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All-Source Exposures To Arsenic For Residents Living Near Historic Gold Mine Areas In Northwest Romania

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All-source exposures to arsenic for residents living near historic gold mine areas in northwest Romania

A Thesis
Presented to the Faculty of the Yale School of Public Health
of
Yale University
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Master of Public Health

By
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Abstract

Background: Roşia Montană gold mine is the site of historic mining operations since Roman times. Contamination of nearby communities by historic mining activities is of serious concern.

Objective: The study objective is to investigate whether living in the vicinity of historic gold mining increases risk of arsenic exposure from the surrounding environment, and if dietary intake from home gardens is associated with reduced risk of arsenic exposure.

Methods: We performed assessments in 64 participants from 57 households within the target communities. All study participants gave written informed consent. The study was approved by the institutional review boards of the regional public health authority of Alba County in Romania. Urine samples were digested and analyzed, while fingernails were washed, digested and analyzed. Data were compiled as geometric means of urinary total arsenic concentration ($\mu\text{g/L}$) and fingernail arsenic concentration ($\mu\text{g/g}$).

Results: 64 participants in the study were divided into 4 clusters based on distance from the historic mine. Urine total arsenic from the closest cluster to the farthest was $30.65 \pm 7.60 \mu\text{g/L}$, $27.19 \pm 14.17 \mu\text{g/L}$, $34.11 \pm 11.54 \mu\text{g/L}$, $28.97 \pm 8.56 \mu\text{g/L}$, respectively, all significantly below the present standard for urinary arsenic $100 \mu\text{g/L}$. In contrast, fingernail arsenic was $10.73 \pm 4.87 \mu\text{g/g}$, $12.91 \pm 3.42 \mu\text{g/g}$, $12.09 \pm 1.08 \mu\text{g/g}$, $9.35 \pm 3.87 \mu\text{g/g}$, all significantly above the present standard of nail arsenic of $1 \mu\text{g/g}$. Overall, we did not find evidence that people who live closer to the mine had higher exposure to arsenic compared to those lived farther away. Drinking water source, dietary intake of leafy vegetable and fish, and cleaning practices did not have significant effects on either urinary total arsenic or fingernail arsenic. Self-reported symptoms (extremity numbness, frequent and reoccurring heartburn, frequent leg/muscle cramps, frequent joint pains) were not found to be correlated with the average distances controlled for self-perceived health status and knowledge of arsenic exposure, suggesting these symptoms were less likely to have a causal relationship or to be associated with gold mining activities. Self-reported frequent leg cramps was associated with increased urinary total arsenic concentration.

Conclusion: Our data demonstrate an apparent low-level, on-going, all-source arsenic exposure together with a high historic arsenic exposure. Our study provides evidence that people living within 38 km from historic gold mines still retain a body burden of arsenic exposure that is not reflected in elevated urine total arsenic. And there is concern of self-reported adverse health effects associated with current low-level on-going exposure in this area. These findings suggest that fingernail arsenic concentration may be a better predictor of arsenic environmental exposure than urinary total arsenic in the area with a specific exposure profile - low-level, on-going, all-source and high historic arsenic exposure. But using both fingernail and urinary arsenic concentrations may offer an opportunity for a more effective approach to capture adverse effects from low-level exposure, and predict and convey information regarding long-term exposure burden after overall environmental arsenic exposure has diminished.

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Introduction

More than 140 million people in over 70 countries have chronic exposure to arsenic worldwide. Arsenic is distributed ubiquitously as a trace constituent in soils and rocks, natural waters and organisms (Irgolic, 1986; WHO, 2001). The main natural occurrences of arsenic are ore deposits (Baur and Onish, 1978). Arsenic dispersion into the environment, which naturally occurs through weathering processes, can be intensified by mining activities, leading to local or regional soil and water pollution. Previous studies have shown that activities related to gold mining, such as grinding, drilling and blasting, smelting, leaching, and tailings were highly correlated with arsenic concentration in the environment (Ferreira da Silva et al., 2004; Inam et al., 2011).

Arsenic and Gold Mining in Roşia Montană

Roşia Montană is an historic mining site in the Apueni Mountains of Transylvania, Romania. Gold mining activities at Roşia Montană Quadrilateral date back as far as Roman times, and nowadays have triggered controversy in Romania, due to the potential environmental pollution caused by these mining activities. In fact, the approval process for the “European’s largest open-pit gold mine operation” has been halted for decades due to nation-wide protest, environmental concerns and the objections of the Romanian parliamentary commission. A decision made by the Romania’s Ministry for Culture early this year to add Roşia Montană to the country’s tentative list of UNESCO World Heritage sites has granted Roşia Montană protection from industrial activities, including mining (David, 2016), hence currently halted mining operation might be held in suspension for good. If this is the case, studies related to Roşia Montană gold mine area might not only focus on its historic mining site aspect, but also evaluate potential risks associated with a closed mine. The stability of the population in the region may then provide a unique opportunity to examine the decay of arsenic body burden depending on on-going bioavailability to arsenic exposure from the historic mining operations.

As a result of historic mining activities, abandoned waste dumps and tailings ponds in the

Roşia Montană area have been left with high levels of metals including zinc, iron, arsenic, lead and cadmium discharged, untreated, into local streams, soils and water (Burja et al., 2010). There have been many attempts to demonstrate health effects of arsenic or other metals in mining areas including abandoned or closed metal mines (Chung et al., 2005; Lynch et al., 2000). Some studies reported that the body burden of arsenic in residents near metal mines was higher than that of non-exposed areas (Basu et al., 2010; Moreno et al., 2010), and that increased urinary arsenic levels were observed in 33% of 275 residents living in the surroundings of abandoned gold mine tailings in Mexico (Colín-Torres, 2014). Both past and current mining activities can contaminate the surrounding environment and nearby communities (Fields, 2003). Because gold- and arsenic-bearing minerals coexist, there is a hazard of mobilizing arsenic during or even after gold mining activities (Garelick et al., 2010).

Multimedia Exposure to Arsenic

Arsenic exposure from gold mining activities occurs via inhalation and ingestion of windblown soil and dust, and ingestion of contaminated water or food (ATSDR 2007; Tchounwou et al., 2012). Dermal absorption occurs to a lesser extent (Rossman, 2007). Many studies show arsenic pollution from soil, water, sediment, and dust around metal mines (Jung, Thornton, & Chon, 2002; Lindberg et al., 2006; Leonardi et al., 2012; Ishinishi et al., 1986).

Distance

One study found elevated levels of arsenic in stream sediments that were sampled in the vicinity of a mine, and decreased with distance from the mine (Jung, Thornton & Chon, 2002). Another study on an abandoned arsenic mine's effects on drinking water resources quality found high concentration of arsenic (about 200 µg/L) near the mine, and a decreasing concentration of arsenic in water with increasing distance from the mine (Hajalilou et al., 2011). Martin and colleagues found inverse trends between particle size and levels of arsenic, and suggested that finer particles are highly susceptible to long-distance transport (Martin et al., 2015).

Drinking Water

Drinking water and food typically account for 99% of the total human arsenic intake (Jones, 2007). Several studies also found a significant correlation between arsenic concentrations in water and urine (Calderon et al., 1999, Ahsan et al., 2000, Lindberg et al., 2006). Lindberg's study also found that smokers and people with higher BMI have higher urinary arsenic concentrations, but that there was no difference of arsenic concentrations in urinary or water among different age groups.

The Arsenic Health Risk Assessment and Molecular Epidemiology (ASHRAM) studies of low-level long-term inorganic arsenic exposure via drinking water in eastern Hungary, western Romania and Slovakia found that total inorganic arsenic levels in water in the two Romanian counties bordering the Alba County to the northwest and southwest were relatively lower than the other study areas, with the median values of 0.70 and 2.1 $\mu\text{g/L}$ (Lindberg et al., 2006; Leonardi et al., 2012), and far below the US EPA drinking water standard for arsenic (10 $\mu\text{g/L}$) (EPA Chemical Contaminant Rules). The concentration range of arsenic in Arad and Bihor Counties (downstream to the study area in Alba County) in west Romania is 0-176 $\mu\text{g/L}$. Estimates suggest that there were approximately one million people exposed to naturally occurring arsenic via drinking water that exceeded the 10 $\mu\text{g/L}$ WHO and EU standards (Gurzau et al., 2001).

Dust and Soil

Incidental soil and dust ingestion, inhalation of soil-born particulate matter, and to a lesser extent, direct dermal contact are the major direct exposure pathways (Bacigalupo et al., 2012).

Airborne arsenic is generally in the form of arsenic trioxide (Ishinishi et al., 1986). When ores are heated in smelters, most of the arsenic goes up the stack and enters the air as a fine dust. Dust containing arsenic can enter the body by inhalation and ingestion, or through absorption from dermal and eye contact, though it is not a major route of exposure compared to ingestion and inhalation exposures (ATSDR, 2007; Martin et al., 2014)

Soil arsenic is also a significant predictor of increased urinary arsenic concentration for

residents living in old mining areas (Hinwood et al., 2006). Elevated arsenic levels were observed in soils around historic gold mining areas (Martin et al., 2013). This study also found a significant correlation between soil arsenic levels and toenail arsenic concentration ($r=0.630$, $p=0.001$), suggesting some systemic absorption associated with periodic exposures.

Dietary intake

Dietary intake, especially leafy vegetables could be protective against arsenic-induced health problems (Kile and Ronnenberg, 2008). For example, Gamble and colleagues (Gamble et al., 2007) showed folic acid lowered blood arsenic levels by 14 percent in a Bangladesh population exposed to arsenic through contaminated drinking water. Folic acid enhances the detoxification (methylation) process of arsenic to MMA and DMA, which are more easily excreted in urine. A randomized control trial of folic acid and creatine supplementations as therapeutic approaches showed an augmented effect of folic acid on the arsenic methylation process in reducing blood arsenic level from baseline when all 662 participants who were previously exposed to drinking water arsenic ($>50 \mu\text{g/L}$) over one year in Bangladesh were exposed to low-level arsenic in drinking water ($<10 \mu\text{g/L}$) using water filter at baseline (Peters et al., 2015). Another animal study comparing arsenic methyltransferase knockout mice to wild-type mice in arsenic body burden and urinary excretion found a 16-20 fold higher body burden of arsenic in lung, liver, kidney, and urinary bladder of the knockout mice (Hughes et al., 2010). The study also found that urinary arsenic level was significantly lower in arsenic methyltransferase knockout mice than in wild-type mice.

On the other hand, home garden vegetables might be a potential exposure route for inorganic arsenic, through incidental soil and dust ingestion (Bacigalupo et al., 2012), contaminated water irrigation (Islam et al., 2016), arsenic-enriched fertilizers and pesticides (ATSDR, 2007), or food preparation (Diaz et al., 2015). Studies have shown arsenic contents in plant samples to vary with species and parts, with higher concentrations in plant leaves and lower concentrations in grains and stalk (Jung, Thornton, & Chon, 2002; Lim et al., 2007). Another

review paper reported that both leafy vegetables and nonleafy vegetables are good accumulators of heavy metals. In nonleafy vegetables, the bioaccumulation was highest in the leaves (Khan et al., 2015).

Acute and Chronic Arsenic Toxicity

Adverse health outcomes associated with exposure to mine wastes have been observed in residents living close to or within mining-affected areas (Plumlee and Morman 2011). Previous epidemiologic studies have shown environmental exposure to arsenic in drinking water or soil has been associated with increased risk of skin lesions, cardiovascular diseases, diabetes and cancers (Brown et al., 2002, Leonardi et al., 2012). Inhalation, ingestion, or dermal exposure to inorganic arsenic has caused peripheral nerve inflammation (neuritis) and degeneration (neuropathy), reduced peripheral circulation, anemia, increased mortality due to cardiovascular failure, and cancers of the skin, lungs and lymphatic system (Rossman, 2007; Ratnaike 2003).

The immediate symptoms of acute arsenic poisoning including vomiting, abdominal pain and diarrhea, which are often followed by extremities numbness and tingling, muscle cramping and even death. Chronic environmental exposure to arsenic has been documented to cause skin lesions, patchy palms and hyperkeratosis. Arsenic and arsenic compounds are classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans, causing skin cancer, lung cancer and bladder cancer. Long-term exposure is also associated diabetes, cardiovascular disease (WHO 2001)

Arsenic levels in blood, urine, hair and nails have been investigated and used as biological indicators of exposure to arsenic. Arsenic is cleared from blood within a few hours (CDC 2009; ATSDR 2007). Arsenic from exposure through inhalation or ingestion is absorbed from the lungs or the gastrointestinal tract, undergoes biomethylation in the liver and excreted in the urine mainly within 1-2 days (ATSDR 2007; Rossman, 2007). Thus measurement of urinary arsenic levels is generally accepted as the most reliable indicator of recent arsenic exposure, and has proved useful in identifying above-average exposures in populations living near industrial

point sources of arsenic (Milham and Strong 1974; Polissar et al. 1990). Also, arsenic tends to accumulate in hair and nails, so measurement of arsenic levels in fingernails is a useful indicator of past 6-12 months exposures (Choucair and Ajax, 1988). Garland et al. (1993) showed that toenail arsenic levels remain relatively constant over 6 years in 127 women. Another study also found toenail measurements of arsenic reproducible over a three to five years period (Michaud et al., 2004). Toenails provide an integrated measure of internal inorganic arsenic exposure and reflect all sources of exposure, including drinking water, diet, and occupation. Normal levels of arsenic in urine is 100µg/L or less, and that in fingernails is 1ppm or less (Valentine et al., 1979; Jenkins et al., 1979; ATSDR, 2007).

Objectives

This study (funded by Jan A. J. Stolwijk Fellowship) was carried out in the historic mining district in the Apuseni Mountains of Transylvania, Romania. Our study is part of a project funded by the United States Environmental Protection Agency, covering an area of 1,000 square kilometers and population of 50,000. The U.S.EPA funded project focuses on geospatial distribution and exposure of multiple heavy metals (arsenic, cadmium, mercury, lead and nickel) in the multimedia environment, including air (indoor and outdoor dust), soil, underground and surface water, and vegetables.

The objective of our study was to assess current exposure to arsenic in local residents in an historic gold mining area. Specifically, the study investigated risk of self-reported health problems and arsenic exposure level in local residents living in the vicinity (within 38 km) of gold mines in Roşia Montană of Transylvania area, northwest Romania.

The seven towns and communes within the sampling area have similar profiles for occupation, dietary habits and lifestyle. The primary hypothesis of this study is that living in the vicinity of the historic gold mining increases risk of arsenic exposure from the surrounding environment, and is associated with self-reported health symptoms. A secondary hypothesis is that dietary intake (e.g. leafy vegetable) from home gardens is associated with reduced risk of

arsenic exposure. To address these hypotheses we evaluated arsenic exposure at the level of each individual (summer 2015) by measuring urinary total arsenic and fingernail arsenic. The existence of potential health problems and household information was obtained by questionnaire.

There have been limited epidemiological studies of arsenic exposure and its implications for human health in the Roşia Montană area, Alba County specifically. Therefore it is worthwhile to undertake such a study to determine the association between living in the vicinity of the gold mine and elevated arsenic concentration in humans and whether this complex exposure is associated with residents' self-reported health symptoms. In contrast to exposure-related information, we know little about the relative source contribution for all potential arsenic exposure sources from the environment, e.g., drinking water (surface water, groundwater and rain water), airborne arsenic, soil, and food combined. Thus this study focuses on how environmental exposure due to vicinity to the mines is associated with the arsenic exposure level, without limiting the information to certain exposure sources or routes of exposure and how arsenic exposure based on biomarkers (source independent) predicts self-reported symptoms.

From a public health perspective, we are also interested in determining if age, gender, BMI, education, income, smoking, dietary intake and cleaning practices would modify the effect the all-source arsenic exposure, and thus which groups of participants may be at greater risk to chronic arsenic exposure in an historic gold mining area.

Materials and Methods

Study area and Participant

Study area

The Roşia Montană gold mining region (Figure 1) is located in the Apuseni Mountains in Alba County, northwest Romania. The study area included the seven towns and communes in the gold mine area. The study area includes two open pits (Cetate and Carnic), 17 waste dumps, and two tailing dams. The processing plant is now decommissioned. Alba County has a population of

342, 376 (data from census INS) and contains 4 municipalities, 7 towns and 67 communes in 2011 (INS, 2012a). The seven communities were selected based on their geographical distances to the Roșia Montană Gold Mine, including two towns (Abrud and Câmpești) and five communes (Arieseni, Bistra, Bucium, Roșia Montană and Vadu Motilor). The distances from residential households to the Roșia Montană Gold Mine range from 2.42 km to 38.59 km. The towns and communes had similar occupational, dietary, and lifestyle profiles. Participants were recruited through their family doctors at the local medical units who manage the primary care of the majority of residents of that town. Each of these towns and communes was served by only one primary care center, which supplied all primary health care and referral. Primary care physicians provided the advertising and recruitment for the study.

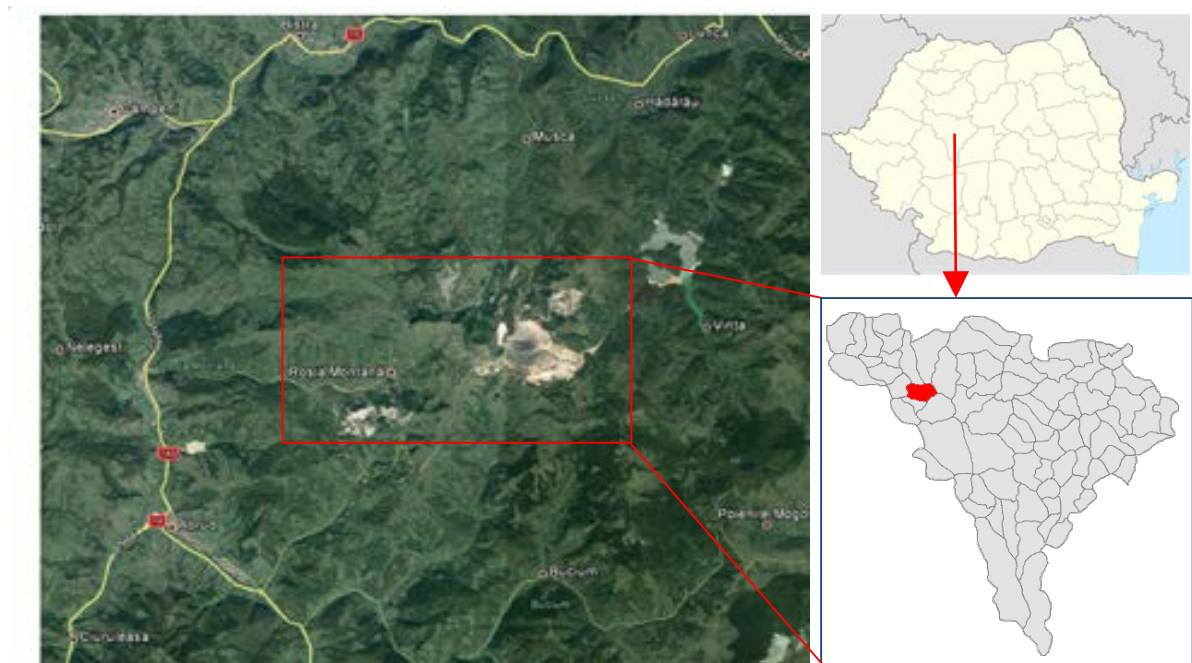


Figure 1 Location of Roșia Montană gold mine (arrow on the left map), the country of Romania (top right), and the gold mine area in Alba County (bottom right)

Clusters

The gold mining area was subdivided for this analysis into four clusters, reflecting distance from the gold mine and therefore risk of exposure to arsenic. The subdivision was based

on the distance from the gold mine area including its surrounding tailing piles, and established towns and communes. Participants were grouped into four clusters, each with small distances among the cluster members, and similar distances to the mine area. The clusters were also divided such that the majority of each cluster's members were from the same administrative division (i.e. same town or commune). Euclidean distances from households to the gold mine were calculated from GPS coordinate values. Household GPS data was collected during home visits. GPS coordinates of the gold mine area were estimated at its geometric center. The four clusters were significantly different based on distance to the gold mine ($p < 0.0001$).

Participants

Eligible participants included those individuals between the age of 18 and 65 years with longstanding residence (≥ 20 years) in the locality. Participants were excluded if they had cardiovascular, diabetic, lung diseases or skin cancers. Those who declined biological sampling (urine, nail, hair or all), follow-up home visit (i.e. including environmental sampling), or who were lost to follow-up also were excluded from this study.

As shown in Figure 2, Family doctors at local medical units made a total of 98 enquiries, to which 84 participants responded and were recruited initially. Following the self-administered questionnaire, fingernail and urine samples were collected at the medical units, except that participants who had shorter fingernails were sampled later during individual house visits. A household visit was scheduled after the initial recruitment and assessment phase. Three participants were excluded from the study analysis because they declined both fingernail and urine sampling. Eighteen participants were excluded for declining a household visit or being lost to follow-up. Participants who did not give consent for household visit were also excluded, even if they were sampled with fingernails and/or urine. 64 participants from a total of 57 households were followed up in a home visit, among whom there were 7 couples from the same households.

Apuseni Recruitment Flow diagram

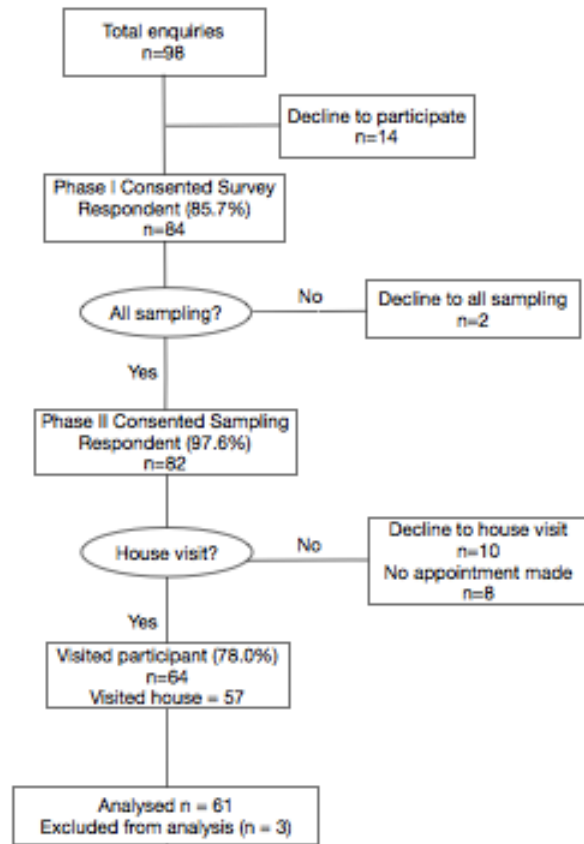


Figure 2 Recruitment Flow Chart

All 84 participants completed the questionnaire. Eighty-one urine samples and 23 fingernail samples were available for assessment, with geographic data from 64 participants. There was no significant difference in urine or fingernail arsenic concentrations between males and females or across the seven different districts. Hence urine and fingernail samples were compared among all participants.

All study participants gave informed written consent. The protocol was approved by the institutional review boards of the regional public health authority of Alba County in Romania, and qualified the exemption requirements of the Human Investigation Committee for Yale University School of Public Health.

Questionnaire

This study used a structured questionnaire collecting information on demographics, lifestyle, residential history, health history, occupational history as well as drinking water and dietary consumption. Self-reported symptoms, home garden and home cleaning practices were also collected through the structured questionnaire.

A Household Visit Checklist was used to collect environmental samples (soil, outdoor and indoor dust, and hand dust wipe), GPS data, and household information (e.g. construction year, construction material, water resources, filtration system, type of stove and heating, and number of floors, bedrooms and residents) at follow-up home visits after participants' initial appointments at the medical units in their towns or communes. The follow-up home visit was performed during the following 1-3 weeks with the consent of the participants.

Sample Collection and Analysis

Urine Sample

Urine samples for all participants were collected as spot urine samples at the time of the study. Urine was analyzed because urinary excretion is the major pathway for eliminating arsenic from the mammalian body (Vahter ME, 1988). Total urinary arsenic was measured. Speciation analysis was not performed for this study.

Sample pretreatment and mineralization: The urine samples are mineralized using a method specific for urine mineralization. They were placed into a microwave digestion system in the presence of nitric acid and hydrogen peroxide as follows: 5 ml urine + 5 ml HNO₃ + 2 ml H₂O₂.

Hydride generation atomic absorption spectrometry (HG-AAS) analysis: After mineralization, the samples were introduced in volumetric flasks which were filled up to 25 ml with ultrapure water. The analysis method was based on the measurement of the metal ion concentration of the sample by atomic absorption spectrometry. Atomic absorption spectrometry with hydride generation was used to determine arsenic concentrations, in which procedure the sample reacts as a column

reaction with sodium borohydride in an acidic environment with volatile metal hydrides forming as a result. The samples were analyzed with a Zeenit 700P atomic absorption spectrometer with a hydride generator system, using a sodium borohydride solution as reducing agent and nitric acid solution as carrier solution. After each determination set, the calibration curve was plotted. For samples analyzed with the technique based on hydrides, the capillary tube was introduced manually into the volumetric flasks with the mineralized samples, they were atomized and their absorbance was measured. After each reading, the capillary tube was rinsed with 0.5 % nitric acid solution.

Fingernail Analysis

Nail inorganic arsenic concentration is considered a stable indicator of arsenic exposure from the recent 6-12 months. Normal levels in nails are 1ppm or less (Choucair and Ajax 1988; Franzblau and Lilis 1989; ATSDR 2007) If exposed, the elevated levels of arsenic may remain 6-12 months (Choucair and Ajax 1988). Extensive washing of nails is required to remove exogenous contamination (Agahian et al., 1990).

Fingernails were clipped either using participants' own nail clippers at their home or with provided clippers. The provided clippers were wiped with cotton swabs and ethanol before and after each participant clipping fingernails. Fingernail samples were collected from all 10 fingernails. Nail clippings were secured in zip bags and transported at ambient temperature.

Sample pretreatment: Before beginning the washing process, any visible impurity from the surface of the nails had to be removed. The nails were washed with distilled water 5 times, then left in acetone for 30 minutes. They were removed from the acetone and rinsed 5 times with distilled water, followed by an overnight drying in a drying stove at 50-60 degrees Celsius (122-140 F), after which they were kept in a desiccator for 2 more hours.

Acid digestion and mineralization: The fingernail samples were weighed and mineralized using a specific method for animal tissue mineralization. The samples were mineralized in a Teflon digestion system, with nitric acid (HNO₃) in a MARS 6 microwave oven, with 600 W power.

HG-AAS analysis: After mineralization, the volumetric flasks were filled up to 50 ml with ultrapure water. The samples were analyzed with a Zeenit 700P atomic absorption spectrometer with a hydride generator system, using a sodium borohydride solution as reducing agent and nitric acid solution as carrier solution.

The analysis method is based on the metallic ion concentration determination in the sample by atomic absorption spectrometry. For arsenic determination, atomic absorption spectrometry with hydride generation is used, resulting in the reaction between the sample and the sodium borohydride in acidic environment and the generation of volatile metal hydrides. After each determination set, the calibration curve was plotted. For samples analyzed with the technique based on hydrides, the capillary tube was introduced manually into the volumetric flasks with the mineralized samples, they were atomized and their absorbance was measured. After each reading, the capillary tube was rinsed with 0.5 % nitric acid solution.

Statistical Analysis

Data were analyzed using SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA). Study population demographic characteristics, dietary intake, drinking water sources, cleaning practices, fingernail arsenic and urinary total arsenic were analyzed across distance-based clusters and tested for significance using chi-square test, Fisher's exact test, and student t-test where appropriate. The arsenic data were analyzed with respect to exposure and the four most frequently self-reported symptoms (numbness and tingling in extremities, frequent or recurring heartburn, frequent leg cramps, constant or frequent joint pains) out of 17 total symptoms in the questionnaire using a multivariate analysis of variance, controlling for age, BMI, sex, education level, income, smoking status, residence type, behavior and diet.

One-way analysis of variance (ANOVA) was used to detect significant differences across clusters. The Tukey-Kramer honest significant difference (HSD) test, based on a significance level of 0.05, was used to determine differences among clusters. Correlations between urinary

total arsenic, fingernail arsenic levels and distance to the mine were performed assuming linear relationship. All p values presented herein were determined with one-way ANOVA. Two-way ANOVA was used to examine the interaction between covariate and control for confounders. Significant differences were considered at an $\alpha=0.05$.

Regression model analysis was performed for each of the four most frequently reported symptoms. Age, gender, income and education were included in the models, regardless of significance, as these are thought to influence both behaviors, dietary and non-dietary intake of arsenic and metabolism of arsenic, although this study did not focus on the metabolic facet of arsenic. Race was not included only because all participants declared the same race-Romanian, though race/ethnicity was inquired in the questionnaire (Romanian, Hungarian, German, Gypsy).

Results

Of the total of 84 participants, we restricted our analysis to the 64 individuals (76.2%) who had complete geographic information and urine samples.

Geographic and Demographic Characteristics

The study used a simplified distance-based clustering approach to divide participants and communities into four clusters. Number of participants and households, and geometric means and standard deviation were presented in Table 1. Each cluster varies significantly in distance from the gold mine area ($p<0.0001$).

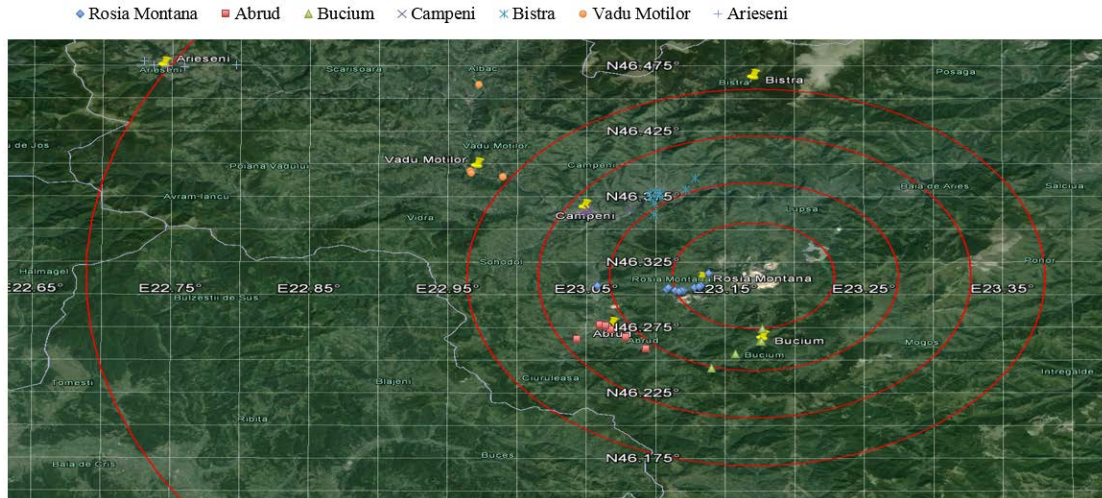


Figure 3 Roșia Montană gold mine site and areas considered in this study: yellow pins were the locations of the seven towns/communes; dots of colors represented participants/households included in the analysis; red circles were different distance radius (4.5km, 8 km, 12 km, 16 km and 37 km, respectively)

Table 1 Geometric mean distances from households to the Roșia Montană gold mine by cluster

	Cluster			
	1	2	3	4
No. of participants	12	23	17	12
No. of households	11	18	17	11
Distances				
Mean	2.42	7.94	9.39	28.46
STD	0.83	1.81	1.10	1.03
Min	2042	4.55	7.62	16.26
Max	4.93	11.16	10.93	38.59

Demographic characteristics are summarized in Table 2 for participants from different distance-based clusters. The participants within each cluster were not significantly different in terms of age, BMI, gender, education level, household income, smoking status, and residence type (house or apartment).

Table 2 Demographics for residents living near gold mine area by distance-based cluster ^a

Characteristics	Distance-based cluster				p ^c
	1 ^b	2 ^b	3 ^b	4 ^b	
Age (Years) ^a	44.2 ± 12.3	46.5 ± 20.8	50.0 ± 17.4	41.2 ± 14.4	0.6192
BMI ^a	23.9 ± 3.0	26.9 ± 4.0	25.6 ± 3.8	27.0 ± 3.8	0.1584
Gender					0.3479
Male	5 (41.7)	9 (39.1)	2 (14.3)	5 (41.7)	
Female	7 (58.3)	14 (60.9)	12 (85.7)	7 (58.3)	
Education level					0.1817
Secondary	0 (0.0)	3 (13.0)	0 (0.0)	0 (0.0)	
High	1 (9.1)	9 (39.1)	6 (46.2)	3 (27.3)	
College	9 (81.8)	11 (47.8)	6 (46.2)	8 (72.7)	
Graduate	1 (9.1)	0 (0.0)	1 (7.7)	0 (0.0)	
Household income					0.2064
<500	0 (0.0)	0 (0.0)	2 (16.7)	2 (16.7)	
500 – 1000	1 (9.1)	2 (13.3)	0 (0.0)	1 (8.3)	
1000 – 2000	1 (9.1)	6 (40.0)	6 (50.0)	3 (25.0)	
>2000	9 (81.8)	7 (46.7)	4 (33.3)	6 (50.0)	
Smoke					0.9982
Current smoker	2 (16.7)	4 (20.0)	2 (14.3)	2 (16.7)	
Former smoker	3 (25.0)	6 (30.0)	4 (28.6)	4 (33.3)	
Never smoked	7 (58.3)	10 (50.0)	8 (57.1)	6 (50.0)	
Residence type					0.1415
House	9 (75.0)	17 (73.9)	13 (92.9)	12 (100.0)	
Apartment	3 (25.0)	6 (26.1)	1 (7.1)	0 (0.0)	

^aTable values are mean ± SD for continuous variables and n (column %) for categorical variables.

^bNumbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.

^cP-value is for ANOVA (continuous variables) or chi-square/Fisher's exact test (categorical variables).

Table 3 summarizes leafy vegetable consumption and drinking water source, as well as whether the participants had a vegetable gardens at their household or not. Reported dietary intake habits (e.g. consumption of leafy vegetable, frequency of vegetable intake, and consumption of fish from local water) were not significantly different among clusters. The major drinking water source differed substantially across clusters (p=0.0112), as shown in Table 3. Participants from cluster 1 predominantly used non-well water as their main drinking water

source, with only two participants reported using all three sources. In contrast, cluster 3 has the lowest proportion (14.3%) of participants reporting use of tap water, but the highest proportion (71.4%) reporting use of well water as main drinking water source. Cluster 2 and cluster 4 have similar proportions of residents who reported using well water (40.9% and 40.0%, respectively). No participants from cluster 3 or 4 reported they used bottled water as a main drinking water source. Main household water supply was not analyzed in this study. Cleaning practices (Table 4) at participants' households were similar among all four clusters, except that floor wet mopping frequency was higher in cluster 2 and 4 (50.0% and 60.0% of participants did mopping everyday, $p=0.024$), and lower in cluster 1 and 3 (10.0% and 15.4% mopping daily).

Table 3 Diet and drinking water for residents living near the gold mine areas by cluster^a

Characteristics	Distance-based cluster				P ^b
	1 ^a	2 ^a	3 ^a	4 ^a	
Vegetable garden					0.1967
Yes	5 (45.4)	9 (50.0)	13 (71.4)	9 (81.8)	
No	6 (54.6)	9 (50.0)	4 (28.6)	2 (18.2)	
Drinking water source					0.0112
Tap water	6 (50.0)	8 (36.4)	2 (14.3)	6 (60.0)	
Well water	0 (0.0)	9 (40.9)	10 (71.4)	4 (40.0)	
Bottled water	4 (33.3)	3 (13.6)	0 (0.0)	0 (0.0)	
All	2 (16.7)	2 (9.1)	2 (14.3)	0 (0.0)	
Eat fish in local water					0.0965
Yes	5 (41.7)	5 (25.0)	9 (69.2)	5 (45.5)	
No	7 (58.3)	15 (75.0)	4 (30.8)	6 (54.6)	
Eat leafy vegetable					0.3293
Yes	11 (91.7)	11 (64.7)	8 (61.5)	8 (72.7)	
No	1 (8.3)	6 (35.3)	6 (38.5)	3 (27.3)	
Vegetable frequency					0.6216
Less often	3 (25.0)	4 (19.1)	6 (42.9)	4 (33.3)	
Monthly	2 (16.7)	1 (4.8)	2 (14.3)	0 (0.0)	
Weekly	3 (25.0)	9 (42.9)	2 (14.3)	4 (33.3)	
Daily	4 (33.3)	7 (33.3)	4 (28.6)	4 (33.3)	

^a Numbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.

^b P-value is for chi-square/Fisher's exact test (categorical variables).

Table 4 Cleaning practices for residents living near the gold mine areas by cluster ^a

Characteristics	Distance-based cluster				p ^b
	1 ^a	2 ^a	3 ^a	4 ^a	
Cleaning frequency					0.3253
Everyday	4 (33.3)	10 (43.5)	7 (50.0)	9 (75.0)	
3 times/ week	5 (41.7)	6 (26.1)	2 (14.3)	1 (8.3)	
Once/week	3 (25.0)	6 (30.4)	5 (28.6)	1 (8.3)	
Less than once/week	0 (0.0)	0 (0.0)	1 (7.1)	0 (8.3)	
Vacuum					0.7811
Yes	11 (91.7)	21 (91.3)	13 (92.9)	12 (100.0)	
No	1 (8.3)	2 (8.7)	1 (7.1)	0 (0.0)	
Vacuum frequency					0.1903
Everyday	1 (8.3)	3 (14.3)	5 (35.7)	1 (8.3)	
3 times/ week	6 (50.0)	6 (28.6)	1 (7.18)	3 (25.0)	
Once/week	4 (33.3)	12 (57.1)	7 (50.0)	8 (66.7)	
Less than once/week	1 (8.3)	0 (0.0)	1 (7.1)	0 (0.0)	
Wet mop floor					0.4345
Yes	11 (100.0)	23 (100.0)	13 (92.9)	11 (91.7)	
No	0 (0.0)	0 (0.0)	1 (7.1)	1 (8.3)	
Wet mop frequency					0.0240
Everyday	1 (10.0)	11 (50.0)	2 (15.4)	6 (60.0)	
3 times/ week	7 (70.0)	5 (22.7)	8 (61.5)	2 (20.0)	
Once/week	2 (20.0)	1 (4.6)	1 (7.7)	0 (0.0)	
Less than once/week	0 (0.0)	5 (22.7)	2 (15.4)	2 (20.0)	
Wet wipe window seal					0.2542
Yes	11 (100.0)	23 (100.0)	14 (100.0)	11 (91.7)	
No	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	
Wet wipe frequency					0.2274
Everyday	1 (8.3)	1 (4.6)	1 (7.1)	0 (0.0)	
3 times/ week	5 (41.7)	16 (72.7)	3 (21.4)	6 (54.6)	
Once/week	3 (25.0)	2 (9.1)	3 (21.4)	1 (9.1)	
Less than once/week	3 (25.0)	3 (13.6)	7 (50.0)	4 (36.4)	
Ventilation frequency					0.5823
Everyday	10 (83.3)	11 (50.0)	8 (61.5)	7 (58.3)	
More than once/ week	2 (16.7)	7 (31.8)	4 (30.8)	3 (25.0)	
Less than once/week	0 (0.0)	2 (9.1)	0 (0.0)	0 (0.0)	
During specific activity	0 (0.0)	2 (9.1)	1 (7.7)	2 (16.7)	

^a Numbers may not sum to total due to missing data. percentages may not sum to 100% due to rounding.

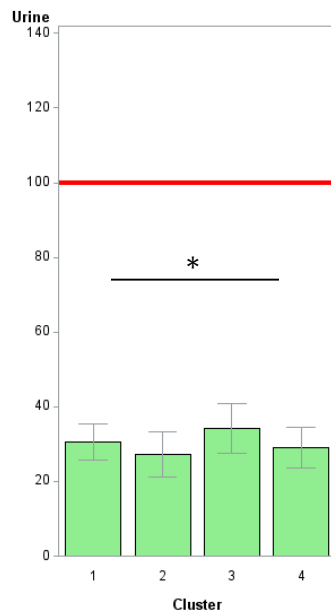
^b P-value is for chi-square/Fisher's exact test (categorical variables).

Urinary and Fingernail Arsenic

In our study, there were 3 urinary total arsenic samples excluded from further analysis due to a larger than two standard deviation on a normal distribution of urinary total arsenic values. The biomarkers indicated recent exposure and prolonged exposure (Figure 4). All urinary

total arsenic levels for the 64 participants tested were below the “normal human level” of 100 $\mu\text{g/L}$ in urine indicated by ATSDR. But all fingernail samples had much higher arsenic levels than the 1 $\mu\text{g/g}$ normal level. Urinary total arsenic concentrations ranged from 3.02 to 53.49 $\mu\text{g/L}$ (geometric mean \pm standard error: 29.81 \pm 11.56 $\mu\text{g/L}$). Fingernail arsenic levels ranged from 3.54 to 18.61 $\mu\text{g/g}$ (11.67 \pm 3.75 $\mu\text{g/g}$), with a normal distribution slightly skewed to the left. Among the 64 participants 93.4% had lower than 50 $\mu\text{g/L}$ urinary arsenic level, which is classified as the “normal category” based on CDC’s National Health and Nutrition Examination Survey (NHANES) urinary arsenic categories (≤ 50 $\mu\text{g/L}$ as normal, >50 to <200 $\mu\text{g/L}$ as high normal, and ≥ 200 $\mu\text{g/L}$ as high) (Flanagan et al., 2012). However, we did not observe a correlation between urinary total arsenic and fingernail arsenic ($n=23$, $r=-0.13$, $p=0.54$).

Average urinary total arsenic level by Cluster
With 95% Confidence Limits



Average fingernail arsenic level by Cluster
With 95% Confidence Limits

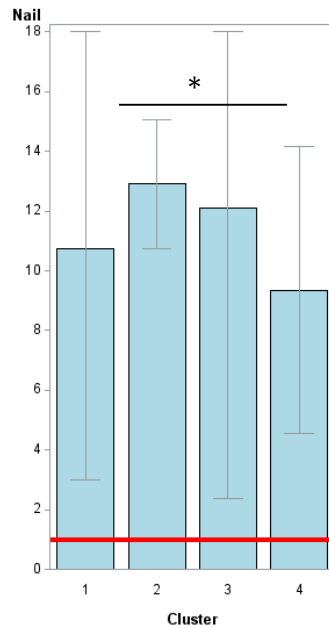


Figure 4 Urinary total arsenic and fingernail arsenic concentrations by Cluster: showing within cluster mean concentration of urinary and fingernail arsenic compared to “Normal human levels” indicated by ATSDR, <math><100\ \mu\text{g/L}</math> in urine and $\leq 1\ \mu\text{g/g}</math> in nail (: $p<0.05</math> for difference between total arsenic and standard value)$$*

Vicinity

Summary statistics of each biological matrix by distance-based cluster are shown in Table 5. Mean urinary total arsenic concentration was the highest in cluster 3, nearly two-fold as high as the mean urinary total arsenic concentration of cluster 2. One-way analysis of variance (ANOVA) was performed, using cluster group as the independent variable and urinary and fingernail arsenic concentrations as dependent variables, to detect significant differences across clusters. The urinary total arsenic ($p=0.36$) and the fingernail arsenic ($p=0.34$) did not vary significantly among clusters. Pearson correlation analysis was also performed, using distance to the mine as a continuous variable. No correlations were observed between distance and either of these measures, urinary total arsenic ($r=-0.06$, $p=0.67$), the fingernail arsenic levels ($r=-0.34$, $p=0.12$). Participants from cluster 2 had the lowest mean urinary total arsenic level ($27.19 \pm 14.17\ \mu\text{g/L}$), and those from cluster 4 had the lowest fingernail arsenic concentrations ($9.35 \pm$

3.87 μ g/g). Neither the total urinary arsenic nor the fingernail arsenic levels varied significantly with age (p=0.62, p=0.49), BMI (p=0.62, p=0.74), sex (p=0.16, p=0.41), education (p=0.51, p=0.95), income (p=0.37, p=0.82), cigarette smoking (p=0.42, p=0.92), residence type (p=0.29, p=0.10), or occupation i.e. whether working on the mine or not (p=0.88).

Table 5 Geometric mean urinary total arsenic (μ g/L) and nail arsenic (μ g/g) by Cluster from the Roşia Montană Gold Mine area (GM \pm STD)

	Distance-based cluster				p
	1	2	3	4	
No. of ppl	12	23	14	12	
Urine	30.65 \pm 7.60	27.19 \pm 14.17	34.11 \pm 11.54	28.97 \pm 8.56	0.36
Fingernail	10.73 \pm 4.87	12.91 \pm 3.42	12.09 \pm 1.08	9.35 \pm 3.87	0.34

Drinking Water

Main drinking water source in this study includes tap water, well water, bottled water and all sources. The urinary and fingernail arsenic values were not significantly different among drinking water sources as shown in Figure 5 (p=0.90 and p=0.089, respectively). Nor was an association observed between urine/nail ratio and the main drinking water source (p=0.62). Participants who used well water as the main drinking water source had the highest fingernail arsenic level (14.0 \pm 2.4 μ g/L), compared to participants who used tap water (12.3 \pm 4.2 μ g/L) and bottled water (8.5 \pm 1.1 μ g/L), and those who reported using all sources (9.5 \pm 1.3 μ g/L). The difference between drinking well water and tap water was more obvious in fingernail arsenic concentrations than urinary arsenic concentrations. The mean urinary arsenic concentration was 30.0 \pm 11.0 μ g/L among those who used tap water, as compared to 29.6 \pm 12.4 μ g/L of those using well water, 33.3 \pm 8.4 μ g/L of those using bottled water and 30.5 \pm 13.0 μ g/L of those using all sources.

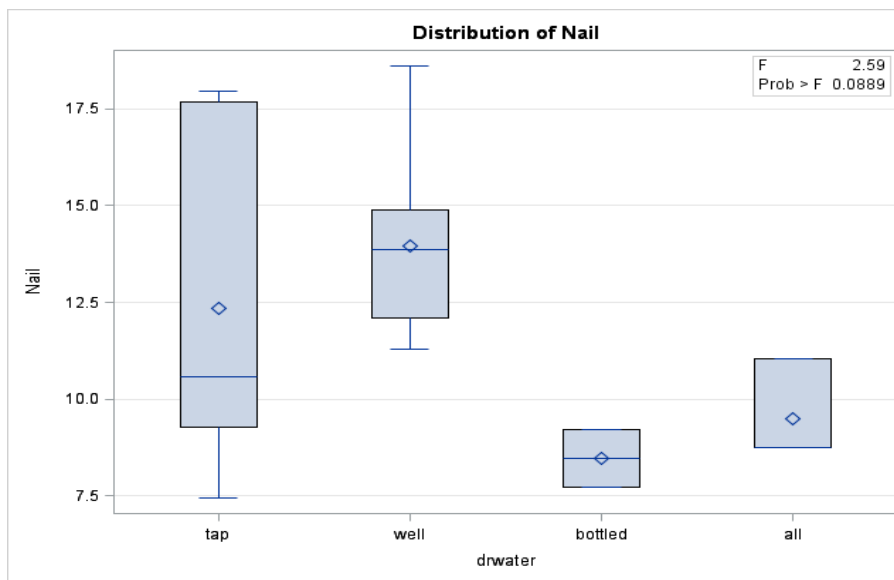
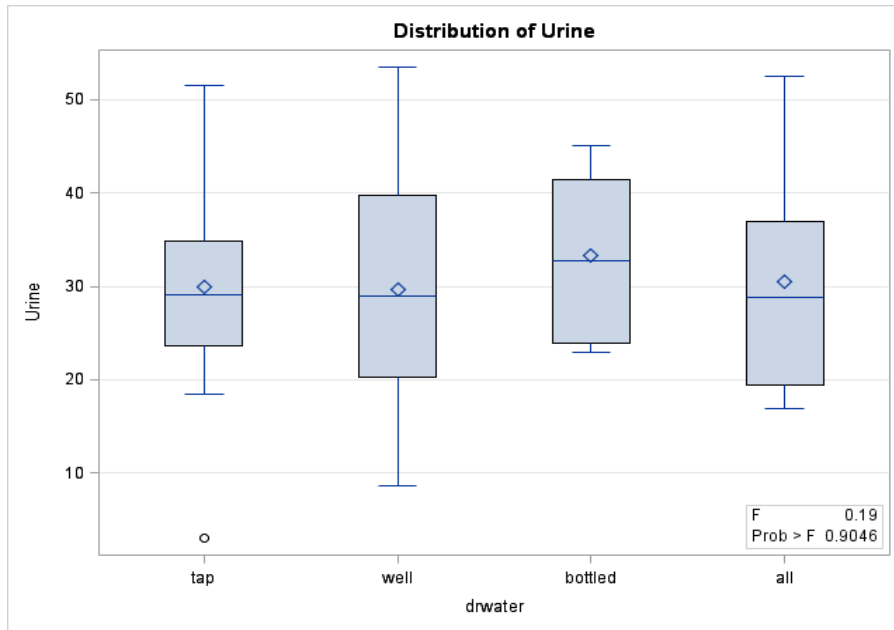


Figure 5 Urinary total arsenic (n=61) and fingernail arsenic (n=23) by drinking water sources: one-way ANOVA showing effect of drinking water source

Diet and Cleaning Practices

The dietary intake of leafy vegetable and fish from local water, as well as vegetable consumption frequency and vegetable washing before eating did not have significant effects on either urinary total arsenic or fingernail arsenic (all p values >0.05). Similarly, the urinary total arsenic and fingernail arsenic concentrations were not influenced by different cleaning practice

habits, including vacuum, floor wet mopping, window seal wet wiping, as well as frequencies of cleaning, vacuum, floor wet mopping, window seal wet wiping and ventilation (all p values >0.05).

Symptoms, Exposure and Vicinity

For this study, all participants were recruited based on overall good health. There were 78.7% of the participants who had a self-perceived above “Good” health status (Good, very good, and excellent). Out of the total twenty-seven symptoms (answered with yes/no) in the questionnaire, four symptoms were most frequently reported among all the participants, including numbness and tingling of the extremities, frequent and reoccurring heartburns, frequent leg and muscle cramps, and frequent pains in the joints. These four symptoms were specifically looked at because of their higher reported frequencies. The number of symptoms was not significantly differentiated between male and female ($p=0.40$), by self-perceived health status ($p=0.12$), or by the self-perceived knowledge level of arsenic exposure ($p=0.38$). The self-reported symptoms did not vary significantly among age ($p=0.08$) and BMI ($p=0.77$) groups, with different education levels ($p=0.92$), occupation ($p=0.16$) or smoking status ($p=0.15$). Although there were no significant differences in terms of the number of self-reported symptoms between male and female groups, a higher percentage of females (72.2%) reported having symptoms compared to men (56.0%) ($p=0.19$).

The multivariate ANOVA was performed using categorized number of self-reported symptoms as the dependent variable (categorized by number of symptoms, i.e. no symptoms, one symptom, and more than two symptoms), and urinary arsenic and fingernail arsenic as independent variables (Table 6). There was no association between the number of symptoms and the arsenic levels in urine or fingernail ($p=0.27$ and $p=0.29$, respectively). Participants who reported experiencing one symptom had both the highest urinary total arsenic ($33.46 \pm 13.30 \mu\text{g}/\text{L}$) and fingernail arsenic ($12.52 \pm 2.47 \mu\text{g}/\text{g}$) levels.

Table 6 Geometric mean urinary total arsenic ($\mu\text{g/L}$) and fingernail arsenic ($\mu\text{g/g}$) by number of symptoms ($GM \pm STD$)

No. of symptoms	No. obs	Urinary arsenic	Nail arsenic
0	25	27.45 \pm 9.57	10.99 \pm 3.52
1	16	33.46 \pm 13.30	14.07 \pm 3.79
≥ 2	20	29.83 \pm 12.18	11.07 \pm 3.91
P value		0.27	0.29

The pattern of the numbers of self-reported symptoms was not substantially different among clusters ($p=0.49$). When using distance as a continuous variable, the means of distances varied significantly as the number of symptoms changed ($p=0.092$). Overall, as the number of symptoms increased, the average distances from residences to the gold mine areas also increased. The distances from households to the mining area, urinary and fingernail arsenic as continuous factors of self-reported symptoms were further analyzed using a simple logistic regression and multivariate logistic regression analysis adjusting for influential factors including age, BMI, sex, education level, income, smoking status, self-perceived health status, and self-perceived knowledge on arsenic exposure risk. Results were expressed as ratios of the least squared means of arsenic concentration ($\mu\text{g/g}$) in Table 7. After controlling for all the other covariates, there was a small, but significantly ($p=0.02$) increased risk of self-reported frequent leg and muscle cramps as the urinary total arsenic level increased (1.14, CI: 1.01,1.29).

Table 7 Odds ratios of self-reported symptoms with urinary total arsenic, fingernail arsenic and distance by the most frequently self-reported symptoms

Symptoms	Mean ^a	Urinary arsenic		Mean	Fingernail arsenic		Mean	Distances	
		Crude ^b	Adjusted ^c		Crude	Adjusted		Crude	Adjusted
Extremity Numbness									
Yes	32.5±16.9	1.00 (0.96,1.05)	1.01 (0.94,1.09)	13.2±4.2	1.09 (0.89, 1.33)	0.97 (0.72, 1.31)	32.5±16.9	1.04(0.99,1.10)	1.13 (0.98, 1.31)
No	35.3±27.1	1.00	1.00	10.9±3.3	1.00	1.00	35.3±27.1	1.00	1.00
Frequent heartburn									
Yes	35.6±20.5	1.02 (0.97, 1.07)	0.98 (0.90,1.06)	11.0±3.5	0.91 (0.72, 1.15)	0.87(0.64, 1.17)	35.6±20.5	1.04(0.98,1.10)	1.04 (0.91, 1.20)
No	33.7±24.6	1.00	1.00	11.8±3.8	1.00	1.00	33.7±24.6	1.00	1.00
Frequent leg cramps									
Yes	36.5±24.3	1.02 (0.97,1.07)	1.14 (1.01,1.29)	12.2±4.2	1.08 (0.87, 1.34)	1.31(0.88, 1.94)	36.5±24.3	1.07(1.01,1.14)	1.01 (0.88,1.16)
No	33.4±23.5	1.00	1.00	11.8±3.8	1.00	1.00	33.4±23.5	1.00	1.00
Constant joint pains									
Yes	31.3±12.8	1.02 (0.96,1.07)	1.02 (0.94,1.10)	10.0±2.5	0.97 (0.77, 1.23)	1.10(0.78, 1.54)	31.3±12.8	1.03(0.97, 1.09)	1.02 (0.88, 1.17)
No	34.9±25.6	1.00	1.00	12.2±3.9	1.00	1.00	34.9±25.6	1.00	1.00
Symptoms (Y/N)									
Yes	35.4±21.0	1.03 (0.99,1.08)	1.04 (0.95,1.13)	12.6±3.9	1.08 (0.89, 1.32)	1.03 (0.74, 1.45)	35.4±21.0	1.03(0.97, 1.09)	1.07 (0.92, 1.23)
No	32.5±27.2	1.00	1.00	11.0±3.5	1.00	1.00	32.5±27.2	1.00	1.00

^a Column values are mean ± SD, p-value for Logistic regression was not presented in this table.

^b Ratio were calculated using No symptom as reference group.

^c Adjusted ratio was adjusted for age (18-35, 36-55, >55), BMI, education, income, smoking status, self-perceived health status, knowledge of arsenic exposure.

Discussion

The Roşia Montană gold and silver mining project in Romania's Apuseni Mountains has been in and out of the environmental headlines in recent years (Egresi et al., 2011). In the community around the historic Roşia Montană gold mine site, exposures to environmental pollutants are of concern. The assessment in this study was based on both short- and long-term biomarkers that inform all-source arsenic exposure from environmental routes such as soil, dust, water, and dietary intake, as well as health behaviors and practices.

In our study, the high arsenic concentrations in fingernail samples are especially of concern due to the levels of fingernail arsenic significantly above the normal human standard of 1 µg/g. Nail arsenic levels greater than 1 µg/g are indicative of excessive long-term exposure to arsenic. However, in contrast to the higher than normal fingernail samples, urinary total arsenic levels were all below the normal standard of 100 µg/L (ATSDR 2007). The urinary total arsenic levels detected from participants in our study were consistent with the 4th National Report on Human Exposure to Environmental Chemicals, which reported that the 95th percentile for total urinary arsenic and the sum of inorganic-related arsenic as 65.4 and 18.9 µg/L respectively for all participants aged 6 years or older, based on the 2003-2004 NHANES survey data (CDC 2009), indicating that there was no high arsenic exposure in the historic mining area.

We did not find a significant correlation between urinary total arsenic and fingernail arsenic concentrations. Instead, our data demonstrate an apparent very low-level, on-going, all-source arsenic exposure together with a high historic arsenic exposure. The data indicate that the extremely elevated concentrations of fingernail arsenic are the result of the cumulative effect from long-term environmental exposure. Total urinary arsenic concentrations suggest less elevated risk of recent and present arsenic exposure from all routes in the participants living in the vicinity of the Roşia Montană gold mine. Previous studies have shown positive significant correlations between urinary and nail arsenic, and environmental measurements and arsenic levels

in urine and fingernails, particularly among those with higher level of arsenic exposure (Hughes et al., 2006; Rapant et al., 2006; Karagas et al., 2001). On the other hand, the fact that the urinary arsenic did not correlate with fingernail arsenic well was also indicative that the environmental exposure was at a low level. Karagas et al. (2001) showed a better correlation between urinary and nail arsenic concentrations when the exposure level was higher ($r=0.42$, $p=0.044$) compared to the low-level exposure ($r=0.25$, $p=0.071$). Her study also showed that the correlation between urinary arsenic and drinking water arsenic was higher with elevated arsenic exposure from drinking water ($r=0.46$, $p=0.029$) as compared to low arsenic concentration in water ($r=0.02$, $p=0.90$). Garland et al. (1993) and Michaud et al. (2004) showed that toenail arsenic levels remained relatively constant over 6 years in 127 women, and reproducible over a three to five years period. Karagas's study also confirms the long-term reproducibility of toenail measures in populations exposed to low levels of arsenic. In this case, fingernail arsenic concentration may be a better predictor of arsenic environmental exposure than urinary total arsenic concentration in this area with a specific exposure profile - low-level, on-going, all-source and high historic arsenic exposure, and may more effectively convey information regarding the key dimension of arsenic exposure in this study area.

Less is known about arsenic concentrations in the environment. Our data do not shed light on the underlying reason that the vicinity to the historic mine was not associated with on-going arsenic exposure, but instead highlight that all of these communities had historically high exposure. People living within 38 km distance to the gold mine area still retain a body burden of arsenic that is reflected in the elevated fingernail arsenic. Our study used residential proximity to the gold mine as the proxy of potential for exposure to arsenic from all sources. It has been well established in studies that proximity to a point source is significantly correlated with the intensity of environmental exposure (Hogervorst et al., 2006; Su et al., 2009). Overall, we did not find evidence that people living closer to the mine had higher exposure to arsenic compared to those who lived farther away. The primary hypothesis of this study that living in the vicinity of the

historic gold mine increased the risk of on-going arsenic exposure from the surrounding environment is not supported. The absence of a decreasing pattern with distance also indicates that there are other risk factors that were not captured in the distance-surrogate exposure scheme. In addition, the inactive mining activities since the 2006 project cessation also explains the present low all-source environmental arsenic exposure and low urine arsenic levels. It is likely that arsenic exposure may have been decreasing longitudinally since the mining activities ceased in 2006, thus arsenic levels in urine and fingernails have been reduced appreciably compared to those during the active mining period. The current study period may function as a washout phase, should the mine start to operate again. Studies in former or current gold mining areas worldwide where there are high environmental arsenic concentrations have reported elevated arsenic levels in biomarkers of both urine and nails (Hinwood et al., 2003; Basu et al., 2011; Rapant et al., 2006).

With the assessment both across clusters and within clusters, drinking water source did not have significant effects on either urinary total arsenic or fingernail arsenic. All the participants have lived over 20 years in the same town/commune at the time of this study, which suggests that they've been consistently exposed to either historically high levels of arsenic or present low levels of arsenic. Previous studies have already proved that level of arsenic exposure by drinking water was a significant predictor of arsenic levels in nail (Schmitt et al., 2005; Slotnick et al., 2007). Elevated fingernail arsenic levels in our study suggest long-term arsenic exposures, likely from the drinking water source. The low-level urinary arsenic concentrations suggest currently there is less likely an excessively high arsenic exposure from the drinking water. Since there are only 23 fingernail samples available for analysis, we do not have the statistical power to prove drinking water as a significant predictor ($p=0.089$). However, we found a decreasing pattern in fingernail arsenic in residents who used well water, tap water and bottled water as their principal drinking water source. Important to our assessment and interpretation of the relationship between various drinking water sources and arsenic concentration in the

biomarkers was the inter-cluster difference in the utility of drinking water sources. Romania started its Municipal Services Project a decade ago, and is still in the process of improving the coverage of municipal water supply and wastewater services (World Bank 2006). Residents from different towns and communes may predominantly use one or two types of drinking water sources, for example, residents in Arieseni, the farthest commune from the gold mine, all use well water as major drinking water source; while residents from Rosia Montana, the closest town to the gold mine, predominantly use tap water and bottled water. This might also explain the absence of exposure decay pattern with distance to the gold mine. A survey study of a population exposed to high concentrations of arsenic in well water in Alaska found significant correlation between well water arsenic levels and levels of arsenic in urine ($r = 0.58, p < 10^{-8}$) among well-water drinkers. Schmitt et al. (2005) found a significant correlation was observed between toenail and well water arsenic ($r=0.84, p<0.0001$). There is some trend in terms of the relationship between fingernail arsenic concentration and drinking water sources, but a significant proportion of the variation in nail arsenic concentration remains unexplained by drinking water source alone. A case control study used cumulative water consumption estimated by multiplying volume and frequency of intake and cumulative fluid consumption by adding up all individual beverage intake, to examine the effect of increased intake, whether it increased exposure or lowered the risk by more frequent urine excretion, especially in low-level arsenic exposure environment (Michaud et al., 2007).

Although dietary intake may influence arsenic level in human fingernails, we did not find a significant effect of dietary intake and habits on arsenic levels in the two biomarkers. Drinking water and food together typically account for 99% of the total human arsenic intake (Jones, 2007). The dietary intake of leafy vegetables can be both protective and risky in terms of arsenic exposure. The uptake of metals from soils by garden vegetables (and their subsequent consumption) has been recognized as a potential indirect human exposure pathway and is a function of both the transfer of arsenic from soil to garden produce, and the consumption rate of

that produce (Bacigalupo et al., 2012). Exposure to arsenic via vegetable consumption can also be increased through cooking (Ramirez-Andreotta et al., 2013). Del Razo et al. (2002) reported that high arsenic concentration in water used for cooking is an additional source of inorganic arsenic exposure. Since little is known about the concentrations of arsenic in soil and water, potential contamination in food, or the speciation of urine arsenic, we do not have the information on the form of arsenic absorbed based on the total urinary arsenic concentration alone. On the other hand, leafy vegetables could be protective against arsenic exposure in that folate enhances the detoxification (methylation) process of arsenic to MMA and DMA, which are more easily excreted in urine (Gamble et al., 2007). Several randomized control trials from the Health Effects of Arsenic Longitudinal Study (HEALS) have proved dietary supplementation with folic acid increased arsenic methylation and lowered blood arsenic concentration (Gamble et al., 2006; Gamble et al., 2007).

In our study, vegetables consumption (and fish consumption) did not demonstrate a significant effect on arsenic levels in the biomarkers. As a population with low seafood consumption, the absolute levels of total urinary arsenic in this study were also in accordance with Norin and Vahter's study of a population with no intake of seafood arsenic, which reported urinary arsenic concentrations of 5-50 $\mu\text{g/L}$ in subjects with no intake of seafood arsenic and no excessive exposure to inorganic arsenic in drinking water or in the working environment (Norin and Vahter 1981). In general, participants living in the study area maintain similar profile in their daily diet, lifestyle, and years of residence in the same region. There was less likely to be significant variations in biomarker arsenic concentrations with dietary intake or cleaning practices. To better understand the inter-individual differences, further speciation analysis and future sample collection are warranted to study the inter-individual differences in the forms of arsenic absorbed, the underlying arsenic detoxification, the metabolites partitioning and the accumulation and excretion rate.

Our study also found a significant increased risk of self-reported frequent leg cramps with the increasing urinary total arsenic concentration. Though the odds ratio was the only one that was significant, and the confidence interval was very close to 1, the incidence of self-reported leg cramps did associate with urine indicated arsenic exposure. Komulainen et al. (1998) study on 35 current users and 12 former users of drilled wells in Finland also found a higher prevalence of muscle cramps among those with high arsenic exposure and an over 3-fold level of urinary total arsenic in current users (Komulainen et al., 1998).

Limitations

Our study was faced with the problem of missing fingernail data. The small sample size may diminish the statistical power of this study. Inaccurate self-reported symptoms may introduce response bias. The environmental exposure data were not available for our study, so this study does not reveal the chemical form of arsenic to which subjects were exposed. Using distance-based cluster as a proxy for risk of arsenic exposure has its inherent limits because of the arbitrarily selected cutoff points. It also limits understanding of the complex interaction among multiple other covariates. Speciation analysis was not performed in this study, which limits the understanding of inter-individual variability, and the underlying metabolic explanation for the high fingernail arsenic and normal urinary arsenic. More data are needed that tie biomarkers of absorbed dose, especially urinary concentrations of arsenic metabolites to arsenic exposure concentrations, tissue concentrations (Gebel et al., 2002; Vahter et al., 2002).

This study used fingernails instead of toenails as indicators of long-term arsenic exposure. Measurement of arsenic in fingernails has limitations when it comes to quantify internal exposure due to non-definitive exclusion of potential external exposure. Thus the major disadvantage of using fingernails as biomarkers of arsenic exposure is that it is difficult to distinguish between arsenic incorporated into the fingernails from systemic circulation and that bound externally, such as from water, dust, and soil. Arsenic level in toenails may be preferred to

assess arsenic exposure, because they are exposed less extensively to outdoor air or water than are fingernails (Karagas et al., 2000). It is normal for some arsenic to be present in nails, as everybody is exposed to trace amounts of arsenic from the normal diet. Arsenic concentrations in fingernails, at least to a small extent and depending upon quality of sample preparation, can be regarded as markers of external exposure as well as markers of absorbed dose. The variation in accumulation in fingernails between individuals is unknown.

In addition, our study did not adjust urinary total arsenic concentration with creatinine concentration. We cannot exclude the possibility of a dilution effect which could result in lowered urinary total arsenic levels possibly caused by large amount of fluid intake before the urine sampling. Urine flow is highly variable, and dependent on multiple factors, such as BMI, body water content, solute intake, physical activity, and diurnal variations (Diamond 1988).

Conclusion

We found high arsenic concentrations in fingernail samples, which were significantly above the normal human standard of 1 $\mu\text{g/g}$. In contrast, urinary total arsenic levels were all below the normal standard of 100 $\mu\text{g/L}$. The finding suggests excessive long-term historic exposure to arsenic, and a current low-level ongoing arsenic exposure. Overall, we did not find evidence that people who live closer to the mine had higher exposure to arsenic compared to those living farther away. Drinking water source, dietary intake of leafy vegetable and fish, vegetable consumption frequency and vegetable washing before eating, and cleaning practices did not have significant effects on either urinary total arsenic or fingernail arsenic. Therefore, the major contributors did not explain the current exposure profile in the Roşia Montană gold mine area. However, our study also provides evidence that people living within 38 km from historic gold mines still retain a body burden of arsenic exposure that is not reflected in elevated urine total arsenic. An increased risk of self-reported frequent leg cramps associated with increased urinary total arsenic level was also noted. This finding may add weight to the concern of current

low-level on-going universal arsenic exposure in the gold mining area. Overall, these findings suggest fingernail arsenic concentration may be a better predictor of arsenic environmental exposure than urinary total arsenic concentration in this area with a specific exposure profile - low-level, on-going, all-source and high historic arsenic exposure. Using both fingernail and urinary arsenic concentrations may offer an opportunity for a more effective approach to capture smaller adverse effects from low-level exposure, and predict and convey information regarding long-term exposure burden after overall environmental arsenic exposure has diminished.

Given the potential for chronic arsenic exposure and a retained body burden of arsenic, arsenic should be monitored regularly and remediation should be addressed. Longitudinal follow-ups of these participants is warranted to ascertain if urinary arsenic concentrations and fingernail concentrations decrease over time as the gold mine remains inactive. Although the study sample size was small, generalizing the study results across the locale is reasonable considering the non-differentiated low all-source arsenic exposures and homogenous demographic characteristics, diet, lifestyle and practices. A systematic testing of drinking water, as well as sources of soil and air contaminations for the presence of arsenic should be a high priority for the public health authority. Determining the underlying metabolic partitioning patterns may help explain inter-individual variation, identify those more highly exposed and the variation in the susceptibility to arsenic-induced health hazards among healthy individuals, and to inform future exposure assessment regardless of the cessation or proceeding of mining activities.

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