, 2006), it may not remove the arsenic that has been securely bound with sulfhydryl groups after external exposure. External contamination may, therefore, remain a confounding factor in obtaining an accurate measure of endogenous arsenic exposure and contribute to the high variability in arsenic concentrations measured worldwide among studies (Signes-Pastor et al., 2021).

Using laser ablation line scans, we revealed variability among nail layers. For these same 35 individuals from the exposed group, there was a consistent pattern of a double-hump distribution with significantly higher concentrations of arsenic in the dorsal and ventral layers. The double-hump distribution of arsenic was less pronounced in the reference group, but the ventral layer still showed the highest concentrations. Disregarding the potential for confounding effects of contamination, elevated concentrations of trace elements (especially chalcophilic, or sulfur-loving, elements like arsenic) in dorsal and ventral surfaces are often attributed to their affinity to form disulfide bonds with free-SH groups (Cui et al., 2013; Pearce et al., 2010; Slotnick & Nriagu, 2006; Wilhelm et al., 2005). The availability of sulfhydryl groups is greater in the outer layers (i.e., dorsal and ventral) of the nail plate, leading to elevated accumulation of trace elements within these layers relative to the intermediate layer, where stable disulfide groups are rich (Cui et al., 2013; Pearce et al., 2010). Rodushkin and Axelsson (2003) measured higher concentrations and variability (42% relative standard deviation) in the dorsal layer compared to the inner layer, which had lower concentrations and only 6% variability in arsenic. They concluded that the marked improvement in variability in the inner layer confirms the impact of external contamination on the dorsal

layer of the nail. Chan et al. (2023) also promote double or "pre" ablation to remove a potentially contaminated surface/dorsal layer.

To the authors' knowledge, this is the first study that investigated the binding affinity of the different nail layers to arsenic. This is an important consideration because if all layers accumulate arsenic equally, then any difference among layers described above would reflect external contamination of layers with higher concentration (i.e., dorsal and ventral). However, the layers had different binding affinities, with the ventral having the greatest and the inner layer the least. Differentiating the endogenous versus external contamination of exposed layers becomes complicated as the high binding affinity of those layers, in combination with exposure to the environment, becomes additive. By examining the relationship of dorsal to inner layers and ventral to inner layers in the unspiked and spiked reference nails, there is a strong relationship that describes how arsenic would theoretically accumulate in nails along an exposure gradient without external contamination. Many of the exposed groups' nails did not follow this relationship, particularly at the lower inner nail layer arsenic concentrations, but rather had higher dorsal and ventral nail layer arsenic concentrations than would be predicted by the model. This suggests that despite washing/sonication of nails, those exposed layers still had external contamination impacting the results.

Based on inner layer arsenic concentrations, only 10 individuals out of the 35 have arsenic concentrations above the reference group range. However, using dorsal or ventral layer concentrations, that number is much higher at 15 and 22, respectively. Interestingly, the bulk concentrations of arsenic in the exposed group are in the concentration range of the inner layer following dosing with a high-concentration arsenic solution. This further suggests that the bulk concentrations obtained in this study and, possibly in other studies, must have incorporated external contamination, unless the exposure of an individual or population resulted in blood arsenic levels as high as those used here for artificial dosing. Our results on micro-distribution and binding affinity provide insight into the impact of external contamination on total arsenic concentrations and show how LA-ICP-MS can access the uncontaminated inner layer of the nail to provide a more accurate result compared to bulk toenail clipping analysis.

Confounding effects from external contamination in the bulk analysis also explain the lack of relationship between the inner layers (and all layers) and bulk nails in this study. There are many uncertainties when comparing the results obtained from the LA-ICP-MS and bulk analysis. LA-ICP-MS uses a single line scan on the big toenail clipping edge, ablating an almost immeasurably small volume compared to the bulk analysis of the remaining nine toenails. Different nails were analyzed between the two types of analyses, and these nails will have different total thicknesses and layer thicknesses (Fleming et al., 2021). The clippings of different nails also would reflect different timeframes (Rodushkin & Axelsson, 2003; Signes-Pastor et al., 2021; Slotnick & Nriagu, 2006), so unless arsenic exposure was chronic and at stable levels over many months, concentrations among nails could vary significantly, causing a dissociation between the two analyses, as observed here. Gault et al. (2008) also suggested a heterogeneous nature to arsenic accumulation in the nail, so ablating a smaller volume using LA-ICP-MS may not be representative of a homogenized bulk sample. Rodushkin and Axelsson (2003) also found considerable variation in elemental concentrations from one nail to another and within a single nail. Therefore, the LA-ICP-MS results can be used to investigate the potential sources of arsenic and improve the understanding of the kinetics of arsenic in toenails. For biomonitoring purposes, bulk analysis may be used as the primary method and perhaps supported by additional analysis by LA-ICP-MS for validation.

Conclusions

Overall, our results confirm the non-uniformity of the chalcophile trace element, arsenic, through the nail plate and point to a combination of differences in binding affinity and exposure to external contamination as twin drivers of this micro-distribution. Our results also highlight the likelihood that bulk toenail arsenic measurements will not only measure the arsenic that is bioaccumulated in the toenail from the blood supply but also arsenic adsorbed from the toenail surface. This makes the appropriateness of using bulk toenail arsenic measurement as a biomarker for the internal dose questionable. Although the inner nail layer has lower binding affinity, it still binds

arsenic at detectable concentrations, particularly in an exposed environment. Additionally, this layer is not readily subjected to external contamination, so it may be the best reflection of endogenous exposure to arsenic compared to both bulk analysis and ventral/dorsal layers. These results suggest that LA-IC-MS can measure arsenic in the specific regions within the nail matrix which is a more accurate biomarker for arsenic exposure monitoring.

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Author contributions J.C.—study design, data analysis, wrote main manuscript text and figures.

G.L.—nail sample preparation and LA-ICP-MS analysis, data processing, reviewing manuscript.

J.S.C.—data collection, data analysis.

R.R.—study design, data collection.

A.M.—study design, reviewed manuscript.

B.L.—study design, reviewed manuscript.

H.M.C.—obtained funding, study design, data collection, reviewed manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are not publicly available due to ethical considerations and participant privacy, but can be made available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The research was conducted following the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and, in particular, Chapter 9, research involving the First Nations, Inuit and Métis Peoples of Canada (Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, Social Sciences and Humanities Research Council of Canada, 2010), and the document entitled: Indigenous Peoples & Participatory Health Research: Planning & Management, Preparing Research Agreements published by the World Health Organization (Fediuk & Kuhnheim, 2003). The study also follows the First Nations principles of Ownership, Control, Access, and Possession (OCAP®) of data (Schnarch, 2004).

The study was approved by the Health Sciences and Sciences Research Ethics Board of the University of Ottawa (http://research.uottawa.ca/ethics/reb) and the Aurora College Research Ethics Committee. In addition, the study has been granted a Scientific Research License from the Aurora Research Institute in Northwest Territories. Individual participation in the project was voluntary and based on informed written consent following an oral and written explanation of each project component.

Competing interests The authors declare no competing interests.

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