Arsenic exposure and early biomarkers of cardiovascular disease and cancer

Ameer, Syeda Shegufta

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Syeda Shegufta Ameer

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Faculty opponent
Ulla Vogel

LUND UNIVERSITY
Division of Occupational and Environmental Medicine
Department of Laboratory Medicine

Syeda Shegufta Ameer

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Abstract: Inorganic arsenic exposure through drinking water is a serious public health concern because of its association to cancer and non-cancer diseases. More than one hundred million people world-wide are exposed to elevated levels of arsenic on a regular basis. Arsenic is classified as class I carcinogen by International Agency for Research on Cancer (IARC). Recent report shows that arsenic exposure via drinking water is associated with increased risk of cardiovascular disease and diabetes. Several mechanisms of arsenic-related toxicity have been suggested, among those are genotoxicity and epigenetic modifications affecting gene expression. The aim of this thesis was to identify early biomarkers for arsenic-related cancer and cardiovascular disease, as well as, to analyse changes in gene expression and DNA methylation related to chronic arsenic exposure.

The study participants were from the Salta Province of northern Argentina, a region known to have areas with arsenic in drinking water. Two groups of study subjects, one residing in Puna area of Andes mountains (~4000 meters above the sea level) here called as Andes, and another residing in Salta plains (~300 meters above sea level) called Chaco, were studied. Arsenic exposure was assessed as the sum of inorganic arsenic and its metabolites, inorganic arsenic (iAs), methylarsonic acid (MMA) and dimethylarsinic acid (DMA) in urine, measured by high performance liquid-chromatography hydride-generation inductively-coupled-plasma-mass-spectrometry (HPLC-HG-ICPMS). The arsenic metabolism efficiency was assessed by the urinary fractions (%) of individual metabolites. To evaluate cardiovascular health, blood pressure was measured and homocysteine concentration and lipid profile were analysed in the plasma as early cardiovascular biomarkers in Andean women. To evaluate arenic realted DNA damage, telomere length and mitochondrial DNA copy number (mtDNAcn) were measured in peripheral blood in both Andes and Chaco study groups. Gene expression and DNA methylation were measured in peripheral blood in the Andes study group.

The arsenic concentrations in water had large ranges in both Andes and Chaco, and the median urinary arsenic concentrations for Andes and Chaco were 196 µg/L and 80 µg/L, respectively. The urinary arsenic metabolites differed significantly between the study groups, the median %iAs and %MMA were higher and the median %DMA was lower in Chaco population compared to the Andes, reflecting a less efficient arsenic metabolism in the Chaco study group. In women from Andes, increased urinary arsenic concentration was associated with decreased diastolic blood pressure and apoB/A, but there were no associations between urinary arsenic and homocysteine, triglycerides or cytokines, suggesting no evident cardiovascular toxicity. In men and women in Andes and Chaco, urinary arsenic was associated with longer telomere length and in Chaco with increased mtDNAcn. When the study groups were stratified according to fraction of inorganic arsenic in urine (%iAs), the associations remained significant in the above %iAs group, i.e. with less efficient arsenic metabolism capacity, for both Andes and Chaco. This suggests that individuals with less efficient arsenic metabolism are more prone to arsenic-related DNA damage and may be at high risk for arsenic-related future diseases. In Andes women, urinary arsenic was associated with decreased gene expression and increased DNA methylation in peripheral blood, indicating that arsenic exposure may have silenced gene expression by increasing DNA methylation. This thesis showed genotoxic and epigenetic, but no adverse cardiovascular, effects of arsenic exposure via drinking water. Future studies are needed to follow-up the study groups for future arsenic-related disease.

Key words Cardiovascular disease, Telomere length, mtDNAcn, gene expression, DNA methylation

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Syeda Shegufta Ameer

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Musarrat Jabeen Rahman

Division of Occupational and Environmental Medicine
Department of Laboratory Medicine
Faculty of Medicine, Lund University
SE-221 85
Sweden

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To my parents

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Ameer SS, Xu Y, Engström K, Li H, Tallving P, Nermell B, Boemo A, Parada LA, Peñaloza LG, Concha G, Harari F, Vahter M, Broberg K. 2016. Exposure to inorganic arsenic is associated with increased mitochondrial DNA copy number and longer telomere length in peripheral blood. Frontiers in Cell and Developmental Biology: in press.

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Broberg K, Engström K, **Ameer SS**. Gene Environment Interaction. In Handbook on the Toxicology of Metals. Nordberg GF, Fowler BA, Nordberg M, Friberg LT. (eds). 4th edition.

1 INTRODUCTION

The present thesis focuses on exposure to inorganic arsenic (iAs) in drinking water and early biomarkers of cardiovascular disease and cancer. Arsenic is classified as a class I carcinogen by the International Agency for Research on Cancer (IARC), as it can cause skin, lung, urinary bladder, kidney, and possibly liver and prostate cancer in humans (IARC, 2012). In addition, exposure to iAs through drinking water has been associated with increased risk of several non-cancer health effects (NRC 2001; WHO 2001; IARC 2012), e.g., hyperkeratosis (thickening of skin) and pigmentation changes in the skin (Karagas et al. 2015), immunosuppression (Ahmed et al. 2011; Ahmed et al. 2014), respiratory problem and non-malignant lung diseases (Argos et al. 2014; Parvez et al. 2013), liver and kidney disorders (Tchounwou et al. 2003; IARC 2004), peripheral and central nervous system and neurodevelopment disorders (Hamadani et al. 2011; Tolins et al. 2014), and cardiovascular diseases including hypertension, peripheral arterial disease, myocardial infarction, diabetes mellitus, and stroke (Kuo et al. 2013; Moon et al. 2012; Moon et al. 2013). Arsenic can pass through the placenta; arsenic exposure during pregnancy is associated with low birth weight, fetal loss and delayed infant development (Tofail et al. 2009). The strong toxicity of arsenic through drinking water makes it a world-wide health concern. It is therefore important to identify early markers related to future arsenic related chronic disease. One of the proposed mechanisms of arsenic related carcinogenicity is by gene regulation via epigenetic modes of action (Kim et al. 2008; Ren et al. 2011; Rossman and Klein 2011). In this thesis, biomarkers of toxicity of chronic arsenic exposure that can be related to early markers for future cardiovascular disease and cancer were studied in a population living in the Salta Province, northern Argentina who is exposed to iAs via drinking water. Further, diseases and biochemical pathways related to arsenic toxicity via gene expression and DNA methylation were elucidated.

1.1 ARSENIC

Arsenic is an element with atomic number 33 and relative atomic mass 74.92 amu. Arsenic is often referred as metalloid, as its physical and chemical properties are intermediate between a metal and a non-metal. From biological and toxicological perspectives, there are three major groups of arsenic compounds:

1. iAs compounds [arsenic trioxide, sodium arsenite and arsenic trichloride are common trivalent arsenic compounds; arsenic pentoxide, arsenic acid and arsenates (e.g. lead arsenate and calcium arsenate) are common pentavalent arsenic compounds]
2. organic arsenic compounds (arsanilic acid, methylarsonic acid, dimethylarsinic acid and arsenobetaine)
3. arsenic gas

In reduced and oxygenated conditions, the trivalent arsenite (AsIII) and the pentavalent arsenate (AsV), respectively, are the most common inorganic forms in water, while arsenobetaine, arsenocholine and arsenosugars are common organic forms in certain foods, particularly in sea food (IARC, 2004). Moreover, plants, such as rice can take up iAs from soil (Zhao et al. 2010).

1.1.1 Use of arsenic

Arsenic is used in pharmaceuticals, wood preservatives, agricultural chemicals and has applications in the mining, metallurgy, glass-making, and semiconductor industries. Organic arsenicals (e.g. roxarsone, arsanilic acid and its derivatives) have been used as feed additives for poultry and swine to increase the rate of weight gain, to improve feed efficiencies, pigmentation, and for disease treatment and prevention (Silbergeld and Nachman 2008; EPA 2000, 2006; FDA 2008a,b). Elemental arsenic is used to manufacture alloys, particularly with lead (e.g. lead acid batteries) and copper. Gallium arsenide, a compound of gallium and arsenic, is used in high-speed semiconductor devices, high-power microwave and millimeter-wave devices, and opto-electronic devices, including fiber-optic sources and detectors, due to its high electron mobility, as well as light-emitting, electromagnetic and photovoltaic properties (IARC 2006). Arsine, a flammable and highly toxic gas, is used as a doping agent to manufacture crystals for computer chips and fiber optics (IARC 2012). Arsenic in different chemical forms is also used in pigments, sheep-dips, leather preservatives, and poisonous baits, as well as in catalysts, pyrotechnics, antifouling agents in paints, pharmaceutical substances, dyes and soaps, ceramics, alloys and electrophotography (IARC 2012).

1.1.2 Environmental arsenic

Arsenic is the 20th most common element in earth's crust. In nature, arsenic is mostly present as the mineral arsenopyrite. Arsenic is released to the environment through volcanic activity from arsenic-rich bedrock as well as industrial activities (Fishbein 1981). Mining, smelting and burning of fossil fuels are the main anthropogenic sources of arsenic contamination of water, soil, and air. The ambient environment has also been contaminated by the historical use of arsenic containing pesticides (WHO 2001). Arsenic is mainly transported in the environment via water from both natural and anthropogenic sources. In some regions of the world, groundwater (used for drinking water) naturally contains arsenic due to arsenic-rich geological sediments and bedrock (Sarkar and Paul 2016). These areas include Bangladesh, China, Taiwan, West Bengal (India), Argentina, Chile, Mexico, Vietnam, Australia and USA. In the affected areas, the levels of arsenic may range from tens to thousands of micrograms per liter (IARC 2012). Figure 1 depicts world-wide documented distribution of arsenic in ground water (>50 µg/L), related to mining and geothermal activities.

Figure 1:
World-wide documented distribution of arsenic in ground water (>50 µg/L), related to mining and geothermal activities. Reproduced with permission of the British Geological Survey © NERC. Smedley, P. L. and Kinniburgh, D.G. 2013. Chapter 12: Arsenic in groundwater and the environment. In: Essentials of Medical Geology, Second Edition. Eds: Selinus, O., Alloway, B., Centeno, J.A., Finkelman, R.B., Fuge, R., Lindh, U. and Smedley, P.L. Springer, pp 279-310. (http://www.bgs.ac.uk/about/copyright/acknowledgements_published.html).

1.1.3 Human exposure

Exposure through drinking water: The primary exposure of arsenic in the general population is via ingestion of arsenic-containing water. Millions of people world-wide are currently exposed to arsenic concentrations above 10 µg/L (IARC 2004; Mukherjee et al. 2006; Vahter 2009), the recommended cutoff of drinking water by World Health Organization (WHO 2004). In Bangladesh the problem of arsenic in groundwater is particularly serious; a UNICEF/DPHE survey of ground waters from 15 upazilas in southern Bangladesh found that out of 316,951 boreholes tested, 66% had arsenic concentrations exceeding the national standard of 50 µg/L for arsenic in drinking water (Rosenboom, 2004).

In South America, it is estimated that several million people are exposed to arsenic from drinking water, and in some regions the exposure has likely occurred for many generations (up to ~7000 years) (Bundschuh et al. 2012). Mummies of approximately 1500 years of age found in the Tarapacá Valley in Chile's Atacama Desert had high concentrations of arsenic in hair, possibly caused by the consumption of arsenic contaminated drinking water and food grown with irrigation using arsenic containing water (Arriaza et al. 2010).

Dietary exposure: Food, mainly rice-based, is an important source of exposure to iAs, particularly in areas where arsenic-rich groundwater is used for irrigation (Carignan et al. 2016; Karagas et al. 2016; Rahman and Hasegawa 2011; Sigrist et al. 2016), or paddy fields located close to mining areas such as in southern China (Yu et al. 2016). People may also be exposed to arsenic via seafood, which mainly contains organic forms of arsenic, such as arsenobetaine, arsenosugars, and arsenocholine. However, these forms are considered to be much less toxic than iAs.

Occupational exposure: Inhalation of arsenic-containing particulates in the areas with industrial emissions may also be a route of exposure to arsenic (Wang et al. 2009).

1.1.4 Arsenic exposure in northern Argentina

In Argentina, elevated (>10 µg/L) concentrations of arsenic in groundwater have been detected particularly in the Puna region in the Andes, the Chaco region, Córdoba, and the Pampean plain (Farias et al. 2003; Francisca and Carro Perez 2009; Nicolli et al. 2012). In these areas arsenic and other toxic elements leaches and weathers from the volcanic bedrock and mixes with ground water, river water and natural spring water which are major sources of drinking water for the inhabitants. M. Vahter from the Unit of Metals and Health, Institute of Environmental Medicine, Karolinska Institutet and coworkers first visited the

Puna region in the Andes back in early 90s and reported elevated arsenic concentration in drinking water in the village San Antonio de los Cobres (Vahter et al. 1995) and, subsequently, local clinicians informed that there were patients with arsenic-related health effects in the Chaco and Pampean plains. In 1999, researchers from the Unit of Metals and Health, Institute of Environmental Medicine, KI, started collecting biological and water samples from the area.

1.1.5 Absorption, metabolism and excretion of arsenic

After ingestion of iAs, approximately 80-90% is absorbed in the gastrointestinal tract in animals, including humans (Marafante et al. 1987; Vahter and Norin 1980). Absorbed pentavalent arsenate is first reduced to trivalent arsenite in blood (Vahter and Envall 1983), where the majority of the arsenic has a half-life of about two to three hours. Arsenite is considerably more toxic than arsenate due to its strong ability to react with thiols [sulfhydryl (SH-) groups], and therefore arsenite can deplete cells of the thiol-containing tripeptide glutathione (Santra et al. 2000).

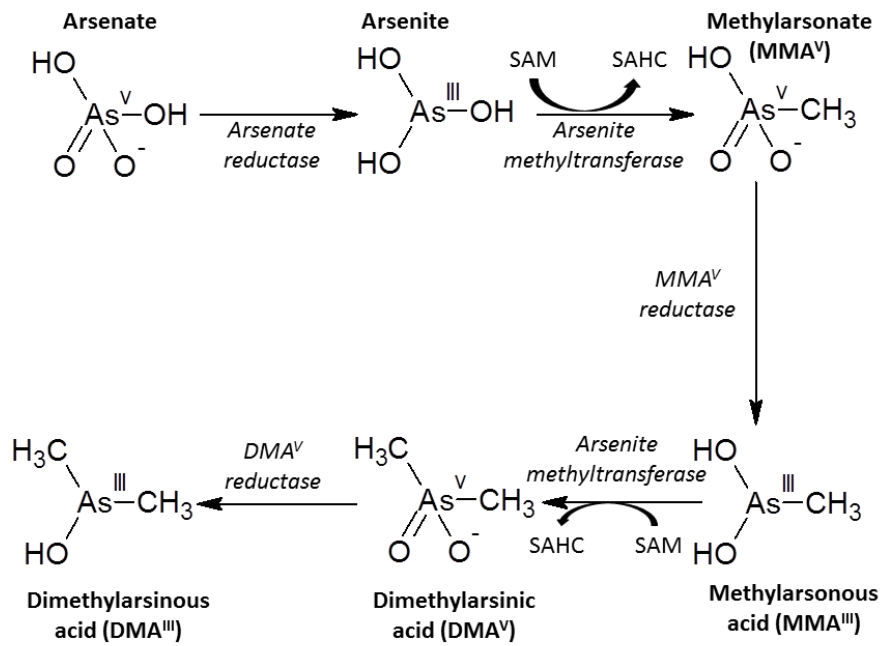

Figure 2:
Proposed schematic of arsenic metabolism. SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine.

The reduced arsenite in blood is taken up by the hepatocytes where it is methylated (Lerman et al., 1983). Arsenic uptake in the cell is most likely mediated by the aquaporins, which transports water, glycerol and other small neutral solutes, as shown in the prokaryotes *Escherichia coli* and *Sinorhizobium meliloti*, and in eukaryotes such as the yeast *Saccaromyces cerevisiae* (Liu et al. 2002; Meng et al. 2004; Sanders et al. 1997; Wysocki et al. 2001; Yang et al. 2005). Experiments in rats indicated uptake of MMA(III) at a higher rate than As(III) by aquaporine 9 (AQP9) in the liver (Liu et al. 2006). In liver, the multi drug resistant protein 2 (MRP2), a member of the adenosine triphosphate binding cassette, ABC, superfamily, extrudes arsenic-glutathione complexes into bile and may be a route of arsenic detoxification in humans (Cui et al. 2004). iAs is mainly metabolized in the liver, by altering reductions and methylations, where S-adenosylmethionine (SAM) acts as the main methyl donor (Marafante and Vahter 1984). In humans, iAs is metabolized through the conversion of AsV to AsIII, followed by methylation to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Vahter 2009; Watanabe and Hirano 2013). The main arsenite methyltransferase is AS3MT which methylates iAs to MMA as well as MMA to DMA (Lin et al. 2002; Pierce et al. 2012; Schlebusch et al. 2015). Figure 2, illustrates the proposed schematic of iAs metabolism to DMA by *AS3MT*, in humans. The major route of elimination of ingested arsenic is via urine. The main metabolites of iAs that are excreted in urine are DMA (60%-80% of total metabolites in urine) and MMA (about 10%-20%). However, the methylation efficiency is not 100% and approximately 10-30% of arsenic in urine is inorganic (Hopenhayn-Rich et al. 1996b; Vahter 2002). The total concentration of these metabolites is used as a measure of exposure to iAs, and the relative proportion of each metabolite is used as a measure of methylation capacity (Vahter 2009).

1.1.6 Variation in arsenic metabolism capacity

The fraction of metabolites excreted via urine varies substantially among individuals and populations (Vahter 2002). For example, populations from some areas of Taiwan have an average %MMA of 20%-30% (Chiou et al. 1997; Hsueh et al. 1998a), while indigenous population in the Andes have an average %MMA of less than 10% (Concha et al. 2006; Hopenhayn-Rich et al. 1996b; Hopenhayn et al. 2003). Individuals who excrete a higher proportion of ingested arsenic as urinary MMA also have a higher risk of arsenic-associated adverse health effects (Antonelli et al. 2014; Engstrom et al. 2015; Huang et al. 2008; Lindberg et al. 2008). Moreover, efficient methylation of iAs to DMA has been associated with lower retention of arsenic in body (Gardner et al. 2011). Factors contributing to differences in arsenic metabolism capacity between individuals include genetics, age, sex and nutritional status.

Genetic background: The degree of methylations of iAs to DMA among individuals varies due to underlining genetic differences of arsenic metabolism capacity. *AS3MT* is the gene showing strongest and most consistent associations with methylation capacity of arsenic (Drobna et al. 2005; Wood et al. 2006). Genome-wide association studies showed that polymorphisms in *AS3MT* were associated with different urinary arsenic metabolite patterns (Pierce et al. 2012; Schlebusch et al. 2015).

Age and sex difference: The arsenic metabolism capacity can be influenced by both age and sex differences. A recent study in rural Bangladesh shows that children have more efficient arsenic metabolism capacity compared to their mothers during pregnancy (Skroder Loveborn et al. 2016). Moreover, studies on humans exposed to arsenic via drinking water reported less %MMA and more %DMA in the urine in women compared to men (Hopenhayn-Rich et al. 1996a; Hsueh et al. 1998a; Hsueh et al. 2003), suggesting that women have higher capacity to methylate ingested arsenic compared to men. Men have been shown to be more affected by arsenic-related skin lesions, including skin cancer (Ahsan et al. 2006; Bhowmick et al. 2014; Chen et al. 2003; Guha Mazumder et al. 1998; Rahman et al. 2006), which may be related to their less efficient arsenic metabolism.

Nutritional status/BMI: The arsenic metabolism efficiency can be influenced by the nutritional status, likely as it determines the amount of methyl groups available for arsenic methylation. The diet is an important source for methyl groups which are components of the one-carbon cycle. Lindberg et al (2007) reported an inverse association between BMI [body weight in kg/(height in meters)^2] and %MMA in the urine among men in the Central Europe (Lindberg et al. 2007). Other studies also reported similar findings (Ahsan et al. 2007; Gomez-Rubio et al. 2011; Li et al. 2008).

1.1.7 Mechanisms of arsenic toxicity

Although numerous studies have examined the mechanisms of arsenic toxicity, the exact pathways are not yet clear. Arsenic manifests its toxicity by different mechanisms depending on dose, duration of exposure, tissue type and metabolism. The arsenate oxyanion is chemically similar to phosphate; one hypothesis in arsenic related toxicity is based primarily on the competitive inhibition of proteins that use phosphate, so it affects many enzymes in metabolism and oxidative phosphorylation (Yang et al. 2012). The arsenite, which is highly reactive, binds preferentially to SH-groups, resulting in inhibition of various enzymes that have SH-groups in their active site (WHO 2001), such as DNA repair enzymes (Walter et al. 2007) and antioxidant related enzymes, e.g. glutathione peroxidase and

thioredoxin reductase (Ganyc et al. 2007). Other proposed mechanisms of arsenic toxicity include immunosuppression, oxidative stress, chromosomal aberrations, micronuclei formation, modification of cellular signaling, induction of apoptosis, altered expression and DNA binding activity of transcription factors and epigenetic modifications (Dangleben et al. 2013; Farzan et al. 2013; Flora 2011; IARC 2004; Vahter 2009).

1.2 ARSENIC ASSOCIATED CARDIOVASCULAR DISEASE

Several studies have reported that arsenic exposure is associated with increased risk of developing cardiovascular diseases, e.g., cardiac heart disease, stroke and peripheral arterial disease (Moon et al. 2012; Moon et al. 2013). A case-control study in inner Mongolia showed association of water arsenic exposure (median: 13.1 μg/L, range: <0.1 to 208.1 μg/L) with acute myocardial infarction, cardiomyopathy and suggestive angina (Wade et al. 2015). Prospective studies have also shown associations between arsenic and risk of cardiovascular disease; a study in Bangladesh showed associations of arsenic (arsenic concentration in water at baseline of the study: 112 μg/L) with ischemic heart disease, cardiovascular disease (CVD) and stroke (Chen et al. 2013a). In another study in Colorado, United States, time-weighted average lifetime arsenic exposure to low level iAs in drinking water (10 to 100 μg/L) was associated with cardiac heart disease (James et al. 2015).

1.2.1 Mechanisms of arsenic-related cardiovascular disease

The effect of arsenic on the cardiovascular system may involve several pathways. It has been shown that arsenic exposure induces oxidative stress-related activation of nuclear factor-κB (Barchowsky et al. 1996), which suppresses cytokine-induced iNOS gene expression and causes reduced NO availability (de Vera et al. 1996; Oda et al. 2000; Tsou et al. 2005). Decreased availability of biologically active NO in the endothelium has been implicated in the pathophysiology of several vascular diseases (Kumagai and Pi 2004) by promoting proliferation of endothelial and smooth-muscle cells, cell adhesion, platelet aggregation, and arterial vasocontraction (States et al. 2009). Arsenic also stimulates the release of reactive oxygen species (ROS) through activation of NADPH oxidase, as shown in *in vitro* studies (Bunderson et al. 2002; Smith et al. 2001). Moreover, chronic arsenic exposure is associated with changes in cardiac electrophysiology, such as QT-interval prolongation, which is a risk factor for sudden cardiac arrest (Chen et al.

2013b; Mordukhovich et al. 2009; Mumford et al. 2007). Studies in humans showed the importance of early-life exposure in arsenic-related cardiovascular disease in Chile: very high arsenic exposure (more than 850 µg/L) *in utero* was associated with increased risk of acute myocardial infarction in both men and women later in life with the most adverse effect observed in men 30-49 years (Yuan et al. 2007).

1.2.2 Arsenic and blood pressure

Arsenic exposure may affect blood pressure in humans (Abhyankar et al. 2012). A review study including 11 cross-sectional studies involving >20,000 subjects reported a pooled odds ratio of 1.27 (95% CI: 1.09, 1.47) for hypertension in highest arsenic exposure category compared to lowest exposure (Abhyankar et al. 2012). In an U.S. cohort study, arsenic exposure was associated with greater increases in blood pressure over the course of pregnancy (Farzan et al. 2015). One cross-sectional study in Bangladesh showed no association between arsenic in drinking water and hypertension, but water arsenic was associated with increased pulse pressure (Islam et al. 2012). A longitudinal study in Bangladesh showed increases in both diastolic and systolic blood pressures over the time among the individuals with the highest exposure to arsenic (median arsenic concentrations in water at baseline for the whole cohort 62 µg/L) with the highest quartile (Jiang et al. 2015).

1.2.3 Arsenic and lipid biomarkers

Studies have evaluated the effect of arsenic exposure on traditional lipid markers: total cholesterol, low-density lipoprotein (LDL), high-density lipoproteins (HDL), and triglycerides (TG). Aberrant profiles of these lipid markers are associated with a high risk of cardiovascular disease (The Emerging Risk Factors Collaboration, 2012). A study from Bangladesh showed that individuals exposed to arsenic (mean arsenic in water 218 µg/L) had significantly lower levels of total cholesterol, HDL and LDL compared to unexposed individuals (Nabi et al. 2005). Another study on Bangladeshi individuals reported that serum levels of total cholesterol, LDL and HDL were lower and oxidized LDL was higher among individuals living in arsenic-endemic area (mean water arsenic 174.5 µg/L) compared to non-endemic areas (mean water arsenic 2.3 µg/L) (Karim et al. 2013). No associations between arsenic exposure and TG were observed in any of these studies. In contrast, one study on Mexican individuals showed that water arsenic concentrations (median = 47.9 µg/L; range = <0.01 – 419.8 µg/L,) were associated with elevated triglycerides, high total cholesterol, diabetes (determined by fasting blood glucose

level and oral glucose tolerance test), and higher HDL concentrations (Mendez et al. 2016), suggesting that associations between arsenic exposure and lipid profiles are inconsistent.

1.2.4 Arsenic and cytokines

Arsenic-related toxicity may be mediated through inflammatory responses. Arsenic exposure increased the expression of several pro-inflammatory genes such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), chemokines and macrophage inflammatory protein-2 in mice exposed to sodium arsenite via drinking water (Wu et al. 2008). A similar study on mice exposed to sodium arsenite through drinking water (0.01-0.1 mg/L) for 30 days showed that in mice consuming higher amount of arsenic, NF-κB activation did not lead to the classical IL-6 upregulation response, indicating immune-suppression (Choudhury et al. 2016). In another study, intestinal epithelial caco-2 cells exposed to 1 μM arsenite, 0.1μM monomethylarsonous acid (MMAIII), and 1 μM dimethylarsinous acid (DMAIII) at different time points increased the expression and release of the pro-inflammatory cytokines (TNFα), IL-6 and IL-8 (Calatayud et al. 2014). Moreover, arsenic exposure during pregnancy in humans has been shown to enhance placental inflammatory responses, reduce placental T-cells, and alter cord blood cytokines concentrations (Ahmed et al. 2011).

1.2.5 Arsenic and hemoglobin

Arsenic exposure may also affect blood hemoglobin levels. One study in Bangladesh found that arsenic exposure (median urinary arsenic >200 μg/L) was negatively associated with hemoglobin concentrations, but only in those individuals with hemoglobin levels below 100 g/L (Heck et al. 2008). A case-control study (arsenic in drinking water 40 μg/L vs. <1 μg/L) on Chilean pregnant women showed that the prevalence of anemia rose more sharply among arsenic exposed (49%) *versus* unexposed women (17%) during pregnancy (Hopenhayn et al. 2006). Recently, a case-control cross-sectional study on Bangladeshi women (median water arsenic 17.2 μg/L) showed that the odds of having arsenic-induced skin lesions were approximately three times higher among anemic women (<120 g/L) compared to women with normal hemoglobin levels, indicating that anemic individuals are more sensitive to arsenic exposure (Kile et al. 2016).

1.3 ARSENIC ASSOCIATED GENOTOXIC EFFECTS

1.3.1 Telomere length

With each round of DNA replication, the chromosome becomes slightly shorter due to the inability of the polymerase to replicate the full length of the chromosome, a phenomenon referred to as the "end replication problem" (Olovnikov 1973). The telomeres, which are 10 base pair (bp) -10 kilo bp of DNA consisting of hundreds to thousands repeats of the same sequence (5′-TTAGGG-3′) at the end of each chromosome, protects the cell from erosion and loss of genetic information during replication. Thus, telomeres play a key role in keeping chromosomal stability by protecting chromosome ends from chromosome fusions or being recognized as strand breaks (Blackburn 2010; Li et al. 2013). In general, during each cell cycle, 50-100 bp of telomeric DNA are deleted from the ends of every chromosome. At a certain telomere length the cells stop dividing, become enlarged and enter into senescence. Telomere length decreases along with age, which has been observed in blood in several population-based studies (Frenck et al. 1998; Svenson et al. 2011). Cancer cells have the ability to overcome senescence, by using mechanisms capable of maintaining telomere lengths by expressing telomerase, which enables cancer cells to divide indefinitely (Kim et al. 1994; Shay et al. 1991).

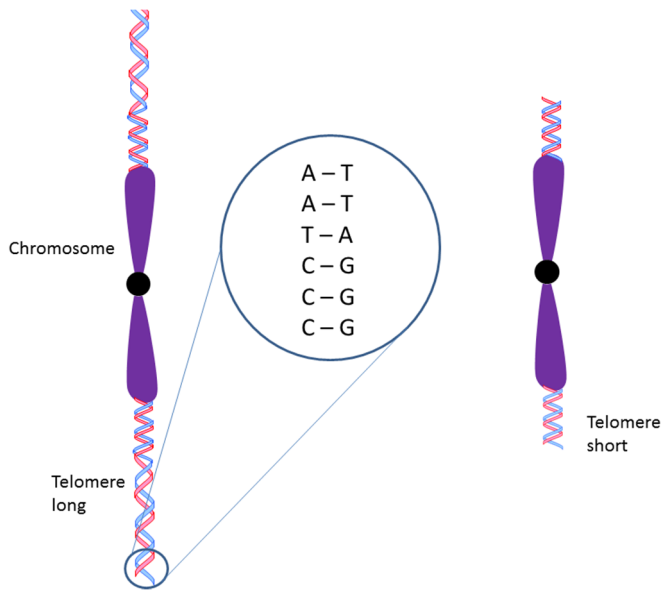

Figure 3:
Schematic diagram of telomeres.

1.3.2 Arsenic telomere length and disease risk

Telomere length in blood has often been used as a measure of individuals' telomere status, as telomere length in blood correlates with the telomere length in other tissues to some extent (Friedrich et al. 2000; Gadalla et al. 2010). Telomere dysfunction, either represented by aberrantly short telomeres or long telomeres, has been associated with disease. Short telomeres in peripheral blood are associated with various age-related diseases, including cardiovascular disease (Brouilette et al. 2007; Khan et al. 2012; Olivieri et al. 2013; Sabatino et al. 2012; Willeit et al. 2010), diabetes (Zhao et al. 2013), and lung diseases, including idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease/emphysema (Gansner and Rosas 2013). Telomere length in blood is also associated with different types of cancer such as bladder, lung, breast, colon, head and neck, renal and skin cancer (Broberg et al. 2005; Gansner and Rosas 2013; Han et al. 2009; Hofer et al. 2011; Ma et al. 2011; McGrath et al. 2007; Seow et al. 2014; Shen et al. 2007; Wu et al. 2003). On the other hand, long telomere length in peripheral blood has been related to the risk of developing melanoma, lung cancer, pancreatic cancer and soft tissue sarcoma (Anic et al. 2013; Lan et al. 2013; Lynch et al. 2013; Xie et al. 2013). Recent epidemiological studies found that chronic arsenic exposure was associated with longer telomeres in peripheral blood leukocytes (Gao et al. 2015; Li et al. 2012) and increased expression of telomerase (Mo et al. 2009).

1.3.3 Mitochondrial DNA copy number (mtDNAcn)

Mitochondria are structures within the cells that, via the electron transport chain, convert the energy from food to adenosine triphosphate (ATP), the form cells can use for energy. Mitochondria also have an important role in the regulation of apoptosis (Lee and Wei 2000), and cell proliferation (Martinez-Outschoorn et al. 2011). Mitochondria contain circular, double-stranded DNA (mtDNA) of 16.6 kilo bp which encodes protein subunits of the electron transport chain complexes I, III, IV, and V, including 22 tRNAs, 2 rRNAs and 13 polypeptides. Proteins involved in mtDNA replication, transcription, translation, and repair are encoded by the nuclear DNA and enters mitochondria from cytoplasm (Lister et al. 2005; Mokranjac and Neupert 2005). MtDNA copy number (MtDNAcn) differs between different tissues; in energy-intensive tissues such as heart and muscle there are approximately 4000-6000 copies of mtDNA per cell, while in liver, kidney and lung tissues there are 500-2000 copies (D'Erchia et al. 2015). Mitochondria generate high levels of reactive oxygen species during energy production. The lack of introns and protective histones, as well as its proximity to the electron transport

chain, makes the mtDNA highly susceptible to oxidative damage (Van Houten et al. 2006).

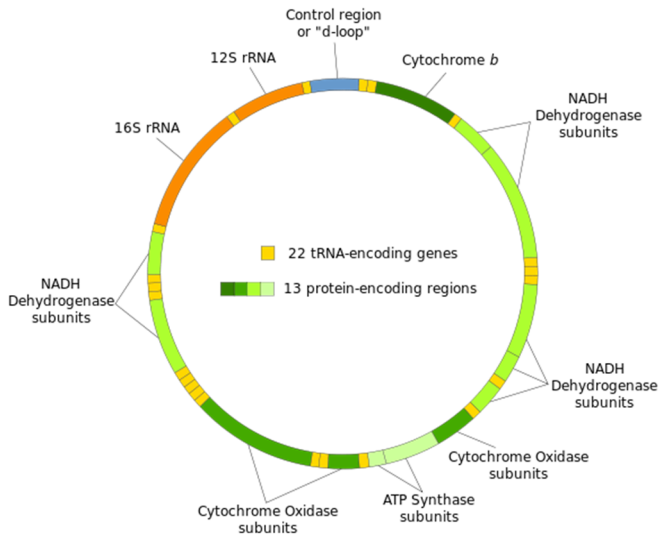

Figure 4:
Human mitochondrial DNA. Creatice Commons: derivative work: Shanel, Mitochondrial DNA de.svg: translation by Knopfkind; layout by jhc. https://commons.wikimedia.org/wiki/File:Mitochondrial_DNA_en.svg

1.3.4 Arsenic, mtDNAcn and disease risk

Mitochondrial dysfunction has been linked to cancer (Seyfried 2015). A recent survey of mtDNAcn variation across 22 tumor types profiled by The Cancer Genome Atlas project reported that mtDNA is depleted in many tumors, especially in bladder, breast, and kidney, compared to matched-normal tissue (Reznik et al. 2016). Alteration in mtDNAcn in peripheral blood has been associated with different types of cancer; such as increased risk of cancer in the breast, head and neck, prostate, kidney and colon/rectum in women (Cheau-Feng et al. 2014; Huang et al. 2014; Lemnrau et al. 2015; Melkonian et al. 2015; Thyagarajan et al. 2013; Zhou et al. 2014). An *in vitro* study reported that arsenic exposure to mammalian cells (up to 1 mg/L) reduced mtDNAcn (Partridge et al. 2007), whereas, another study of arsenic-induced Bowen's disease, also known as squamus cell carcinoma *in situ*, showed increased mitochondrial biogenesis and increased mtDNAcn in skin biopsies. This report also found that *in vitro* mtDNAcn increased in a dose dependent manner by 20-60% in keratinocytes after 0.1-1.0 µmol/L arsenic treatment (Lee et al. 2011). Alterations in mtDNAcn has also been associated with non-cancer health effects such as cardiovascular disease (Chen et al. 2014; Huang et al. 2016) and diabetes (Xu et al. 2012).

1.4 ALTERATIONS IN GENE EXPRESSION AND EPIGENETIC MODIFICATION

Epigenetic modifications are defined as changes affecting gene-regulation and gene-expression that occur without alterations in the DNA sequence. Major epigenetic modifications include DNA methylation, histone modifications (through acetylation, methylation, phosphorylation, ubiquitinylation) and non-coding RNAs. Dysregulation of epigenetic processes, such as aberrant DNA methylation, may lead to chronic diseases, including cancer, inflammatory and cardiovascular diseases (Ngollo et al. 2014), as well as obesity, metabolic syndrome, and diabetes mellitus (Whayne 2015). The most widely studied epigenetic modification is cytosine methylation. DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs), which can add methyl groups to the C5 position of cytosines in DNA (Okano et al. 1998), where a cytosine nucleotide is followed by a guanine nucleotide in 5´-3´ direction (CpG site). DNA methylation is usually associated with transcriptional silencing of genes (Figure 5) (Robertson 2005). In carcinogenesis, global DNA methylation is generally decreased, whereas specific regions of DNA containing tumor suppressor genes often show hypermethylation (Deng et al. 2006).

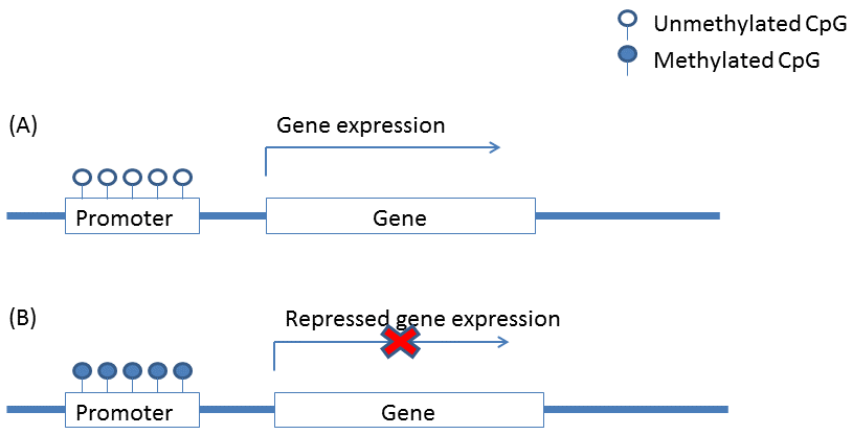

Figure 5:
General model for DNA methylation in promoter regions and gene expression. Gene expression can continue when the promoter region is unmethylated (A). Gene expression is repressed when the CpG sites are methylated (B).

1.4.1 Arsenic and DNA methylation

The most commonly studied epigenetic mechanism affected by arsenic exposure is DNA methylation. One early study showed that exposing human lung adenocarcinoma A549 cells to sodium arenite (0.08 to 2μM) and sodium arsenate (30 to 300 μM) causes dose-dependent hypermethylation of promoter of the tumour suppressor protein p53 (Mass and Wang 1997). Mexican women exposed to arsenic from drinking water (mean water arsenic concentration 110 μg/L) with arsenic-associated skin lesions showed CpG hypermethylation in 182 genes compared to unexposed individuals with no skin lesions (Smeester et al. 2011). Another study on the same individuals as in Smeester et al. (2011) (mean urinary arsenic concentration 10.7 μg/L) showed that subjects with different arsenic metabolites fractions in urine had different DNA methylation profiles, suggesting that arsenic metabolism efficiency may affect the DNA methylation pattern (Bailey et al. 2013). In a Bangladeshi study, higher arsenic exposure during pregnancy (median urinary arsenic in gestational week 30: 89 μg/L) was associated with lower methylation in CpG sites in cord blood, mainly in boys, suggesting that arsenic exposure can effect *de novo* methylation (Broberg et al. 2014).

1.4.2 Arsenic and gene expression

Human renal carcinoma cells exposed to sodium arsenite showed increased gene expression of the multi drug resistance gene (*MDR1)* of up to seven to eight times compared to unexposed cells (Chin et al. 1990). Mice exposed to arsenic (80 mg/L via drinking water for ten weeks) had altered expression of genes involved in cell proliferation, acute phase proteins and metabolic enzymes in the liver (Xie et al. 2004). Arsenic may, through inhibition of DNA repair mechanisms, cause increased number of mutations. In a population based case-control study in the U.S. it has been shown that toe nail arsenic levels were inversely correlated with expression of nucleotide excision repair complex *ERCC1* in lymphocytes, suggesting that inhibition of DNA repair capacity is a potential mechanism arsenic-related carcinogenicity (Andrew et al. 2003).

2 AIMS OF THE THESIS

Overall aim:

The overall aim of this thesis was to identify early health effects of chronic arsenic exposure through drinking water. We specifically evaluated early markers of cardiovascular disease, such as homocysteine, triglycerides, apoA and apoB. We also evaluated the effect of arsenic on early markers of cancer, such as telomere length and mitochondrial DNA, and considered the role of arsenic metabolism efficiency in modifying arsenic toxicity.

Specific aims:

1. To elucidate the effect of arsenic on blood pressure and early risk markers for cardiovascular disease such as homocysteine, triglycerides, apoA and apoB. We also evaluated the association of arsenic and hemoglobin.
2. To evaluate the effect of arsenic on mitochondrial DNA content and telomere length in peripheral blood.
3. To investigate the effect of arsenic exposure and arsenic methylation efficiency on gene regulation in peripheral blood.

3 MATERIALS AND METHODS

This section is a summary of the materials and methods used in this thesis. For further details, the reader is referred to the individual papers (Papers I-III).

3.1 STUDY AREA

The study areas of this thesis are the Puna (referred to as Andes) and Chaco areas of the Salta province in the north-western part of Argentina (Figure 6).

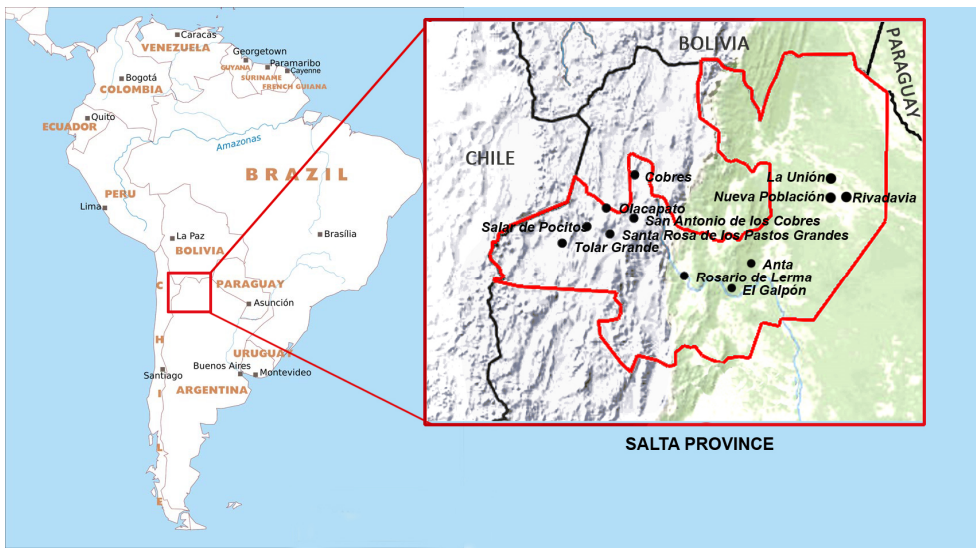

Figure 6:
The study areas of this thesis are the Puna (referred to as Andes) and Chaco of Salta province in the north-western part of Argentina.

Andes: The Puna area is an arid highland region located at ~4000 meters above the sea level and surrounded by the Andes mountain belt. The majority of our study participants were living in the main village San Antonio de los Cobres, but study participants were also recruited from surrounding villages including Tolar Grande, Salar de Pocitos, Olacapato, Santa Rosa de los Pastos Grandes, Cobres and

Rosario de Lerma. The total population of San Antonio de los Cobres and surrounding villages is about 8,000 these days and the individuals are mainly of indigenous origin (Kolla ethnicity). The population is poor and the local economy is based on trading (e.g. handcrafts), breeding of llamas, goats and sheep, mine work and tourism (Concha et al. 2010). The diet is largely of animal origin (meat and some dairy products, but essentially no fish or sea food) but the diet also consists of vegetables, potatoes and corn. The source of drinking water in San Antonio de los Cobres is a natural spring ("Agua de Castilla") located approximately 10 km away from the village. The water is first pumped to a series of sand filters and transferred to a chlorination station before being distributed throughout the village (Concha et al. 2010).

Figure 7:
San Antonio de los Cobres, the main village in the Andes, Salta Province, where the studies of the thesis took place. Photo courtesy: Karin Broberg

Chaco: The Chaco region is a semiarid area in the Salta plains at ~300 meters above the sea level. The study participants of Chaco area were from the Anta county: El Rincón, Los Rosales and other villages; Rivadavia county: Rivadavia, La Unión and and Nueva Población; and Metán county: El Galpón. The total population in Anta is ~8,000, El Galpón ~8,400, and Rivadavia and La Unión ~6,400 inhabitants. The people are mostly 'creoles', i.e. of Spanish/European origin, and to a lesser extent of indigenous origin (Wichí).

Figure 8:
The hospital in El Galpon, Chaco area, Salta Province. Photo courtesy: Pia Tallving.

The local economy is based on production of cattle and collection of logs of native trees from forest. In Anta there is a large production of cattle, but they also cultivate soybean, corn, wheat, safflower, sorghum, chickpea, cotton and fruits, like watermelon and melon, in the semiarid regions. The diet is mainly of animal origin (meat and some dairy products, very little amount of fish from the market but essentially no sea food) but the diet also consists of rice, pasta, corn, vegetables and potatoes (personal communication, Analia Boemo, Facultad de Ciencias Exactas & Consejo de Investigación, Universidad Nacional de Salta, Salta, Argentina).

3.2 SAMPLING AND DATA COLLECTION

Andes: The hospital "Dr. Nicolás Cayetano Pagano" is located in San Antonio de los Cobres and this hospital provides basic health service to the residents. Our research group performed field studies in 2008 and 2011 at the hospital where we

examined and collected biological samples of relatives or attendants of patients admitted to the hospital. We also examined individuals recruited through help of primary health care workers. Further, we went to surrounding villages to San Antonio de los Cobres and examined in the field village inhabitants that wanted to participate in the study. One questionnaire was filled in for each participant with questions about age, ancestry, drinking water sources, dietary habits, medical history, duration of residence ion the study area, smoking, alcohol consumption, coca chewing, as well as parity (for women). Weight and height were measured and used for calculation of body mass index (BMI).

Chaco: Professor Marie Vahter and Dr. Gabriela Concha, Unit of Metals and Health, Institute of Environmental Medicine, Karolinska Institutet started working in the Andes area of the northern Argentinian Andes in the 1990s. The local health authority invited the Unit of Metals and Health to conduct a study in the Chaco area with the aim to compare the populations from the Andes and the Chaco regions for arsenic metabolism and toxicity. One field study was performed in Anta including Rosario de Lerma during 1999, and one in El Galpón, Los Rosales and El Rincón during 2013. Our collaborators at the University of Salta performed a field study in Rivadavia, La Unión and and Nueva Población between 2013 and 2015, according to the standard protocol developed by the Unit of Metals and Health, Karolinska Institutet, that was used for the field study in the Andes. The people in Chaco were informed about the campaign by radio announcements, by asking individuals randomly in the lines at the post-office, and through the primary health care workers. In the Rivadavia hospital the patients' attendants were asked to participate and those who wanted to volunteer were included in the study.

Blood pressure was measured and urine samples were collected from all study participants in Andes and Chaco areas. Urine samples were collected from spot mid-stream urine in plastic urine collection cups, transferred to 24 mL polyethylene vials, and kept at -20 °C until transport to Sweden. Peripheral blood samples were collected in two K_2EDTA tubes (Vacuette, Greiner Bio-One GmbH, Greiner, Germany) per person. One blood sample was immediately kept in -20 ºC for later DNA extraction. Another blood sample was, after 10 minutes of waiting, separated by 10 minutes centrifugation to obtain the plasma fraction. Blood samples were also collected in PAX tubes (Becton Dickinson, Franklin Lakes, NJ) according to the instructions of the manufacturer. All blood and urine samples were transported with cooling blocks to Sweden and stored at -80 ºC until further analyses.

3.3 EXPOSURE ASSESSMENT

We measured total arsenic concentrations in water samples collected from the Andes and Chaco regions by inductively coupled plasma mass spectrometry (ICPMS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) as previously described (Concha et al. 2010). Briefly, water arsenic was analysed by atomic absorption spectrophotometry (AAS), following dry ashing (Vahter and Lind 1986). We did not perform speciation of inorganic arsenic in water, since the arsenite is oxidized to arsenate rapidly in presence of oxygen. Therefore, the speciation needs to be performed very soon after sampling (performed in a way to avoid oxygenation of the water), which was not possible in our study.

We used arsenic in urine as our biomarker of exposure. Urinary arsenic was determined based on the sum of concentrations of iAs and its metabolites (MMA + DMA) in urine (U-As). We assessed the efficiency of arsenic metabolism by the fractions (percentages) of iAs, MMA, and DMA in urine (Vahter 2002). Arsenic metabolites in urine were measured by high-performance liquid chromatography (HPLC: Agilent 1100 series system, Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and ICPMS (HPLC-HG-ICPMS) as described previously (Gardner et al. 2011; Lindberg et al. 2007). Urine samples were shaken, and approximately 0.5 mL of each urine sample was filtered with 0.20 μm syringe filter and transferred to HPLC-HG-ICPMS system. The HPLC separates the iAs species and HG system introduces the metabolites to the ICP-MS, where it detects the iAs and its metabolites. Urine samples from Anta county were analysed for arsenic metabolites by HPLC-AAS, as previously described (Concha et al. 2006).

To compensate for variation in dilution of urine samples, the arsenic concentrations were adjusted to the mean specific gravity (1.020), measured by a hand refractometer (Atago, Japan) or a digital refractometer (EUROMEX RD 712 clinical refractometer; EUROMEX, Arnhem, the Netherlands).

3.4 OUTCOMES

3.4.1 Blood pressure

Blood pressure was measured in all individuals after around five minutes rest in supine position (Paper I). An aneroid sphygmomanometer (AB Henry Eriksson, Stockholm, Sweden) for adults (size 12x35 cm) was used and the first phase of Korotkoff sound was recorded as systolic blood pressure and the fifth phase of

Korotkoff sound was recorded as diastolic blood pressure. Blood pressure was measured only once for each individual.

3.4.2 Lipid markers and homocysteine

For evaluation of potential cardiovascular effects of arsenic exposure, homocysteine, a marker for cardiovascular disease (Conri et al. 2000) was measured in EDTA-plasma. The formation of homocysteine is a part of one-carbon metabolism cycle (Anguera et al. 2006), which produces methyl groups for a variety of substrates in the body, including substrates for arsenic methylation. We centrifuged the blood samples after 10 minutes of incubation at room temperature in order to standardize the effect of time before plasma separation on homocysteine concentrations. For evaluation of other potential cardiovascular effects of arsenic, the lipid markers apolipoproteins A and B (Tognon et al. 2012), and triglycerides (Miller et al. 2011) were measured in EDTA-plasma as well. ApoA is major protein component in high density lipoprotein (HDL), and apoB is the protein component of low density lipoprotein (LDL) in plasma. All these were measured as part of routine analyses at Clinical Chemistry, Lund University Hospital, Sweden (Paper I).

3.4.3 Cytokines

Inflammation can induce atherosclerosis (States et al. 2009), and we analyzed a panel of TH1 and TH2 cytokines (interleukins, IL: IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-15 and IL-21) in EDTA-plasma using Luminex XMAP technology on a Bio-plex 200 platform (Bio-Rad, Hercules, CA, US), according to instructions from the manufacturer (Paper I).

3.4.4 Hemoglobin

Venous blood samples were collected in K_2EDTA tubes (Vacuette, Greiner Bio-One GmbH, Greiner, Germany). Hemoglobin was measured in blood from the K_2EDTA tube by using a HemoCue (HemoCue, Hb 201+, Ängelholm, Sweden; Paper I).

3.4.5 Telomere length

Telomere length was measured using real-time quantitative PCR (qPCR) according to the method developed by Cawthon (Cawthon 2002). The telomere length for Andes was reported previously (Li et al. 2012). In this thesis we measured telomere length from Chaco population (Paper II). The basic principle of the method is estimation of relative telomere length by calculating the ratio of telomere repeat products (T) and the product single copy gene (S, in our experiment the single copy gene is the Hemoglobin Subunit beta, *HBB*) for each subject, by the formula $T/S = 2^{-\Delta Ct}$, where Δ^{Ct} = Ct(telomere) – Ct(*HBB*). The ratio is then compared with the ratio of the reference DNA to adjust for differences between runs. The relative telomere length is an arbitrary value. The coefficient of variance (CV) of the reference DNA was 9.8% for Chaco (Paper II) and 10% for Andes (Li et al. 2012).

The telomere length for individuals in the Andes and Chaco were measured at two different time-points and with different reference DNA. To make the telomere length data comparable, we measured the ratio between the two reference DNAs used at the different time-points and adjusted the values of Andes by this ratio, so that all data were based on the same reference DNA. For that, we took reference DNA A (reference DNA used in Li et al. 2012) in the standard curve, and ref DNA B (reference DNA used in the present study) as a sample. We then multiplied the telomere values of Andes with the ratio of ref A/ref B (Paper II, Andes population). The calculation is described below:

Relative TL Andes (prev.) * ref DNA A=relative TL Andes (present) * ref DNA B

Relative TL Andes (present)=relative TL Andes (prev.) * ref DNA A/ ref DNA B

3.4.6 Mitochondrial DNA copy number (mtDNAcn)

Relative mtDNAcn was measured by qPCR according to a method developed by Hou and colleagues (Hou et al. 2010). MtDNA regions are highly polymorphic and may also contain common deletions. Thus, the primers were chosen from a region where mtDNA deletions rarely occur: the mitochondrial $tRNA^{Leu\ (UUR)}$ gene region is rarely deleted and contains only a few rare single nucleotide polymorphisms. Relative mtDNA was measured following a similar principle as for relative telomere length. The relative mtDNAcn was the quotient of the quantity of mtDNAcn and *HBB*, and thus, it is an arbitrary value. The coefficient of variation (CV) of the control sample included in every run was 12% (Paper II).

3.4.7 DNA Methylation

DNA was isolated form peripheral blood using Qiagen DNA Blood Midi kit (Qiagen, Hilden, Germany). DNA quality was evaluated on a NanoDrop spectrophotometer and a Bioanalyzer 2100. The DNA samples showed good quality as observed by a 260nm (DNA) and 280 nm (protein) ratio of >1.80, which indicates relatively pure DNA. DNA was bisulfite treated using EZ DNA Methylation kit (Zymo, CA, USA) according to manufacturer's standard protocol. Approximately 200 ng of bisulfite-treated DNA was taken for hybridization to the Infinium Human-Methylation 450K BeadChip (Illumina, WI, USA) and the analysis was performed at the SCIBLU facility in Lund. Through the 450K BeadChip array it is possible to interrogate 485,000 methylation sites. The array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. It covers 96% of CpG islands, with additional coverage in island shores and the regions flanking them. The 450K BeadChip employs both Infinium I and Infinium II assays, enhancing its breadth of coverage. Infinium, I assay design employs two bead types per CpG locus, one each for the methylated and unmethylated states. The Infinium II design uses one bead type, with the methylation state determined at the single base extension step after hybridization.

Image processing, background correction, quality control, filtering, and normalization (by the SWAN procedure) were performed in the R package minfi. All samples performed well, since they all had at least >98% of the CpGs with detection p-value below 0.01. We removed CpGs for which more than 20% of the samples had a detection p-value above 0.01 (N=871). Furthermore, the following probes were removed: rs probes [probes that measure single nucleotide polymorphisms (SNPs)] and CpH probes (representing nonCpG methylation) (N=3,091), probes with *in silico* nonspecific binding (N = 27,384) (Chen et al. 2013) and probes with common SNPs (according to the function dropLociWithSnps in minfi; N=17,217). In total 436,949 probes were left for further analysis (Paper III). All beadchips were from the same batch.

3.4.8 Gene expression

Blood samples collected in PAX tubes were used for RNA isolation by PAX Gene Blood RNA kit (PreAnalytiX, Qiagen). RNA concentrations and purity were measured by a Nanodrop spectrophotometer (Wilmington, DE, USA) and RNA integrity (RIN) was evaluated on a Bioanalyzer 2100 (Agilent, CA, USA). The RNA samples showed good quality (RIN > 7.5) (Engstrom et al. 2013). A microarray based system using the BeadArray technology (Illumina, CA, USA) was used for gene expression analysis. Total RNA was reverse transcribed to

double-stranded cDNA, followed by an amplification step (*in vitro* transcription) to generate biotin-labelled cRNA. The labelled RNA strand was hybridized to the beads on the DirectHyb HumanHT-12 v4.0 (Illumina) BeadChip containing the complementary gene-specific sequence. After overnight hybridization and washing, the samples were bound to the hybridized BeadChip, which allowed for differential detection of signals when the BeadChips were scanned. The Illumina scanning systems measures fluorescence intensity at each addressed bead location. The intensity of the signal corresponds to the quantity of the respective transcript in the original sample. The experimental analysis was performed at the SCIBLU facility, Lund University.

Gene expression data (fluorescence intensities of the hybridisation probes on the gene expression array) was quantile normalized by log2-transformation in the GenomeStudio software (Illumina). In the beginning, data for 47,323 probes were included in the array before filtering. Probes with a detection p-value below 0.1 (i.e., p-value for the difference between the array and the background noise) in 90% or less of the samples were selected for further analyses, resulting in 14,290 (30%) probes (Paper III).

3.4.9 Pyrosequencing

We validated the results from the 450K BeadChip analyses by measuring DNA methylation for three CpG sites from the genes calcium voltage-gated channel subunit alpha A (*CACNA1A*) and myelin oligodendrocyte glycoprotein (*MOG)* by pyrosequencing. One µg of DNA was bisulfite-treated using the EPITect® kit (Qiagen), eluted in 25 µl and stored at -20 ºC until further use. Of the bisulfite treated DNA, 0.6 to 1.0 µl was used in the PCR. Pyrosequencing assays were designed using Assay design (version 2.0, Qiagen) software. The PCR was performed using pyromark PCR kit (Qiagen) and the pyrosequencing was performed as described previously (Hossain et al. 2015) (Paper III).

3.4.10 Pathway analysis

Paper III: We evaluated both up- and downregulated genes associated to arsenic with q-value (false discovery rate adjusted p-value) < 0.1 using Ingenuity Pathway Analysis (IPA, Qiagen, CA, USA) to identify relevant pathways, diseases and networks. IPA analysis was performed using primary cells, tissues and cells of human blood and organs in the platform, but not cell lines. Arsenic was highlighted in casual networks in order to score master regulators for relationships to arsenic exposure.

3.5 ETHICAL CONSIDERATION

This study was approved by the Health Ministry of Salta, Salta, Argentina; and the Ethics Committee of the Karolinska Institute, Stockholm, Sweden (Papers I-III).

Oral and written informed consents were obtained from participants before they joined the study. Study subjects were only identified by a number assigned to them in the study. None of the participants received feedback of individual results of arsenic concentration in the urine, concentrations of lipid markers, homocysteine, cytokines, telomere length, mtDNA, gene expression or DNA methylation. The participants did, however, receive immediate information about the measurements that were done in the field, which included height, weight, blood pressure and hemoglobin and results from urine strip test (including measurements of presence of red blood cells, protein, glucose and bacteria; Medi-Test Combi 10 SGL Urine Test Strips, Medisave, UK).

On a regular basis, Unit of Metals and Health, IMM, Karolinska Institutet reported and discussed the measured concentrations of arsenic and different trace elements in drinking water with the hospital in San Antonio de los Cobres, the primary health care clinics in the surrounding villages and the Ministry of Health in Salta. Also, results of the published articles have been reported to the Ministry of Health in Spanish. Based on the repeated reporting by the Unit of Metals and Health demonstrating elevated concentration of arsenic in the area, the private company that supplies water in the main village San Antonio de los Cobres has recently (around 2012) installed an arsenic treatment plant in the water purification system.

3.6 STATISTICAL ANALYSES

Papers I-II: Statistical analyses were performed using IBM SPSS software, version 22 (IBM, Chicago, IL, USA). Statistical significance refers to $p < 0.05$.

The associations between the outcomes, U-As, and covariates were evaluated using Spearman's rank correlation coefficient. Mann-Whitney U test was used to determine differences between two study groups (Paper II) and Kruskal-Wallis tests to determine differences among the tertiles of urinary arsenic concentrations (Papers I and II). The linearity between U-As and the outcomes were inspected using scatter plots. In order to improve linearity of associations, U-As, homocysteine and triglycerides were log2-transformed in Paper 1, and mtDNAcn was log2-transformed for both Andes and Chaco in Paper II.

Effects of U-As exposure on outcomes were evaluated by multi-variable adjusted models (linear regression and logistic regression) (Papers I and II). The influence

of efficiency of arsenic metabolism on the associations between U-As and log2-mtDNAcn or U-As and telomere length were analyzed by including an interaction term between U-As and the fraction of each metabolite separately (defined as above and below median of %iAs, %MMA, or %DMA). We also performed linear regression analyses with individuals stratified by median values of the U-As metabolites (Paper II). The statistical models were adjusted for covariates that were associated with exposure and outcomes and also based on the knowledge from published literature (Papers I and II).

Several sensitivity analyses were performed to examine if the associations between U-As exposure and outcomes were influenced by outliers (Paper I and II). To perform sensitivity analyses in Paper I, we excluded women with heart problem, high blood pressure, diabetes, pregnancy and those that were on medication, as well as, outliers for cardiovascular-related markers. In Paper II, we adjusted the outcome variables (mtDNAcn and telomere length) for the cell composition in peripheral blood for both Andes and Chaco study groups. Since none of the study participants from Chaco chewed coca-leaves, we adjusted only the Andes population for chewing coca leaves. Further, few individuals in Chaco reported hyperkeratosis, we also performed sensitivity analyses excluding those individuals to see the effect of arsenic on mtDNAcn and telomere length in the rest of the Chaco population.

Paper III: Statistical analyses were performed using R (v.3.1.3) and SPSS software, version 22 (IBM, Chicago, IL, USA). Principal component analysis (PCA) was performed to evaluate the influence of technical and biological variables on gene expression and DNA methylation. Associations between variables and principal components were evaluated using linear regression analyses. For the PCA, we employed the universally applicable singular value decomposition. In the downstream analyses, we adjusted for cell type proportions with the strongest influence on gene expression or DNA methylation according to the PCA; we did not adjust for all cell types, since the cell type proportions showed strong correlations between the different cell types ($r_s > 0.23$).

In the analyses for the associations between U-As and 1) gene expression or 2) DNA methylation, individuals were grouped into quartiles according to their concentrations of U-As, due to skewed distributions of raw data (also when U-As was natural log transformed) as evident from inspection of histograms. The quartiles for U-As were used as continuous variables (labelled 1 – 4). The association between U-As and gene expression or DNA methylation was evaluated by fitting a robust linear regression model to each array using the R package limma (with empirical Bayes smoothing was applied to the standard errors) adjusting for age, coca chewing and estimated fractions of granulocytes, monocytes and CD4+ cells (models for gene expression) or age, coca, granulocyte

and natural killer cells (models for DNA methylation) based on the respective PCA plots. The influence of efficiency of arsenic metabolism on the associations between U-As and gene expression/DNA methylation was analysed by stratifying the linear regression analyses for below and above median %MMA or %iAs. P-values were adjusted for multiple comparisons by the Benjamini-Hochberg false rate discovery (FDR) method (Benjamini and Hochberg 1995) to obtain q-values. A q-value of 0.05 or lower was considered statistically significant.

4 RESULTS AND DISCUSSION

This section summarizes the main findings of the thesis, with reference to the actual papers. For further details about the results and discussion, the reader is referred to the individual papers (Papers I-III). Figure 9, illustrates the number of study individuals of each Paper from the two study groups Andes and Chaco.

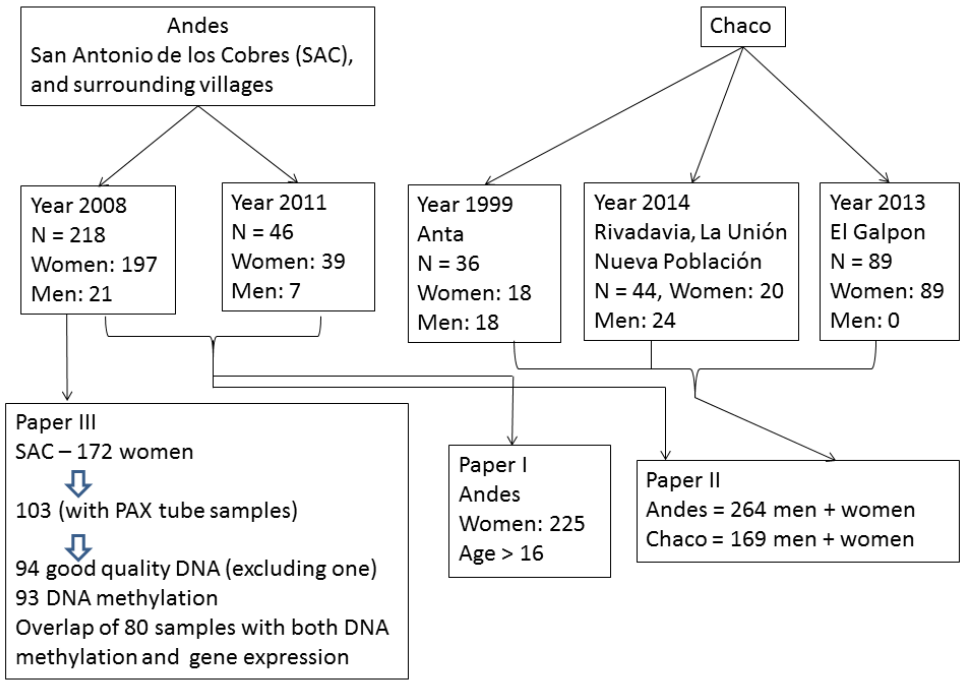

Figure 9:
Schematic diagram of subjects and inclusion in the different scientific papers of the thesis. Both Andes and Chaco belongs to the Province of Salta in Northern Argentina

4.1 EXPOSURE ASSESSMENT OF ARSENIC (Papers I-III)

Urinary arsenic reflects exposure to inorganic arsenic from all sources, i.e. both from drinking water and food (Vahter et al. 2006). Our study participants were chronically exposed to arsenic through drinking water. It is therefore likely that the excretion of arsenic and its metabolites in urine had reached a steady state level. Besides, they had very limited access to fish or sea-food, and their staple food is also not rice like some other arsenic exposed populations (Bangladesh, India and Taiwan) in the world. Thus, the urinary arsenic concentrations would reflect chronic ongoing exposure to inorganic arsenic through drinking water. Indeed, in Andes and Chaco, the water and urinary arsenic concentrations were highly correlated (r_S = 0.72, p<0.001 in Andes and r_S = 0.87, p <0.001 in Chaco), implying that the arsenic exposure in both study groups were predominately through drinking water (Table 1). Figure 10 shows the villages of Andes and Chaco with different water arsenic concentrations.

Table 1: Concentrations of arsenic in drinking water and urine of women in Andes and Chaco.

Study groups	*n*	Arsenic in water (µg/L)	*n*	Urinary arsenic (µg/L)
Andes				
San Antonio de los Cobres	2	208	192	260 (20 – 1251)
Tolar Grande	2	3.5	26	25 (10 – 121)
Salar de Pocitos	2	72	5	73 (27 – 117)
Olacapato	2	12	10	31 (14 – 56)
Santa Rosa de los Pastos Grandes	1	31	15	71 (33 – 118)
Cobres	1	3.5	13	47 (22 – 149)
Rosario de Lerma	3	0.67	3	68 (64 – 147)
Chaco				
Anta	7	212	36	315 (70 – 1843)
El Rincón	2	13	25	43 (17 – 97)
Los Rosales	1	72	22	150 (60 – 877)
El Galpón	7	4.7	42	20 (6.5 – 236)
Rivadavia	2	243	26	346 (76 – 585)
La Unión	1	23	12	53 (27 – 71)
Nueva Población	1	983	6	1943 (623 – 2258)

*The arsenic concentrations in water and urine were analyzed in our laboratory at Karolinska Institutet. The arsenic concentrations in water and urine for Andes population were previously reported in Concha et al. (Concha et al., 2010).

In some studies, due to the strong affinity of arsenic to keratin, arsenic in hair and nails has been used as long-term biomarkers of inorganic arsenic exposure (Hughes 2006). However, analysis of hair and nails were not considered as arsenic biomarkers in our studies of Andes and Chaco, because of the risk of external arsenic contamination of hair and nails (washing and bathing in arsenic-contaminated water).

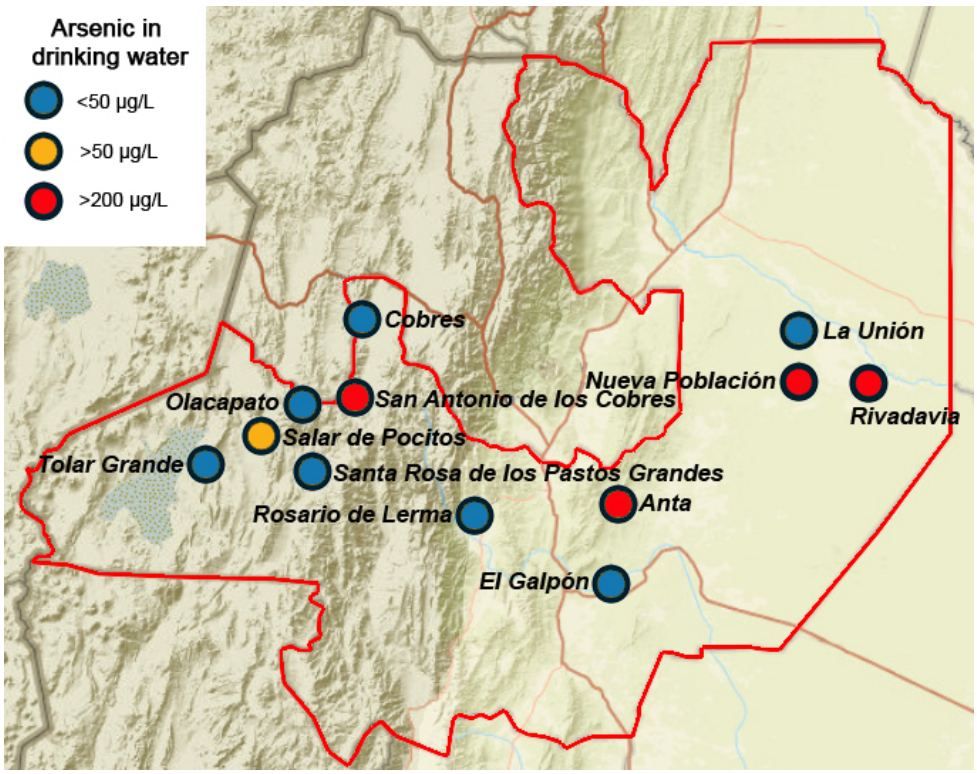

Figure 10:
Study areas in Andes and Chaco showing different water arsenic concentrations. Courtesy: Emilie Stroh

The median water arsenic concentration was ~200 µg/L in San Antonio de los Cobres, the main village of the Andes study group, and ~72 (range: 4.7 to 983) µg/L in Chaco. Water arsenic range in the surrounding villages of San Antonio de los Cobres was 3.5-70 µg/L. In Bangladesh, the median concentration of arsenic in drinking water from 5,966 tube-wells was 62 µg/L (range 0.1 to 864 µg/L) (Jiang et al. 2015). In the US, median concentration of arsenic in drinking water from 2,233 household wells was 2.0 µg /L (range <1.0 to 3,100 µg/L (Knobeloch et al. 2006). In the south-west coast of Taiwan, the median arsenic concentration of artisan well water ranged from 700 to 930 µg /L. After implementing a tap water

system in mid 1970s, the median arsenic concentration in shallow well water became 40 µg /L (range: 0-300 µg /L) (Tseng et al. 1997). These data suggest that the populations studied in this thesis were exposed to moderate arsenic level from drinking water compared to the studied populations from the US, Bangladesh and Taiwan.

4.2 ARSENIC EXPOSURE, BLOOD PRESSURE AND EARLY CARDIOVASCULAR MARKERS (Paper I)

In Paper I, we found that increasing arsenic exposure, measured as the sum of arsenic metabolites in urine (U-As), was associated with decreasing diastolic blood pressure among Andeans (Ameer et al. 2015). This is in contrast to previous cross-sectional and case-control studies among Bangladeshi and Mongolian populations that found positive associations between arsenic and hypertension (Huda et al. 2014, Guo et al. 2007, Rahman et al. 1999). Longitudinal studies have also shown an increase in both systolic and diastolic blood pressures in relation to arsenic exposure among Bangladeshi population (Jiang et al. 2015) and in an US pregnancy cohort (Farzan et al. 2015). *In vitro* studies have shown that arsenic exposure alters vascular tone in blood vessels by suppressing vasorelaxation (Lee et al. 2003). In animal models it has been shown that arsenic exposure increases superoxide accumulation and impaired nitric oxide formation in endothelial cells (Barchowsky et al. 1996; Barchowsky et al. 1999; Lee et al. 2005). However, one cross-sectional study in US reported no association with low arsenic exposure and blood pressure (Jones et al. 2011). The Andes study participants were apparently healthy and with better arsenic metabolism capacity compared to the other arsenic exposed populations (Engstrom et al. 2011), which would suggest less arsenic-related toxicity among Andeans. It would be interesting to conduct a longitudinal study with the same population and examine systolic and diastolic blood pressure in relation to arsenic exposure over time.

In Paper I, we also found that U-As concentrations were inversely associated with the ratio apoB/A (Figure 11), and positively associated with apoA. Further, there were inverse association between U-As and triglycerides, and U-As and homocysteine though the associations were not statistically significant. Studies from Bangladesh showed that patients with arsenicosis (N = 115, mean water arsenic = 218 µg/L) individuals had significantly lower levels of total cholesterol, HDL and LDL as compared with unexposed individuals (N = 120) (Nabi et al. 2005); another study from Bangladesh reported individuals from arsenic-endemic area (N = 218, mean water arsenic = 175 µg/L) had lower levels of total cholesterol, LDL, HDL but higher oxidized LDL compared to individuals form

non-endemic area (N = 108, mean water arsenic = 2.3 μg/L) (Karim et al. 2013). Arsenic exposure was not associated with triglyceride levels in these studies. Also, a study on Mexican individuals showed that moderate water arsenic concentrations (25.5 – 47.8 μg/L) were associated with elevated triglycerides, high total cholesterol, diabetes, and higher HDL (Mendez et al. 2016). Thus, data on the associations between arsenic exposure and lipid profiles are inconsistent, and whether these lipid markers may serve as causal intermediates between arsenic exposure and cardiovascular disease are not clear. For instance, in a study of arsenic-hyper endemic (water arsenic = 700-930 μg/L) villages of southwestern Taiwan, lipid profiles (total cholesterol, triglycerides, HDL, LDL, apoA and apoB) did not differ significantly between arsenic exposed individuals (N = 533) and arsenic exposed individuals with peripheral vascular disease (N = 63) (Tseng et al. 1997). Another study with same study populations from same area in Taiwan reported dose response relationship between duration of arsenic exposure and ischemic heart disease, and ischemic heart disease with hypertension, but no significant association between ischemic heart disease and lipid profiles (Hsueh et al. 1998b). However, a study from same area in Taiwan reported a dose-response relationship between arsenic exposure (duration, average concentration, cumulative exposure) and prevalence of carotid atherosclerosis. Moreover, they reported a dose response association between carotid atherosclerosis and serum total cholesterol, triglycerides, HDL and LDL (Wang et al. 2002). Taking these studies into consideration it can be implied that aberrant lipid profile is associated with different kinds of cardiovascular diseases, but the association between arsenic exposure and aberrant lipid profile is inconsistent.

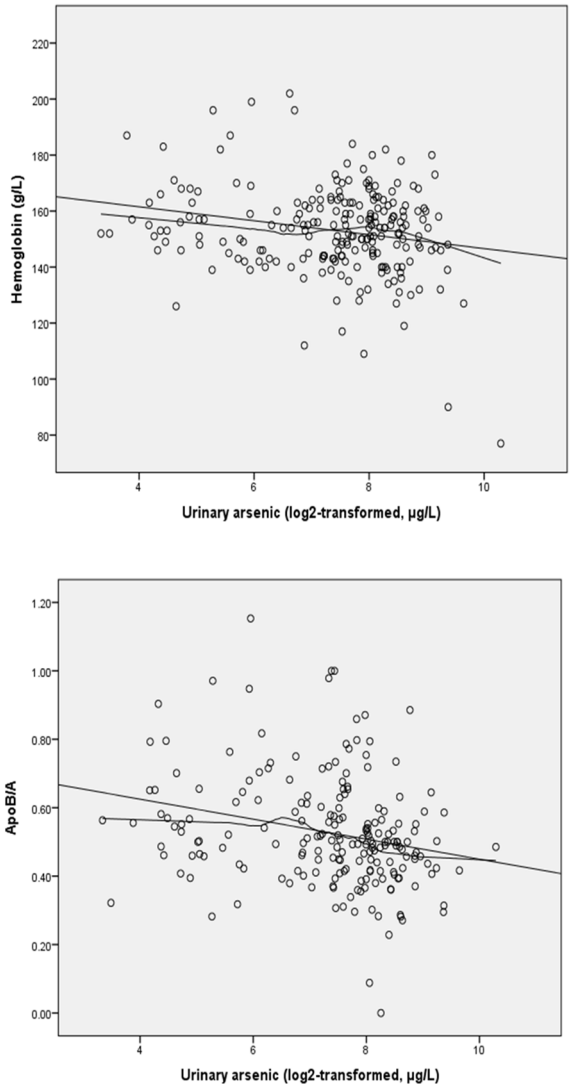

Figure 11:
Scatter plots depicting associations between urinary arsenic concentrations and hemoglobin (top) and arsenic and apoB/A (bottom). Effect estimates and p-values of adjusted linear regression models between arsenic, hemoglobin and apoB/A are presented in Paper I.

In paper I we also reported that U-As concentrations were inversely associated with hemoglobin concentrations (Figure 11). Our study participants had high hemoglobin concentrations (median: 154 g/L; range: 77-202 g/L), which probably is due to a diet rich in animal proteins, and the fact that these indigenous people live in high altitudes for long time, their cardiovascular system has adapted to the

low oxygen pressure (Frisancho 2013). A study on Pakistani pregnant women living in high altitudes showed higher hemoglobin levels compared to pregnant women living in low altitude, suggesting hemoglobin concentrations increases with elevated altitudes (Umar et al. 2015). Another study on pregnant women living in high altitudes in Peru showed that the hemoglobin levels are usually elevated among populations living in high altitudes, so the cut-off for normal hemoglobin concentrations needs to be increased for those populations. Therefore, the inverse association between arsenic and hemoglobin in Paper I (in spite of high hemoglobin concentrations), may be an arsenic-associated adverse effect in Andean women.

Though studies have shown arsenic-related immune-suppression (Ahmed et al. 2014; Choudhury et al. 2016), our study could not find a clear association between arsenic exposure and cytokine response. Among the cytokines analyzed, only anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine IL-12 showed a weak correlation (both positively) with U-As concentrations, suggesting that in the Andes arsenic was not associated with inflammation of the cardiovascular system.

4.3 ARSENIC METABOLISM DIFFERED BETWEEN TWO STUDY GROUPS (Paper II)

In Paper II, we found that %iAs, %MMA were lower and %DMA were higher for the individuals living in Andes compared to Chaco, reflecting better arsenic metabolism among Andeans compared to Chaco (Figure 12). Andeans are mainly indigenous in origin whereas Chaco participants are mainly of Spanish/European descent, which means that they have different genetic background. Previously, Engström et al. reported that polymorphisms related to better arsenic metabolism capacity is much more prevalent among Andean compared with Bangladeshi women (Engstrom et al. 2011). Recently, studies have shown that Andeans have adapted to an arsenic rich environment through positive selection of genetic variants related to an efficient metabolism (Schlebusch et al. 2013; Schlebusch et al. 2015).

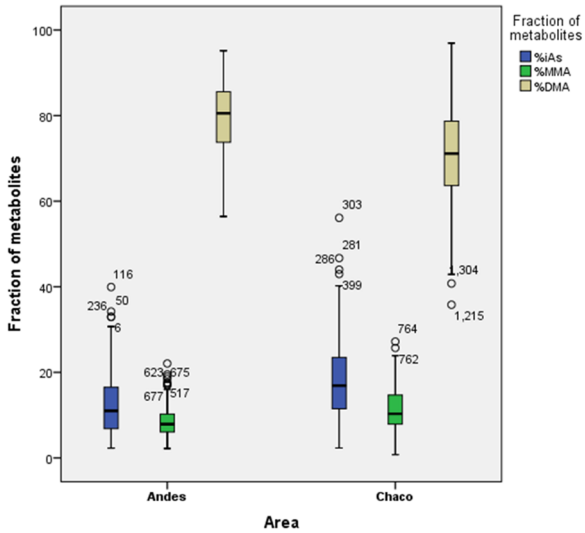

Figure 12:
Urinary arsenic metabolites (percentages of iAs, MMA and DMA) in Andes and Chaco, respectively.

4.4 ARSENIC AND TELOMERE LENGTH (Paper II)

In Paper II we analyzed arsenic exposure (U-As) in relation to telomere length in peripheral blood and we found that arsenic was associated with longer telomeres in both Andes and Chaco study groups. Our findings were consistent with previous reports on arsenic exposure and telomere length, both *in vivo* and *in vitro*. Gao *et al.* reported that in Bangladeshi population with arsenic exposure ranging 0.1 to 864 µg/L (median: 50 µg/L), higher arsenic was associated with longer telomeres compared to very low exposure (Gao et al. 2015). The effect of arsenic on telomeres may be mediated by stimulating the expression of telomerase reverse transcriptase *TERT* (main telomere-maintaining enzyme). In a population in Inner Mongolia, arsenic exposure was found to be related to the expression of *TERT*, and there was also an association between expression of *TERT* and the severity of arsenic-related hyperkeratosis (Mo et al. 2009). In human cord blood cells, sub-nM arsenite exposure increased *TERT* gene and protein expression *in vitro*, which maintained the telomere length, whereas at 1 µM arsenite exposure, *TERT* expression and telomere length decreased (Ferrario et al. 2009). Analyzing these reports on arsenic and telomere length it can be hypothesized that long-term or chronic arsenic exposure at moderate level can extend the life span of cells by activation of *TERT* resulting in elongation of telomere length, but at higher exposure arsenic might affect the telomere length by shortening it.

4.5 ARSENIC AND MITOCHONDRIAL DNA COPY NUMBER (Paper II)

In Paper II we reported that arsenic exposure was associated with increasing mtDNAcn in peripheral blood, particularly in participants in Chaco and to a lesser extent in participants in Andes. Arsenic in relation to mtDNAcn has been studied to a very little extent before. Arsenic related toxicity has been related to competitive inhibition of phosphates that interact with enzymes, thereby affecting, among others, enzymes involved in oxidative phosphorylation in mitochondria (Yang et al. 2012). One study reported that arsenic-induced skin cancer patients had increased mitochondrial biogenesis and function compared to healthy subjects (Lee et al. 2011). Whereas, another study found that human-hamster hybrid A(L) cells treated with high concentrations of sodium arsenite (0.5 -1 mg/L), showed decreased mtDNAcn along with increased large heteroplasmic mtDNA deletions (Partridge et al. 2007). Hou et al. suggested that increased oxidative stress from toxic compounds has dual response on mtDNA (Hou et al. 2010): mild stress, such as low-to moderate arsenic exposure, might stimulate mtDNA synthesis and increase the number of mitochondria to fulfil the need for cellular respiratory capacity, however, excessive oxidative stress, such as high arsenic exposure, may cause decreased mtDNA synthesis, possibly due to increased defects in mitochondria resulting in apoptosis and cell death.

We also found significant positive correlations between telomere length and mtDNAcn in peripheral blood in both study groups. *TERT* maintains telomere length under moderate oxidative stress, but in a time and dose-dependent manner, *TERT* gets excluded from the nucleus and localizes in the mitochondria, giving protection to mitochondria by decreasing mitochondrial superoxide production and cellular peroxide levels (Ahmed et al. 2008). This may explain our finding of longer telomere length and increased mtDNAcn in both study groups. We hypothesize, that arsenic exposure increases oxidative stress that stimulates expression of *TERT* (Figure 13). Expression of *TERT* elongates telomere length and after that it moves to mitochondria to give protecting from oxidative stress, which in turn results in increase in mtDNAcn.

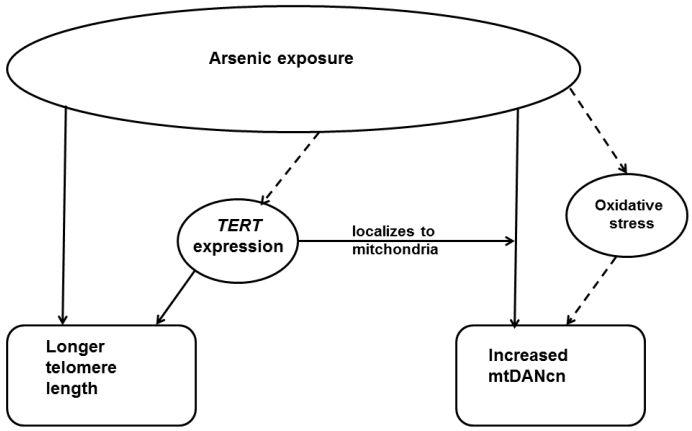

Figure 13:
Proposed hypothesis of arsenic exposure related TERT expression, which in turn can induce longer telomere length and increased mtDNAcn.

4.6 MODIFICATION BYARSENIC METABOLISM EFFICIENCY, MtDNAcn, TELOMERE LENGTH AND DISEASE RISK

In Paper II, we observed that the arsenic metabolism efficiency modified the relation between arsenic and both mtDNAcn and telomere length for both study groups. After dividing the metabolites (%iAs, %MMA and %DMA) concentrations in two groups (based on their median), the group with above median %iAs had stronger effect estimates and significant associations compared with below median %iAs group for mtDNAcn and telomere length in both populations. The trend was similar for %DMA stratified data, where the group below median %DMA had stronger effect estimate and significant associations for mtDNAcn compared to the above median group, though for telomere length the trend was similar but not significant. The interactions were significant for mtDNAcn but not for telomere length in both populations.

In prospective studies, longer telomeres in blood have been associated with increased risk for lung (Seow et al. 2014; Shen et al. 2011), and pancreatic cancer (Lynch et al. 2013). In prospective studies higher mtDNAcn in blood have been associated with breast cancer (Lemnrau et al. 2015; Thyagarajan et al. 2013) and in case-control studies higher mtDNAcn has been associated with head and neck

cancer (Cheau-Feng Lin et al. 2014), prostate cancer (Zhou et al. 2014) and chronic lymphocytic lymphoma (Hosnijeh et al. 2014). Arsenic is known to cause lung cancer and has also been associated with, kidney, breast and prostate cancers (Bardach et al. 2015; Ferreccio et al. 2000; Lopez-Carrillo et al. 2014; Wu et al. 1989). Since the associations between U-As and mtDNAcn and U-As and telomere length were observed particularly among individuals with inefficient arsenic metabolism, it can be hypothesized that the inefficient metabolizers are at high risk for arsenic-associated cancer. Arsenic metabolism differs between individuals and populations (Vahter 2002), and inefficient metabolism is a marker for increased susceptibility for different diseases including cancer (Antonelli et al. 2014; Engstrom et al. 2015). Short or long telomere length has been proposed as a biological marker for different tumor type and malignancies (Svenson and Roos 2009). In this thesis we propose mtDNAcn and telomere length as promising arsenic-related malignancy marker for future cancer risk.

The findings of age being inversely associates with telomere length were similar to many reported studies (D'Mello et al. 2015; Haycock et al. 2014; Ormseth et al. 2016; Weischer et al. 2012). However, all of these studies also reported that shorter telomere length is associated with cardiovascular disease. We can hypothesize that in Andes study group, the longer telomeres is giving protection to cells by delaying cellular ageing, resulting no apparent adverse effect in cardiovascular health.

4.7 ARSENIC, DNA METHYLATION AND GENE EXPRESSION (Paper III)

In Paper III, in Andean women increasing arsenic in urine was associated with lower gene expression and higher DNA methylation in peripheral blood, suggesting that the arsenic-related down-regulation of gene expression is potentially mediated by DNA hypermethylation. Previous work in mice support our findings: mice exposed to arsenic via drinking water had increased DNA methylation and decreased gene expression of genes with low baseline methylation levels (Boellmann et al. 2010). In humans, arsenic exposure has been associated with global DNA hypermethylation in blood in adults (Niedzwiecki et al. 2013; Pilsner et al. 2007). Still, we could not link arsenic-related changes in gene expression to changes in DNA methylation for individual genes. An explanation to this may be due to the limited coverage on the arrays for some gene regions. Arsenic metabolism efficiency seemed to modify the associations between arsenic exposure and gene expression or DNA methylation to some extent: different pathways were enriched in efficient and inefficient metabolizers

of arsenic, suggesting that different metabolism efficiency results in differences in toxicity of arsenic or biological response to arsenic (Engstrom et al. 2015). However, it is important to note that the women in this population have an efficient arsenic metabolism, likely through adaptation to arsenic (Eichstaedt et al. 2015; Schlebusch et al. 2013; Schlebusch et al. 2015) and that the effects of arsenic may differ in men between populations due to differences in arsenic metabolism efficiency.

We plotted four genes significantly associated with arsenic exposure (not using U-As quartiles) and gene expression, the associations were mostly linear (Figure 14). Therefore, using linear regression model for the associations were justified, nevertheless we may have missed non-linear associations between arsenic and gene expression or DNA methylation

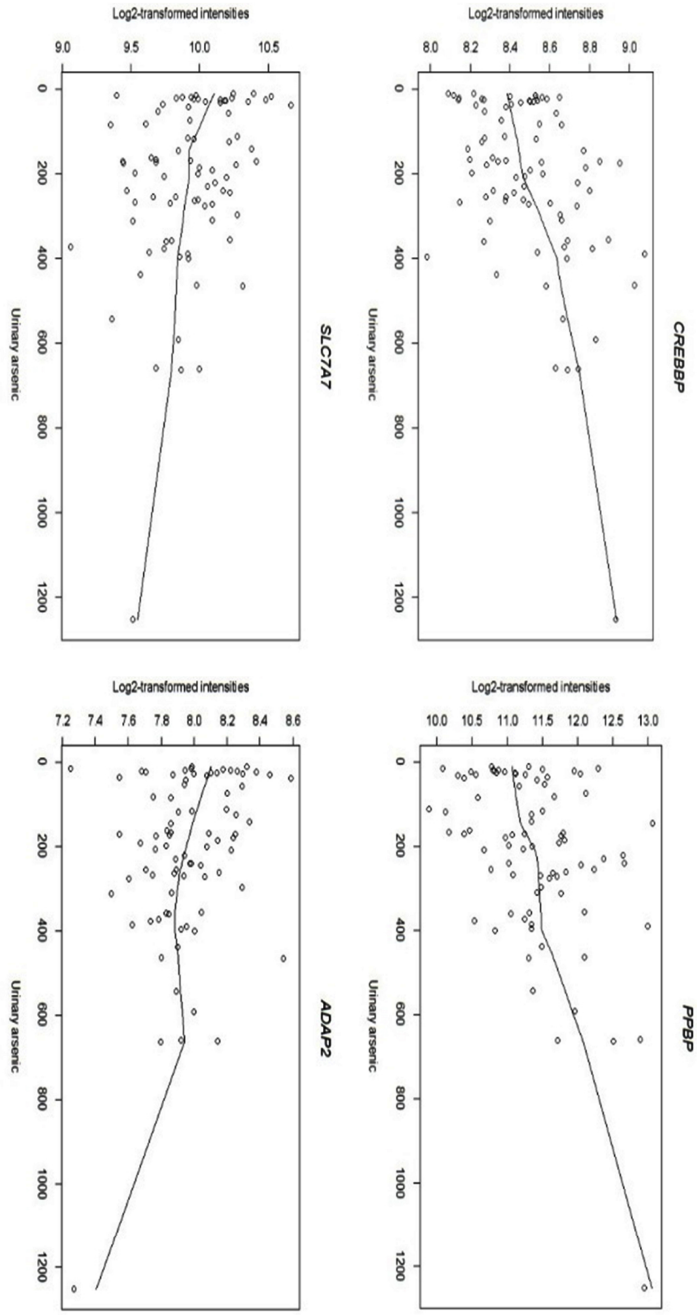

Figure 14:
Scatter plot depicting association between arsenic and gene expression of *CREBBP, PPBP, SLC7A7* and *ADAP2*. Urinary arsenic concentrations were in µg/L.

Pathways and networks upregulated with increasing arsenic exposure have previously been related to arsenic, such as DNA damage by ATM (ataxia-telangiectasia mutated) signaling, and NRF-2 (nuclear factor-E2 related factor 2) mediated oxidative response. DNA damage responsive kinases ATM appears to play a crucial role in arsenite-induced G2/M phase arrest by inducing cell death or decreasing proliferation. The NRF-2 pathway and early antioxidant response, a key regulator of defense to increased oxidative stress, were upregulated in hepatic and kidney cells, spleen, thymus, as well as peripheral blood in arsenic-exposed (5-20 mg/kg) mice.

The association between arsenic exposure and gene expression seemed to differ depending on metabolism efficiency. In the above median %MMA group, more stress related pathways were identified compared with the below median %MMA group. In the above median %MMA group, U-As was associated with networks and pathways related to NRF2 mediated oxidative stress, xenobiotic metabolism signaling, mitochondrial dysfunction, HIF-1α signaling and granzyme A signaling. In the below median %MMA strata, U-As was associated with networks related to DNA damage signaling, DNA repair and suppression of tumor formation; such as chronic myeloid leukemia signaling, glioma signaling, role of *BRCA1* in DNA damage response, AMPK signaling and ATM signaling. In above median %MMA group, genes were associated with mitochondria associated energy production cycle. Arsenic related toxicity has been suggested to be related to competitive inhibition of phosphates that binds with enzymes, so it affects many enzymes in metabolism and oxidative phosphorylation (Yang et al. 2012).

Most CpG sites showed increased DNA methylation in relation to arsenic exposure: among top 1000 arsenic-associated CpGs, 87% of the CpGs had increased methylation. Pathway analysis showed that CpGs present in genes that were mainly associated with cancer and to a lesser extent with developmental and hereditary disorders. Top canonical pathways associated were D-myo-inositol (1,4,5)-trisphosphate degradation, iD-myo-inositol Hexakisphosphate Biosynthesis II, and D-myo-inositol (1,3,4)-trisphosphate Biosynthesis. The gene associated with these pathways is *INPP5A1*, regulate regulator of PI3K/AKT signaling, insulin signaling, endocytosis, vesical trafficking, cell migration, proliferation and apoptosis (Hakim et al. 2012). Other pathways were enriched, however, this was based on elevated methylation in only one CpG site in gene, such as *IDH3* linked to the TCA cycle and maturity onset diabetes of young (MODY) signaling, involving the calcium channel gene *CACNA1A*. Further evidence that arsenic targets *INPP5A* comes from a study on arsenic-related skin cancer where genetic variation in *INPP5A* appears to have a role in susceptibility (Seow et al. 2015).

For the association between arsenic exposure and DNA methylation, genes present in both below and above median %MMA groups were associated with cancer and

endocrine system disorder. In the above median %MMA group, type II diabetes mellitus signaling was enriched. Arsenic exposure has previously been associated with diabetes (Kuo et al. 2013). Further, in the above median %MMA group, U-As was associated with pathways related to PPAR/RXR activation, UVA-induced MAPK signaling, sphingomyelin metabolism, sphingosine-1-phosphate signaling and type 2 diabetes mellitus signaling, whereas, in the below median %MMA group U-As was mainly associated with dermatological diseases and conditions, gastrointestinal disease and metabolic disease.

In the pathway analysis, cancer was the predominant arsenic-related disease for both gene expression and DNA methylation. However, there were no genes for which U-As were statistically significantly associated with both gene expression and DNA methylation. We further validated methylation levels measured by 450K by pyrosequencing. Methylation of *CACNA1A* and MOG measured by pyrosequencing were strongly correlated with methylation measured by 450K (*CACNA1A* $r_S = 0.92$; $p < 0.001$; *MOG* $r_S = 0.84$; $p < 0.001$).

Though we found arsenic exposure was associated with longer telomeres (Paper I), we did not find association between arsenic exposure and *TERT* in gene expression (Paper III). However, Li et al. found a positive association with arsenic exposure and *TERT* expression using the same global gene expression data (Li et al. 2012), the association went away after background correction in my study (Paper III). This may be due to the fact that *TERT* expression is restricted notably to germ cells and stem or progenitor cells in adults (Flores et al. 2006), whereas we evaluated gene expression and DNA methylation in peripheral blood. Moreover, we have found arsenic exposure is associated with increased mtDNAcn (Paper II), and we hypothesized that this is due to oxidative stress generated both by arsenic exposure and during oxidative phosphorylation. In Paper III, we found further support for this, since the association between arsenic and gene expression canonical pathways related to xenobiotic metabolism signaling and NRF-2 mediated oxidative stress predominated. Also among the inefficient metabolizers canonical pathways related to oxidative stress (NRF2-mediated oxidative stress, xenobiotic metabolism signaling, mitochondrial dysfunction and oxidative stress) predominated and mitochondrial phosphorylation related genes such as down regulation of *ATP5A1* (encodes a subunit for ATP synthase), *NDUFA11* (encodes NADH dehydrogenase enzyme in complex I in mitochondria) predominated indicating disruption in mitochondrial phosphorylation and generation of increased oxidative stress which cells may have tried to compensate through generation of increased mtDNAcn (Paper II). Also our prediction for future cancer risk related to these two DNA markers in the population was also validated since cancer came up as the most predominant disease in relation to arsenic exposure and gene expression and arsenic exposure and DNA methylation (Paper III).

5 CONCLUSIONS

In this thesis we report that chronic exposure to arsenic via drinking water was not associated with adverse effects on early markers of cardiovascular disease. However, arsenic exposure was associated with genotoxic effect, as well as gene expression and DNA methylation.

1. Arsenic exposure was inversely associated diastolic blood pressure and apolipoprotein B/A among Andean women but there was no association between arsenic and other risk markers for cardiovascular disease. These findings indicate that arsenic is not associated with adverse effects on early cardiovascular markers in this population. Arsenic exposure was inversely associated with hemoglobin concentrations in Andean women.
2. Arsenic exposure was associated with longer telomere length in Andes and Chaco study groups, suggesting that arsenic exposure can affect *TERT* expression leading to increase in telomere length.
3. Arsenic was associated with increased mtDNAcn in Chaco. We hypothesize that arsenic may increase mtDNAcn to compensate with arsenic-related oxidative stress.
4. The effect of arsenic on telomere length and mtDNAcn was particularly evident among inefficient arsenic metabolizers indicating that this group is at risk for future disease, especially cancer.
5. Arsenic exposure was associated with increased DNA methylation and decreased gene expression among Andean women. More oxidative stress and arsenic-toxicity related pathways were evident among the inefficient arsenic metabolizers, suggesting increase disease risk for them.

6 FUTURE RESEARCH

Based on the conclusions the future research should be aimed to understand the following aspects.

1. We found that arsenic exposure via drinking water was not associated with adverse effects on biomarkers of cardiovascular health. This can be followed up in Chaco study group.
2. To better understand the variability in fractions of arsenic metabolites between Andes and Chaco study group, further studies are required to evaluate the association between arsenic metabolism and genetic polymorphisms in arsenic metabolizing genes in Chaco study group.
3. We found that arsenic exposure was associated with longer telomere length. Telomere length is influenced by *TERT* expression. Studies are needed to better understand arsenic exposure related *TERT* expression and *TERT* regulated effects on telomere and mtDNAcn.
4. Follow-up studies are needed to validate alterations in DNA damage markers and its relation to future diseases, particularly cancer.
5. Future follow-up studies are needed to establish arsenic-toxicity related health effects in relation to arsenic-related gene expression and DNA methylation.

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8 REFERENCES

Abhyankar LN, Jones MR, Guallar E, Navas-Acien A. 2012. Arsenic exposure and hypertension: A systematic review. Environ Health Perspect 120:494-500.

Ahmed S, Passos JF, Birket MJ, Beckmann T, Brings S, Peters H, et al. 2008. Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. J Cell Sci 121:1046-1053.

Ahmed S, Mahabbat-e Khoda S, Rekha RS, Gardner RM, Ameer SS, Moore S, et al. 2011. Arsenic-associated oxidative stress, inflammation, and immune disruption in human placenta and cord blood. Environ Health Perspect 119:258-264.

Ahmed S, Moore SE, Kippler M, Gardner R, Hawlader MD, Wagatsuma Y, et al. 2014. Arsenic exposure and cell-mediated immunity in pre-school children in rural Bangladesh. Toxicol Sci 141:166-175.

Ahsan H, Chen Y, Parvez F, Zablotska L, Argos M, Hussain I, et al. 2006. Arsenic exposure from drinking water and risk of premalignant skin lesions in Bangladesh: Baseline results from the health effects of arsenic longitudinal study. Am J Epidemiol 163:1138-1148.

Ahsan H, Chen Y, Kibriya MG, Slavkovich V, Parvez F, Jasmine F, et al. 2007. Arsenic metabolism, genetic susceptibility, and risk of premalignant skin lesions in Bangladesh. Cancer Epidemiol Biomarkers Prev 16:1270-1278.

Ameer SS, Engstrom K, Harari F, Concha G, Vahter M, Broberg K. 2015. The effects of arsenic exposure on blood pressure and early risk markers of cardiovascular disease: Evidence for population differences. Environ Res 140:32-36.

Andrew AS, Karagas MR, Hamilton JW. 2003. Decreased DNA repair gene expression among individuals exposed to arsenic in United States drinking water. Int J Cancer 104:263-268.

Anic GM, Sondak VK, Messina JL, Fenske NA, Zager JS, Cherpelis BS, et al. 2013. Telomere length and risk of melanoma, squamous cell carcinoma, and basal cell carcinoma. Cancer Epidemiol 37:434-439.

Anguera MC, Field MS, Perry C, Ghandour H, Chiang EP, Selhub J, et al. 2006. Regulation of folate-mediated one-carbon metabolism by 10-formyltetrahydrofolate dehydrogenase. J Biol Chem 281:18335-18342.

Antonelli R, Shao K, Thomas DJ, Sams R, 2nd, Cowden J. 2014. *AS3MT*, *GSTO*, and *PNP* polymorphisms: Impact on arsenic methylation and implications for disease susceptibility. Environ Res 132:156-167.

Argos M, Parvez F, Rahman M, Rakibuz-Zaman M, Ahmed A, Hore SK, et al. 2014. Arsenic and lung disease mortality in Bangladeshi adults. Epidemiology 25:536-543.

Arriaza B, Amarasiriwardena D, Cornejo L, Standen V, Byrne S, Bartkus L, Bandak B. 2010. Exploring chronic arsenic poisoning in pre-Columbian Chilean mummies. J Archaeol Sci 37:1274-1278.

Bailey KA, Wu MC, Ward WO, Smeester L, Rager JE, Garcia-Vargas G, et al. 2013. Arsenic and the epigenome: Interindividual differences in arsenic metabolism related to distinct patterns of DNA methylation. J Biochem Mol Toxicol 27:106-115.

Barchowsky A, Dudek EJ, Treadwell MD, Wetterhahn KE. 1996. Arsenic induces oxidant stress and NF-kappa B activation in cultured aortic endothelial cells. Free Radic Biol Med 21:783-790.

Barchowsky A, Roussel RR, Klei LR, James PE, Ganju N, Smith KR, et al. 1999. Low levels of arsenic trioxide stimulate proliferative signals in primary vascular cells without activating stress effector pathways. Toxicol Appl Pharmacol 159:65-75.

Bardach AE, Ciapponi A, Soto N, Chaparro MR, Calderon M, Briatore A, et al. 2015. Epidemiology of chronic disease related to arsenic in Argentina: A systematic review. Sci Total Environ 538:802-816.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological):289-300.

Bhowmick S, Halder D, Nriagu J, Guha Mazumder DN, Roman-Ross G, Chatterjee D, et al. 2014. Speciation of arsenic in saliva samples from a population of West Bengal, India. Environ Sci Technol 48:6973-6980.

Blackburn EH. 2010. Telomeres and telomerase: The means to the end (nobel lecture). Angew Chem Int Ed Engl 49:7405-7421.

Boellmann F, Zhang L, Clewell HJ, Schroth GP, Kenyon EM, Andersen ME, et al. 2010. Genome-wide analysis of DNA methylation and gene expression changes in the mouse lung following subchronic arsenate exposure. Toxicol Sci 117:404-417.

Broberg K, Bjork J, Paulsson K, Hoglund M, Albin M. 2005. Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer. Carcinogenesis 26:1263-1271.

Broberg K, Ahmed S, Engstrom K, Hossain MB, Jurkovic Mlakar S, Bottai M, et al. 2014. Arsenic exposure in early pregnancy alters genome-wide DNA methylation in cord blood, particularly in boys. J Dev Orig Health Dis 5:288-298.

Brouilette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, et al. 2007. Telomere length, risk of coronary heart disease, and statin treatment in the west of Scotland primary prevention study: A nested case-control study. Lancet 369:107-114.

Bunderson M, Coffin JD, Beall HD. 2002. Arsenic induces peroxynitrite generation and cyclooxygenase-2 protein expression in aortic endothelial cells: Possible role in atherosclerosis. Toxicol Appl Pharmacol 184:11-18.

Bundschuh J, Litter MI, Parvez F, Roman-Ross G, Nicolli HB, Jean JS, et al. 2012. One century of arsenic exposure in Latin America: A review of history and occurrence from 14 countries. Sci Total Environ 429:2-35.

Calatayud M, Gimeno-Alcaniz JV, Velez D, Devesa V. 2014. Trivalent arsenic species induce changes in expression and levels of proinflammatory cytokines in intestinal epithelial cells. Toxicol Lett 224:40-46.

Carignan CC, Punshon T, Karagas MR, Cottingham KL. 2016. Potential exposure to arsenic from infant rice cereal. Ann Glob Health 82:221-224.

Cawthon RM. 2002. Telomere measurement by quantitative pcr. Nucleic Acids Res 30:e47.

Cheau-Feng Lin F, Jeng YC, Huang TY, Chi CS, Chou MC, Chin-Shaw Tsai S. 2014. Mitochondrial DNA copy number is associated with diagnosis and prognosis of head and neck cancer. Biomarkers 19:269-274.

Chen S, Xie X, Wang Y, Gao Y, Xie X, Yang J, et al. 2014. Association between leukocyte mitochondrial DNA content and risk of coronary heart disease: A case-control study. Atherosclerosis 237:220-226.

Chen Y, Wu F, Liu M, Parvez F, Slavkovich V, Eunus M, et al. 2013a. A prospective study of arsenic exposure, arsenic methylation capacity, and risk of cardiovascular disease in Bangladesh. Environ Health Perspect 121:832-838.

Chen Y, Wu F, Parvez F, Ahmed A, Eunus M, McClintock TR, et al. 2013b. Arsenic exposure from drinking water and QT-interval prolongation: Results from the Health Effects of Arsenic Longitudinal Study. Environ Health Perspect 121:427-432.

Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, et al. 2013. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics 8:203-209.

Chen YC, Guo YL, Su HJ, Hsueh YM, Smith TJ, Ryan LM, et al. 2003. Arsenic methylation and skin cancer risk in southwestern Taiwan. J Occup Environ Med 45:241-248.

Chin KV, Tanaka S, Darlington G, Pastan I, Gottesman MM. 1990. Heat shock and arsenite increase expression of the multidrug resistance (*MDR1*) gene in human renal carcinoma cells. J Biol Chem 265:221-226.

Chiou HY, Hsueh YM, Hsieh LL, Hsu LI, Hsu YH, Hsieh FI, et al. 1997. Arsenic methylation capacity, body retention, and null genotypes of glutathione S-transferase M1 and T1 among current arsenic-exposed residents in Taiwan. Mutat Res 386:197-207.

Choudhury S, Gupta P, Ghosh S, Mukherjee S, Chakraborty P, Chatterji U, et al. 2016. Arsenic-induced dose-dependent modulation of the NF-κB/IL-6 axis in thymocytes triggers differential immune responses. Toxicology 357-358:85-96.

Concha G, Nermell B, Vahter M. 2006. Spatial and temporal variations in arsenic exposure via drinking-water in northern Argentina. J Health Popul Nutr 24:317-326.

Concha G, Broberg K, Grander M, Cardozo A, Palm B, Vahter M. 2010. High-level exposure to lithium, boron, cesium, and arsenic via drinking water in the Andes of northern Argentina. Environ Sci Technol 44:6875-6880.

Conri C, Constans J, Parrot F, Skopinski S, Cipriano C. 2000. Homocysteinemia: Role in vascular disease. Presse Med 29:737-741.

Cui X, Kobayashi Y, Hayakawa T, Hirano S. 2004. Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats. Toxicol Sci 82:478-487.

D'Erchia AM, Atlante A, Gadaleta G, Pavesi G, Chiara M, De Virgilio C, et al. 2015. Tissue-specific mtDNA abundance from exome data and its correlation with mitochondrial transcription, mass and respiratory activity. Mitochondrion 20:13-21.

D'Mello MJ, Ross SA, Briel M, Anand SS, Gerstein H, Pare G. 2015. Association between shortened leukocyte telomere length and cardiometabolic outcomes: Systematic review and meta-analysis. Circ Cardiovasc Genet 8:82-90.

Dangleben NL, Skibola CF, Smith MT. 2013. Arsenic immunotoxicity: A review. Environ Health 12:73.

de Vera ME, Wong JM, Zhou JY, Tzeng E, Wong HR, Billiar TR, et al. 1996. Cytokine-induced nitric oxide synthase gene transcription is blocked by the heat shock response in human liver cells. Surgery 120:144-149.

Deng G, Nguyen A, Tanaka H, Matsuzaki K, Bell I, Mehta KR, et al. 2006. Regional hypermethylation and global hypomethylation are associated with altered chromatin conformation and histone acetylation in colorectal cancer. Int J Cancer 118:2999-3005.

Drobna Z, Waters SB, Devesa V, Harmon AW, Thomas DJ, Styblo M. 2005. Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase. Toxicol Appl Pharmacol 207:147-159.

Eichstaedt CA, Antao T, Cardona A, Pagani L, Kivisild T, Mormina M. 2015. Positive selection of *AS3MT* to arsenic water in Andean populations. Mutat Res 780:97-102.

Emerging Risk Factors Collaboration: Di Angelantonio E, Gao P, Pennells L, Kaptoge S, Caslake M, et al. 2012. Lipid-related markers and cardiovascular disease prediction. JAMA 307:2499-2506.

Engstrom K, Vahter M, Mlakar SJ, Concha G, Nermell B, Raqib R, et al. 2011. Polymorphisms in arsenic (+iii oxidation state) methyltransferase (*AS3MT*) predict gene expression of *AS3MT* as well as arsenic metabolism. Environ Health Perspect 119:182-188.

Engstrom KS, Hossain MB, Lauss M, Ahmed S, Raqib R, Vahter M, et al. 2013. Efficient arsenic metabolism--the *AS3MT* haplotype is associated with DNA methylation and expression of multiple genes around *AS3MT*. PloS One 8:e53732.

Engstrom KS, Vahter M, Fletcher T, Leonardi G, Goessler W, Gurzau E, et al. 2015. Genetic variation in arsenic (+3 oxidation state) methyltransferase (*AS3MT*), arsenic metabolism and risk of basal cell carcinoma in a European population. Environmental Mol Mutag 56:60-69.

EPA. 2000. National primary drinking water regulations: arsenic and clarifications to compliance and new source contaminants monitoring; proposed rule [40 CFR parts 141 and 142] Fed Regist 65:38888-38983.

EPA. 2006. Revised registration eligibility decision form MSMA, DSMA, CAMA, and cacodylic acid (EPA 738-R-06-021.

Farias SS, Casa VA, Vazquez C, Ferpozzi L, Pucci GN, Cohen IM. 2003. Natural contamination with arsenic and other trace elements in ground waters of Argentine Pampean plain. Sci Total Environ 309:187-199.

Farzan SF, Karagas MR, Chen Y. 2013. In utero and early life arsenic exposure in relation to long-term health and disease. Toxicol Appl Pharmacol 272:384-390.

Farzan SF, Chen Y, Wu F, Jiang J, Liu M, Baker E, et al. 2015. Blood pressure changes in relation to arsenic exposure in a U.S. Pregnancy cohort. Environ Health Perspect 123:999-1006.

FDA. 2008a. Food and Drug Administration: arsanilic acid (21CFR558.62), 413-414.

FDA. 2008b. Food and Drug Administration: roxarsone (21CFR558.530), 500-503.

Ferrario D, Collotta A, Carfi M, Bowe G, Vahter M, Hartung T, et al. 2009. Arsenic induces telomerase expression and maintains telomere length in human cord blood cells. Toxicology 260:132-141.

Ferreccio C, Gonzalez C, Milosavjlevic V, Marshall G, Sancha AM, Smith AH. 2000. Lung cancer and arsenic concentrations in drinking water in Chile. Epidemiology 11:673-679.

Fishbein L. 1981. Sources, transport and alterations of metal compounds: An overview. I. Arsenic, beryllium, cadmium, chromium, and nickel. Environ Health Perspect 40:43-64.

Flora SJ. 2011. Arsenic-induced oxidative stress and its reversibility. Free Radic Biol Med 51:257-281.

Francisca FM, Carro Perez ME. 2009. Assessment of natural arsenic in groundwater in Cordoba province, Argentina. Environ Geochem Health 31:673-682.

Friedrich U, Griese E, Schwab M, Fritz P, Thon K, Klotz U. 2000. Telomere length in different tissues of elderly patients. Mech Ageing Dev 119: 89-99

Frenck RW, Jr., Blackburn EH, Shannon KM. 1998. The rate of telomere sequence loss in human leukocytes varies with age. Proc Natl Acad Sci U S A 95:5607-5610.

Frisancho AR. 2013. Developmental functional adaptation to high altitude: Review. Am J Hum Biol 25:151-168.

Gadalla S M, Cawthon R, Giri N, Alter BP, Savage SA. 2010. Telomere length in blood, buccal cells, and fibroblasts from patients with inherited bone marrow failure syndromes. Aging 2:867-74.

Gansner JM, Rosas IO. 2013. Telomeres in lung disease. Transl Res 162:343-352.

Ganyc D, Talbot S, Konate F, Jackson S, Schanen B, Cullen W, et al. 2007. Impact of trivalent arsenicals on selenoprotein synthesis. Environ Health Perspect 115:346-353.

Gao J, Roy S, Tong L, Argos M, Jasmine F, Rahaman R, et al. 2015. Arsenic exposure, telomere length, and expression of telomere-related genes among Bangladeshi individuals. Environ Res 136:462-469.

Gardner RM, Nermell B, Kippler M, Grander M, Li L, Ekstrom EC, et al. 2011. Arsenic methylation efficiency increases during the first trimester of pregnancy independent of folate status. Reprod Toxicol 31:210-218.

Gomez-Rubio P, Roberge J, Arendell L, Harris RB, O'Rourke MK, Chen Z, et al. 2011. Association between body mass index and arsenic methylation efficiency in adult women from southwest U.S. And northwest Mexico. Toxicol Appl Pharmacol 252:176-182.

Guha Mazumder DN, Haque R, Ghosh N, De BK, Santra A, Chakraborty D, et al. 1998. Arsenic levels in drinking water and the prevalence of skin lesions in West Bengal, India. Int J Epidemiol 27:871-877.

Guo JX, Hu L, Yand PZ, Tanabe K, Miyatalre M, Chen Y. 2007. Chronic arsenic poisoning in drinking water in inner Mongolia and its associated health effects. J Environ Sci Health A Tox Hazard Subst Environ Eng 42:1853-1858.

Hakim S, Bertucci MC, Conduit SE, Vuong DL, Mitchell CA. 2012. Inositol polyphosphate phosphatases in human disease. Curr Top Microbiol Immunol 362:247-314.

Hamadani JD, Tofail F, Nermell B, Gardner R, Shiraji S, Bottai M, et al. 2011. Critical windows of exposure for arsenic-associated impairment of cognitive function in pre-school girls and boys: A population-based cohort study. Int J Epidemiol 40:1593-1604.

Han J, Qureshi AA, Prescott J, Guo Q, Ye L, Hunter DJ, et al. 2009. A prospective study of telomere length and the risk of skin cancer. J Invest Dermatol 129:415-421.

Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A, Willeit P. 2014. Leucocyte telomere length and risk of cardiovascular disease: Systematic review and meta-analysis. BMJ 349:g4227.

Heck JE, Chen Y, Grann VR, Slavkovich V, Parvez F, Ahsan H. 2008. Arsenic exposure and anemia in Bangladesh: A population-based study. J Occup Environ Med 50:80-87.

Hofer P, Baierl A, Feik E, Fuhrlinger G, Leeb G, Mach K, et al. 2011. MNS16A tandem repeats minisatellite of human telomerase gene: A risk factor for colorectal cancer. Carcinogenesis 32:866-871.

Hopenhayn-Rich C, Biggs ML, Kalman DA, Moore LE, Smith AH. 1996a. Arsenic methylation patterns before and after changing from high to lower concentrations of arsenic in drinking water. Environ Health Perspect 104:1200-1207.

Hopenhayn-Rich C, Biggs ML, Smith AH, Kalman DA, Moore LE. 1996b. Methylation study of a population environmentally exposed to arsenic in drinking water. Environ Health Perspect 104:620-628.

Hopenhayn C, Huang B, Christian J, Peralta C, Ferreccio C, Atallah R, et al. 2003. Profile of urinary arsenic metabolites during pregnancy. Environ Health Perspect 111:1888-1891.

Hopenhayn C, Bush HM, Bingcang A, Hertz-Picciotto I. 2006. Association between arsenic exposure from drinking water and anemia during pregnancy. J Occup Environ Med 48:635-643.

Hosnijeh FS, Lan Q, Rothman N, San Liu C, Cheng WL, Nieters A, et al. 2014. Mitochondrial DNA copy number and future risk of B-cell lymphoma in a nested case-control study in the prospective EPIC cohort. Blood 124:530-535.

Hossain MB, Li H, Hedmer M, Tinnerberg H, Albin M, Broberg K. 2015. Exposure to welding fumes is associated with hypomethylation of the *F2RL3* gene: A cardiovascular disease marker. Occup Environ Med 72:845-851.

Hou L, Zhu ZZ, Zhang X, Nordio F, Bonzini M, Schwartz J, et al. 2010. Airborne particulate matter and mitochondrial damage: A cross-sectional study. Environ Health 9:48.

Hsueh YM, Huang YL, Huang CC, Wu WL, Chen HM, Yang MH, et al. 1998. Urinary levels of inorganic and organic arsenic metabolites among residents in an arseniasis-hyperendemic area in Taiwan. J Toxicol Environ Health A 54:431-444.

Hsueh YM, Wu WL, Huang YL, Chiou HY, Tseng CH, Chen CJ. 1998b. Low serum carotene level and increased risk of ischemic heart disease related to long-term arsenic exposure. Atherosclerosis 141:249-257.

Hsueh YM, Ko YF, Huang YK, Chen HW, Chiou HY, Huang YL, et al. 2003. Determinants of inorganic arsenic methylation capability among residents of the lanyang basin, Taiwan: Arsenic and selenium exposure and alcohol consumption. Toxicol Lett 137:49-63.

Huang B, Gao YT, Shu XO, Wen W, Yang G, Li G, et al. 2014. Association of leukocyte mitochondrial DNA copy number with colorectal cancer risk: Results from the Shanghai women's health study. Cancer Epidemiol Biomarkers Prev 23:2357-2365.

Huang J, Tan L, Shen R, Zhang L, Zuo H, Wang DW. 2016. Decreased peripheral mitochondrial DNA copy number is associated with the risk of heart failure and long-term outcomes. Medicine 95:e3323.

Huang YK, Huang YL, Hsueh YM, Yang MH, Wu MM, Chen SY, et al. 2008. Arsenic exposure, urinary arsenic speciation, and the incidence of urothelial carcinoma: A twelve-year follow-up study. Cancer Causes Control 19:829-839.

Huda N, Hossain S, Rahman M, Karim MR, Islam K, Mamun AA, et al. 2014. Elevated levels of plasma uric acid and its relation to hypertension in arsenic-endemic human individuals in Bangladesh. Toxicol Appl Pharmacol 281:11-18.

Hughes MF. 2006. Biomarkers of exposure: A case study with inorganic arsenic. Environ Health Perspect 114:1790-1796.

IARC. 2004. Some drinking-water disinfectants and contaminants, including arsenic. IARC Monogr Eval Carcinog Risks Hum 84:41-267.

IARC. 2006. Cobalt in hard metals and cobalt sulfate, gallium arsenide, indium phosphide and vanadium pentoxide. IARC Monogr Eval Carcinog Risks Hum 86: 1-294.

IARC. 2012. Arsenic, metals, fibres and dusts. IARC Monogr Eval Carcinog Risks Hum 100 (pt C): 11-465

Islam MR, Khan I, Attia J, Hassan SM, McEvoy M, D'Este C, et al. 2012. Association between hypertension and chronic arsenic exposure in drinking water: A cross-sectional study in Bangladesh. Int J Environ Res Public Health 9:4522-4536.

James KA, Byers T, Hokanson JE, Meliker JR, Zerbe GO, Marshall JA. 2015. Association between lifetime exposure to inorganic arsenic in drinking water and coronary heart disease in Colorado residents. Environ Health Perspect 123:128-134.

Jiang J, Liu M, Parvez F, Wang B, Wu F, Eunus M, et al. 2015. Association between arsenic exposure from drinking water and longitudinal change in blood pressure among HEALS cohort participants. Environ Health Perspect 123:806-812.

Karagas MR, Gossai A, Pierce B, Ahsan H. 2015. Drinking water arsenic contamination, skin lesions, and malignancies: A systematic review of the global evidence. Curr Environ Health Rep 2:52-68.

Karagas MR, Punshon T, Sayarath V, Jackson BP, Folt CL, Cottingham KL. 2016. Association of rice and rice-product consumption with arsenic exposure early in life. JAMA Pediatr 170:609-616.

Karim MR, Rahman M, Islam K, Mamun AA, Hossain S, Hossain E, et al. 2013. Increases in oxidized low-density lipoprotein and other inflammatory and adhesion molecules with a concomitant decrease in high-density lipoprotein in the individuals exposed to arsenic in Bangladesh. Toxicol Sci 135:17-25.

Khan S, Chuturgoon AA, Naidoo DP. 2012. Telomeres and atherosclerosis. Cardiovasc J Afr 23:563-571.

Kile ML, Faraj JM, Ronnenberg AG, Quamruzzaman Q, Rahman M, Mostofa G, et al. 2016. A cross sectional study of anemia and iron deficiency as risk factors for arsenic-induced skin lesions in Bangladeshi women. BMC Public Health 16:158.

Kim KJ, Park MC, Choi SJ, Oh YS, Choi EC, Cho HJ, et al. 2008. Determination of three-dimensional structure and residues of the novel tumor suppressor AIMP3/p18 required for the interaction with ATM. J Biol Chem 283:14032-14040.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.

Knobeloch LM, Zierold KM, Anderson HA. 2006. Association of arsenic-contaminated drinking-water with prevalence of skin cancer in wisconsin's fox river valley. J Health Popul Nutr 24:206-213.

Kumagai Y, Pi J. 2004. Molecular basis for arsenic-induced alteration in nitric oxide production and oxidative stress: Implication of endothelial dysfunction. Toxicol Appl Pharmacol 198:450-457.

Kuo CC, Moon K, Thayer KA, Navas-Acien A. 2013. Environmental chemicals and type 2 diabetes: An updated systematic review of the epidemiologic evidence. Curr Diab Rep 13:831-849.

Lan Q, Cawthon R, Gao Y, Hu W, Hosgood HD, 3rd, Barone-Adesi F, et al. 2013. Longer telomere length in peripheral white blood cells is associated with risk of lung cancer and the rs2736100 (*CLPTM1L-TERT*) polymorphism in a prospective cohort study among women in China. PloS One 8:e59230.

Lee CH, Wu SB, Hong CH, Liao WT, Wu CY, Chen GS, et al. 2011. Aberrant cell proliferation by enhanced mitochondrial biogenesis via mtTFA in arsenical skin cancers. Am J Pathol 178:2066-2076.

Lee HC, Wei YH. 2000. Mitochondrial role in life and death of the cell. J Biomed Sci 7:2-15.

Lee MY, Jung BI, Chung SM, Bae ON, Lee JY, Park JD, et al. 2003. Arsenic-induced dysfunction in relaxation of blood vessels. Environ Health Perspect 111:513-517.

Lee PC, Ho IC, Lee TC. 2005. Oxidative stress mediates sodium arsenite-induced expression of heme oxygenase-1, monocyte chemoattractant protein-1, and interleukin-6 in vascular smooth muscle cells. Toxicol Sci 85:541-550.

Lemnrau A, Brook MN, Fletcher O, Coulson P, Jones M, Tomczyk K, et al. 2015. Mitochondrial DNA copy number in peripheral blood cells and risk of developing breast cancer. Cancer Res 75:2844-50.

Lerman SA, Clarkson TW, Gerson RJ. 1983. Arsenic uptake and metabolism by liver cells is dependent on arsenic oxidation state. Chem Biol Interact 45:401-406.

Li H, Engstrom K, Vahter M, Broberg K. 2012. Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. Chem Res Toxicol 25:2333-2339.

Li H, Hilmarsen HT, Hossain MB, Bjork J, Hansteen IL, Albin M, et al. 2013. Telomere length and LINE1 methylation is associated with chromosomal aberrations in peripheral blood. Genes Chromosomes Cancer 52:1-10.

Li L, Ekstrom EC, Goessler W, Lonnerdal B, Nermell B, Yunus M, et al. 2008. Nutritional status has marginal influence on the metabolism of inorganic arsenic in pregnant Bangladeshi women. Environ Health Perspect 116:315-321.

Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davis KM, et al. 2002. A novel S-adenosyl-L-methionine:Arsenic(iii) methyltransferase from rat liver cytosol. J Biol Chem 277:10795-10803.

Lindberg AL, Kumar R, Goessler W, Thirumaran R, Gurzau E, Koppova K, et al. 2007. Metabolism of low-dose inorganic arsenic in a Central European population: Influence of sex and genetic polymorphisms. Environ Health Perspect 115:1081-1086.

Lindberg AL, Rahman M, Persson LA, Vahter M. 2008. The risk of arsenic induced skin lesions in Bangladeshi men and women is affected by arsenic metabolism and the age at first exposure. Toxicol Appl Pharmacol 230:9-16.

Lister R, Hulett JM, Lithgow T, Whelan J. 2005. Protein import into mitochondria: Origins and functions today (review). Mol Membr Biol 22:87-100.

Liu Z, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, Rosen BP. 2002. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci U S A 99:6053-6058.

Liu Z, Styblo M, Rosen BP. 2006. Methylarsonous acid transport by aquaglyceroporins. Environ Health Perspect 114:527-531.

Lopez-Carrillo L, Hernandez-Ramirez RU, Gandolfi AJ, Ornelas-Aguirre JM, Torres-Sanchez L, Cebrian ME. 2014. Arsenic methylation capacity is associated with breast cancer in northern Mexico. Toxicol Appl Pharmacol 280:53-59.

Lynch SM, Major JM, Cawthon R, Weinstein SJ, Virtamo J, Lan Q, et al. 2013. A prospective analysis of telomere length and pancreatic cancer in the alpha-tocopherol beta-carotene cancer (ATBC) prevention study. Int J Cancer 133:2672-2680.

Ma H, Zhou Z, Wei S, Liu Z, Pooley KA, Dunning AM, et al. 2011. Shortened telomere length is associated with increased risk of cancer: A meta-analysis. PloS One 6:e20466.

Marafante E, Vahter M. 1984. The effect of methyltransferase inhibition on the metabolism of [74As]arsenite in mice and rabbits. Chem Biol Interact 50:49-57.

Marafante E, Vahter M, Norin H, Envall J, Sandstrom M, Christakopoulos A, et al. 1987. Biotransformation of dimethylarsinic acid in mouse, hamster and man. J Appl Toxicol 7:111-117.

Martinez-Outschoorn UE, Pavlides S, Sotgia F, Lisanti MP. 2011. Mitochondrial biogenesis drives tumor cell proliferation. Am J Pathol 178:1949-1952.

Mass MJ, Wang L. 1997. Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: A model for a mechanism of carcinogenesis. Mutat Res 386:263-277.

McGrath M, Wong JY, Michaud D, Hunter DJ, De Vivo I. 2007. Telomere length, cigarette smoking, and bladder cancer risk in men and women. Cancer Epidemiol Biomarkers Prev 16:815-819.

Melkonian SC, Wang X, Gu J, Matin SF, Tannir NM, Wood CG, et al. 2015. Mitochondrial DNA copy number in peripheral blood leukocytes and the risk of clear cell renal cell carcinoma. Carcinogenesis 36:249-255.

Mendez MA, Gonzalez-Horta C, Sanchez-Ramirez B, Ballinas-Casarrubias L, Ceron RH, Morales DV, et al. 2016. Chronic exposure to arsenic and markers of cardiometabolic risk: A cross-sectional study in Chihuahua, Mexico. Environ Health Perspect 124:104-111.

Meng YL, Liu Z, Rosen BP. 2004. As(iii) and Sb(iii) uptake by GlpF and efflux by ArsB in escherichia coli. J Biol Chem 279:18334-18341.

Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, et al 2011. Triglycerides and cardiovascular disease: a scientific statement from the American Heart Asociation. Circulation 123:2292-333.

Mo J, Xia Y, Ning Z, Wade TJ, Mumford JL. 2009. Elevated human telomerase reverse transcriptase gene expression in blood cells associated with chronic arsenic exposure in Inner Mongolia, China. Environ Health Perspect 117:354-360.

Mokranjac D, Neupert W. 2005. Protein import into mitochondria. Biochem Soc Trans 33:1019-1023.

Moon K, Guallar E, Navas-Acien A. 2012. Arsenic exposure and cardiovascular disease: An updated systematic review. Curr Aatheroscler Rep 14:542-555.

Moon KA, Guallar E, Umans JG, Devereux RB, Best LG, Francesconi KA, et al. 2013. Association between exposure to low to moderate arsenic levels and incident cardiovascular disease. A prospective cohort study. Ann Inter Med 159:649-659.

Mordukhovich I, Wright RO, Amarasiriwardena C, Baja E, Baccarelli A, Suh H, et al. 2009. Association between low-level environmental arsenic exposure and QT interval duration in a general population study. Am J Epidemiol 170:739-746.

Mukherjee A, Sengupta MK, Hossain MA, Ahamed S, Das B, Nayak B, et al. 2006. Arsenic contamination in groundwater: A global perspective with emphasis on the Asian scenario. J Health Popul Nutr 24:142-163.

Mumford JL, Wu K, Xia Y, Kwok R, Yang Z, Foster J, et al. 2007. Chronic arsenic exposure and cardiac repolarization abnormalities with QT interval prolongation in a population-based study. Environ Health Perspect 115:690-694.

Nabi AH, Rahman MM, Islam LN. 2005. Evaluation of biochemical changes in chronic arsenic poisoning among Bangladeshi patients. Int J Environ Res Public Health 2:385-393.

Ngollo M, Dagdemir A, Karsli-Ceppioglu S, Judes G, Pajon A, Penault-Llorca F, et al. 2014. Epigenetic modifications in prostate cancer. Epigenomics 6:415-426.

Nicolli HB, Bundschuh J, Blanco Mdel C, Tujchneider OC, Panarello HO, Dapena C, et al. 2012. Arsenic and associated trace-elements in groundwater from the Chaco-Pampean plain, Argentina: Results from 100 years of research. Sci Total Environ 429:36-56.

Niedzwiecki MM, Hall MN, Liu X, Oka J, Harper KN, Slavkovich V, et al. 2013. A dose-response study of arsenic exposure and global methylation of peripheral blood mononuclear cell DNA in Bangladeshi adults. Environ Health Perspect 121:1306-1312.

NRC. 2001. Arsenic in drinking water. Update. Washington, DC, National Academy Press.

Oda M, Sakitani K, Kaibori M, Inoue T, Kamiyama Y, Okumura T. 2000. Vicinal dithiol-binding agent, phenylarsine oxide, inhibits inducible nitric-oxide synthase gene expression at a step of nuclear factor-kappaB DNA binding in hepatocytes. J Biol Chem 275:4369-4373.

Okano M, Xie S, Li E. 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19:219-220.

Olivieri F, Recchioni R, Marcheselli F, Abbatecola AM, Santini G, Borghetti G, et al. 2013. Cellular senescence in cardiovascular diseases: Potential age-related mechanisms and implications for treatment. Curr Pharm Des 19:1710-1719.

Olovnikov AM. 1973. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol 41:181-190.

Ormseth MJ, Solus JF, Oeser AM, Bian A, Gebretsadik T, Shintani A, et al. 2016. Telomere length and coronary atherosclerosis in rheumatoid arthritis. J Rheumatol 43:1469-1474.

Partridge MA, Huang SX, Hernandez-Rosa E, Davidson MM, Hei TK. 2007. Arsenic induced mitochondrial DNA damage and altered mitochondrial oxidative function: Implications for genotoxic mechanisms in mammalian cells. Cancer Res 67:5239-5247.

Parvez F, Chen Y, Yunus M, Olopade C, Segers S, Slavkovich V, et al. 2013. Arsenic exposure and impaired lung function. Findings from a large population-based prospective cohort study. Am J Respir Crit Care Med 188:813-819.

Pierce BL, Kibriya MG, Tong L, Jasmine F, Argos M, Roy S, et al. 2012. Genome-wide association study identifies chromosome 10q24.32 variants associated with arsenic metabolism and toxicity phenotypes in Bangladesh. PLoS Genet 8:e1002522.

Pilsner JR, Liu X, Ahsan H, Ilievski V, Slavkovich V, Levy D, et al. 2007. Genomic methylation of peripheral blood leukocyte DNA: Influences of arsenic and folate in Bangladeshi adults. Am J Clin Nutr 86:1179-1186.

Rahman M, Tondel M, Ahmad SA, Chowdhury IA, Faruquee MH, Axelson O. 1999. Hypertension and arsenic exposure in bangladesh. Hypertension 33:74-78.

Rahman M, Vahter M, Wahed MA, Sohel N, Yunus M, Streatfield PK, et al. 2006. Prevalence of arsenic exposure and skin lesions. A population based survey in Matlab, Bangladesh. J Epidemiol Community Health 60:242-248.

Rahman MA, Hasegawa H. 2011. High levels of inorganic arsenic in rice in areas where arsenic-contaminated water is used for irrigation and cooking. Sci Total Environ 409:4645-4655.

Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. 2011. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect 119:11-19.

Reznik E, Miller ML, Senbabaoglu Y, Riaz N, Sarungbam J, Tickoo SK, et al. 2016. Mitochondrial DNA copy number variation across human cancers. Elife 5.

Robertson KD. 2005. DNA methylation and human disease. Nat Rev Genet 6:597-610.

Rosenboom JW. 2004. Arsenic in 15 upazilas of Bangladesh: water supplies, health and behaviour- and analysis of available data. DPHE, DFID, UNICEF.

Rossman TG, Klein CB. 2011. Genetic and epigenetic effects of environmental arsenicals. Metallomics 3:1135-1141.

Sabatino L, Picano E, Andreassi MG. 2012. Telomere shortening and ionizing radiation: A possible role in vascular dysfunction? Int J Radiat Biol 88:830-839.

Sanders OI, Rensing C, Kuroda M, Mitra B, Rosen BP. 1997. Antimonite is accumulated by the glycerol facilitator GlpF in escherichia coli. J Bacteriol 179:3365-3367.

Santra A, Maiti A, Chowdhury A, Mazumder DN. 2000. Oxidative stress in liver of mice exposed to arsenic-contaminated water. Indian J Gastroenterol 19:112-115.

Sarkar A, Paul B. 2016. The global menace of arsenic and its conventional remediation - a critical review. Chemosphere 158:37-49.

Schlebusch CM, Lewis CM, Jr., Vahter M, Engstrom K, Tito RY, Obregon-Tito AJ, et al. 2013. Possible positive selection for an arsenic-protective haplotype in humans. Environ Health Perspect 121:53-58.

Schlebusch CM, Gattepaille LM, Engstrom K, Vahter M, Jakobsson M, Broberg K. 2015. Human adaptation to arsenic-rich environments. Mol Biol Evol 32:1544-1555.

Seow WJ, Cawthon RM, Purdue MP, Hu W, Gao YT, Huang WY, et al. 2014. Telomere length in white blood cell DNA and lung cancer: A pooled analysis of three prospective cohorts. Cancer Res 74:4090-4098.

Seow WJ, Pan WC, Kile ML, Tong L, Baccarelli AA, Quamruzzaman Q, et al. 2015. A distinct and replicable variant of the squamous cell carcinoma gene inositol polyphosphate-5-phosphatase modifies the susceptibility of arsenic-associated skin lesions in bangladesh. Cancer 121:2222-2229.

Seyfried TN. 2015. Cancer as a mitochondrial metabolic disease. Front Cell Dev Biol 3:43.

Shay JW, Wright WE, Werbin H. 1991. Defining the molecular mechanisms of human cell immortalization. Biochim Biophys Acta 1072:1-7.

Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM. 2007. Short telomere length and breast cancer risk: A study in sister sets. Cancer Res 67:5538-5544.

Shen M, Cawthon R, Rothman N, Weinstein SJ, Virtamo J, Hosgood HD, 3rd, et al. 2011. A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of lung cancer. Lung Cancer 73:133-137.

Sigrist M, Hilbe N, Brusa L, Campagnoli D, Beldomenico H. 2016. Total arsenic in selected food samples from Argentina: Estimation of their contribution to inorganic arsenic dietary intake. Food Chem 210:96-101.

Silbergeld EK, Nachman K. 2008. The environmental and public health risks associated with arsenical use in animal feeds. Ann N Y Acad Sci 1140:346-357.

Skroder Loveborn H, Kippler M, Lu Y, Ahmed S, Kuehnelt D, Raqib R, et al. 2016. Arsenic metabolism in children differs from that in adults. Toxicol Sci 152:29-39.

Smeester L, Rager JE, Bailey KA, Guan X, Smith N, Garcia-Vargas G, et al. 2011. Epigenetic changes in individuals with arsenicosis. Chem Res Toxicol 24:165-167.

Smith KR, Klei LR, Barchowsky A. 2001. Arsenite stimulates plasma membrane NADPH oxidase in vascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 280:L442-449.

States JC, Srivastava S, Chen Y, Barchowsky A. 2009. Arsenic and cardiovascular disease. Toxicol Sci 107:312-323.

Svenson U, Roos G. 2009. Telomere length as a biological marker in malignancy. Biochim Biophys Acta 1792:317-323.

Svenson U, Nordfjall K, Baird D, Roger L, Osterman P, Hellenius ML, et al. 2011. Blood cell telomere length is a dynamic feature. PloS One 6:e21485.

Tchounwou PB, Yedjou CG, Dorsey WC. 2003. Arsenic trioxide-induced transcriptional activation of stress genes and expression of related proteins in human liver carcinoma cells (HepG2). Cell Mol Biol (Noisy-le-grand) 49:1071-1079.

Thyagarajan B, Wang R, Nelson H, Barcelo H, Koh WP, Yuan JM. 2013. Mitochondrial DNA copy number is associated with breast cancer risk. PloS One 8:e65968.

Tofail F, Vahter M, Hamadani JD, Nermell B, Huda SN, Yunus M, et al. 2009. Effect of arsenic exposure during pregnancy on infant development at 7 months in rural Matlab, Bangladesh. Environ Health Perspect 117:288-293.

Tognon G, Berg C, Mehlig K, Thelle D, Strandhagen E, Gustavsson J, et al. 2012. Comparison of apolipoprotein (apoB/apoA-I) and lipoprotein (total cholesterol/HDL) ratio determinants. Focus on obesity, diet and alcohol intake. PloS One 7:e40878.

Tolins M, Ruchirawat M, Landrigan P. 2014. The developmental neurotoxicity of arsenic: Cognitive and behavioral consequences of early life exposure. Ann Glob Health 80:303-314.

Tseng CH, Chong CK, Chen CJ, Tai TY. 1997. Lipid profile and peripheral vascular disease in arseniasis-hyperendemic villages in Taiwan. Angiology 48:321-335.

Tsou TC, Yeh SC, Tsai EM, Tsai FY, Chao HR, Chang LW. 2005. Arsenite enhances tumor necrosis factor-alpha-induced expression of vascular cell adhesion molecule-1. Toxicol Appl Pharmacol 209:10-18.

Umar Z, Rasool M, Asif M, Karim S, Malik A, Mushtaq G, et al. 2015. Evaluation of hemoglobin concentration in pregnancy and correlation with different altitude: A study from balochistan plateau of pakistan. Open Biochem J 9:7-14.

Vahter M, Norin H. 1980. Metabolism of 74as-labeled trivalent and pentavalent inorganic arsenic in mice. Environ Res 21:446-457.

Vahter M, Envall J. 1983. In vivo reduction of arsenate in mice and rabbits. Environ Res 32:14-24.

Vahter M, Concha G, Nermell B, Nilsson R, Dulout F, Natarajan AT. 1995. A unique metabolism of inorganic arsenic in native Andean women. Eur J Pharmacol 293:455-462.

Vahter M. 2002. Mechanisms of arsenic biotransformation. Toxicology 181-182:211-217.

Vahter M, Lind B. 1986. Concentrations of arsenic in urine of the general population in sweden. Sci Total Environ 54:1-12.

Vahter ME, Li L, Nermell B, Rahman A, El Arifeen S, Rahman M, et al. 2006. Arsenic exposure in pregnancy: A population-based study in matlab, bangladesh. J Health Popul Nutr 24:236-245.

Vahter M. 2009. Effects of arsenic on maternal and fetal health. Annu Rev Nutr 29:381-399.

Van Houten B, Woshner V, Santos JH. 2006. Role of mitochondrial DNA in toxic responses to oxidative stress. DNA Rep 5:145-152.

Venegas V, Halberg MC. 2012. Quantification of mtDNA mutation heteroplasmy (ARMS qPCR). Methods Mol Biol 837:313-326.

Wade TJ, Xia Y, Mumford J, Wu K, Le XC, Sams E, et al. 2015. Cardiovascular disease and arsenic exposure in Inner Mongolia, China: A case control study. Environ Health 14:35.

Walter I, Schwerdtle T, Thuy C, Parsons JL, Dianov GL, Hartwig A. 2007. Impact of arsenite and its methylated metabolites on PARP-1 activity, PARP-1 gene expression and poly(ADP-ribosyl)ation in cultured human cells. DNA Rep 6:61-70.

Wang CH, Jeng JS, Yip PK, Chen CL, Hsu LI, Hsueh YM, et al. 2002. Biological gradient between long-term arsenic exposure and carotid atherosclerosis. Circulation 105:1804-1809.

Wang JP, Maddalena R, Zheng B, Zai C, Liu F, Ng JC. 2009. Arsenicosis status and urinary malondialdehyde (MDA) in people exposed to arsenic contaminated-coal in china. Environ Int 35:502-506.

Watanabe T, Hirano S. 2013. Metabolism of arsenic and its toxicological relevance. Arch Toxicol 87:969-979.

Weischer M, Bojesen SE, Cawthon RM, Freiberg JJ, Tybjaerg-Hansen A, Nordestgaard BG. 2012. Short telomere length, myocardial infarction, ischemic heart disease, and early death. Arterioscler Thromb Vasc Biol 32:822-829.

Whayne TF. 2015. Epigenetics in the development, modification, and prevention of cardiovascular disease. Mol Biol Rep 42:765-776.

WHO. 2001. Arsenic and arsenic compounds. Geneva, World Health Organization, International Programme on Chemical Safety.

WHO. 2004. Guidelines for drinking-water quality. 3rd edition, volume 1. Geneva, World Health Organization.

Willeit P, Willeit J, Brandstatter A, Ehrlenbach S, Mayr A, Gasperi A, et al. 2010. Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. Arterioscler Thromb Vasc Biol 30:1649-1656.

Wood TC, Salavagionne OE, Mukherjee B, Wang L, Klumpp AF, Thomae BA, et al. 2006. Human arsenic methyltransferase (*AS3MT*) pharmacogenetics: Gene resequencing and functional genomics studies. J Biol Chem 281:7364-7373.

Wu J, Liu J, Waalkes MP, Cheng ML, Li L, Li CX, et al. 2008. High dietary fat exacerbates arsenic-induced liver fibrosis in mice. Exp Biol Med (Maywood) 233:377-384.

Wu MM, Kuo TL, Hwang YH, Chen CJ. 1989. Dose-response relation between arsenic concentration in well water and mortality from cancers and vascular diseases. Am J Epidemiol 130:1123-1132.

Wu X, Amos CI, Zhu Y, Zhao H, Grossman BH, Shay JW, et al. 2003. Telomere dysfunction: A potential cancer predisposition factor. J Natl Cancer Inst 95:1211-1218.

Wysocki R, Chery CC, Wawrzycka D, Van Hulle M, Cornelis R, Thevelein JM, et al. 2001. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in Saccharomyces cerevisiae. Mol Microbiol 40:1391-1401.

Xie H, Wu X, Wang S, Chang D, Pollock RE, Lev D, et al. 2013. Long telomeres in peripheral blood leukocytes are associated with an increased risk of soft tissue sarcoma. Cancer 119:1885-1891.

Xie Y, Liu J, Liu Y, Klaassen CD, Waalkes MP. 2004. Toxicokinetic and genomic analysis of chronic arsenic exposure in multidrug-resistance mdr1a/1b(-/-) double knockout mice. Mol Cell Biochem 255:11-18.

Xu FX, Zhou X, Shen F, Pang R, Liu SM. 2012. Decreased peripheral blood mitochondrial DNA content is related to HbA1c, fasting plasma glucose level and age of onset in type 2 diabetes mellitus. Diabet Med 29e:47-54

Yang HC, Cheng J, Finan TM, Rosen BP, Bhattacharjee H. 2005. Novel pathway for arsenic detoxification in the legume symbiont sinorhizobium meliloti. J Bacteriol 187:6991-6997.

Yang HC, Fu HL, Lin YF, Rosen BP. 2012. Pathways of arsenic uptake and efflux. Curr Top Membr 69:325-358.

Yu HY, Ding X, Li F, Wang X, Zhang S, Yi J, et al. 2016. The availabilities of arsenic and cadmium in rice paddy fields from a mining area: The role of soil extractable and plant silicon. Environ Pollut 215:258-265.

Yuan Y, Marshall G, Ferreccio C, Steinmaus C, Selvin S, Liaw J, et al. 2007. Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950 to 2000. Am J Epidemiol 166:1381-1391.

Zhao FJ, McGrath SP, Meharg AA. 2010. Arsenic as a food chain contaminant: Mechanisms of plant uptake and metabolism and mitigation strategies. Annu Rev Plant Biol 61:535-559.

Zhao J, Miao K, Wang H, Ding H, Wang DW. 2013. Association between telomere length and type 2 diabetes mellitus: A meta-analysis. PloS One 8:e79993.

Zhou W, Zhu M, Gui M, Huang L, Long Z, Wang L, et al. 2014. Peripheral blood mitochondrial DNA copy number is associated with prostate cancer risk and tumor burden. PloS One 9:e109470.

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The effects of arsenic exposure on blood pressure and early risk markers of cardiovascular disease: Evidence for population differences

Syeda Shegufta Ameer [a], Karin Engström [a], Florencia Harari [b], Gabriela Concha [c], Marie Vahter [b], Karin Broberg [b,*]

[a] Department of Laboratory Medicine, Section of Occupational and Environmental Medicine, Lund University, Lund, Sweden
[b] Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden
[c] Science Department, Risk Benefit Assessment Unit, National Food Agency, Uppsala, Sweden

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ABSTRACT

Background: Exposure to inorganic arsenic has been identified as a risk factor for elevated blood pressure and cardiovascular disease. Our aim with this study was to elucidate effects of arsenic on blood pressure and early risk markers of cardiovascular disease in a population with efficient arsenic metabolism that can modify other arsenic-related health effects.
Methods: The study included 225 women in the northern Argentinean Andes. Exposure to arsenic was assessed by the sum of arsenic metabolite concentrations in urine. Blood pressure was measured in the supine position. Blood samples were collected for measurement of hemoglobin, homocysteine, triglycerides, apolipoproteins A and B, and cytokines in separated plasma.
Results: The median arsenic concentration in urine was 200 μg/L (range 22–545 μg/L). Unexpectedly, urinary arsenic concentrations were inversely associated with both systolic ($p=0.081$), and diastolic ($p=0.002$) blood pressure, and with the ratio of apolipoproteins B/A ($p<0.001$). There was no clear sign of increased inflammation, measured as cytokine concentrations, in relation to arsenic. Furthermore, urinary arsenic was associated with low hemoglobin concentrations ($p<0.001$).
Conclusions: Our results show that arsenic exposure was not associated with elevated levels of early risk markers for cardiovascular disease in this population. This provides evidence that the effects of arsenic on risk of cardiovascular disease differ between populations, which needs to be taken into account in risk assessment.

1. Introduction

Several epidemiological studies have associated exposure to arsenic in drinking water with a higher risk of cardiovascular disease and mortality, coronary heart disease, and peripheral arterial heart disease; for a review, see Moon et al. (2012, 2013). A recent prospective study also showed an association of arsenic concentrations with high blood pressure (Shiue and Hristova, 2014). Mechanisms suggested for arsenic-related impairment of the cardiovascular health include increased inflammation, disruption of lipid metabolism, endothelial dysfunction and activation of the complement system (Balakumar and Kaur, 2009; States et al., 2009).

Indigenous populations in northern Chile (Hopenhayn-Rich et al., 1996) and northern Argentina (Vahter et al., 1995) have efficient arsenic metabolism, despite often quite high arsenic exposure from drinking water, at exposure levels known to impair arsenic metabolism (Vahter, 2002). The aim of this study was to elucidate how arsenic may affect blood pressure and early risk markers of cardiovascular disease in a population from the northern Argentinian Andes.

2. Materials and methods

2.1. Study area and study participants

The study individuals ($N=225$; 187 recruited in 2008, and 38 in 2011) were women, 18–65 years of age, in the Andean plateau (3800 m above sea level) in northern Argentina. The women were

Abbreviations: apoA, apolipoprotein A; apoB, apolipoprotein B; BMI, body mass index; DMA, dimethylarsinic acid; ICPMS, inductively coupled plasma mass spectrometry; IL, interleukin; HDL, high density lipoprotein; HG, hydride generation; HPLC, high-performance liquid chromatography; MMA, methylarsonic acid; LDL, low density lipoprotein; LOD, limit of detection

* Corresponding author. Fax: +46 08 336981.
E-mail address: karin.broberg@ki.se (K. Broberg).

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mainly living in the village San Antonio de los Cobres [water arsenic ~200 μg/L (Concha et al., 2006, 2010)] and in small surrounding villages with lower water arsenic concentrations (10–149 μg/L). Besides arsenic, the drinking water had elevated concentrations of lithium, boron, and cesium with small variations in concentrations over time (Concha et al., 2010). Interviews with the study participants revealed that they were mostly indigenous and 97% of the women reported that they had lived in the area for longer than two years (mean residence time=25 years). Their diets consisted primarily of meat and milk, as well as vegetables, rice, potatoes, and corn, as described previously (Hossain et al., 2012).

The Health Ministry of Salta (Argentina) and the Ethics Committee of Karolinska Institutet (Sweden) approved the study. Oral and written informed consent was obtained from all study participants.

2.2. Exposure assessment

Spot urine samples were collected in plastic urine collection cups the same way in 2008 and 2011, transferred to 24 mL polyethylene vials, and kept at −20 °C until transport to Sweden. The remaining urine sample was used to measure urinary glucose levels with Combur-7 test strips (Roche Diagnostics, Mannheim, Germany). Exposure to inorganic arsenic was determined based on the sum of concentrations of inorganic arsenic and its metabolites (iAs+methylarsonic acid, MMA+dimethylarsinic acid, and DMA) in urine. Arsenic metabolites in urine were measured using high-performance liquid chromatography (HPLC: Agilent 1100 series system, Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and inductively coupled plasma mass spectrometry (ICPMS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) (HPLC-HG–ICPMS), as described previously (Fangstrom et al., 2009). The lithium and boron concentrations in the drinking water and urine samples were assessed by ICPMS, after dilution 1:10 with 1% nitric acid (65% w/w, ppb-trace analysis grade, Scharlau, Scharlab S.L., Sentmenat, Spain) as described previously (Concha et al., 2010). To compensate for variations in dilution of urine, the arsenic and lithium concentrations were adjusted to the mean specific gravity (1.019), measured by a hand refractometer (Atago, Japan), as factors such as age and meat consumption have little effect on the specific gravity of urine (Nermell et al., 2008).

2.3. Measurement of blood pressure and cardiovascular effect markers

Blood pressure was measured once from each subject's right arm with an aneroid sphygmomanometer for adults (size 12×35 cm^2, AB Henry Eriksson, Stockholm, Sweden) after around 5 minutes' rest in supine position, in an standardized way for all individuals. The first phase of Korotkoff sound was recorded as systolic blood pressure and the fifth phase of Korotkoff sound was recorded as diastolic blood pressure.

Venous blood samples were collected in K_2EDTA tubes (Vacuette, Greiner Bio-One GmbH, Greiner, Germany). After 10 min, blood samples were centrifuged for 10 min to obtain the plasma fraction. For evaluation of potential cardiovascular effects of arsenic, the following markers were measured in plasma: homocysteine, apolipoproteins A and B, triglycerides, and cytokines IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-15, and IL-21.

Homocysteine is a marker of the one-carbon metabolism cycle, which transfers methyl groups to a variety of substrates in the body. Elevated concentrations of total homocysteine in plasma, reflecting the inability to re-methylate homocysteine to methionine, have been associated with multiple adverse health effects, including cardiovascular disease (Joseph and Loscalzo, 2013). Total homocysteine was measured by the use of Cobas (Roche Diagnostic, UK). The limit of detection was 2.5 μmol/L.

The ratio of apolipoprotein B/apolipoprotein A (apoB/A; corresponding to LDL/HDL) has been suggested to be the most accurate early marker of cardiovascular disease (Tognon et al., 2012). Apolipoproteins A and B were measured by immunochemistry and turbidimetry (Cobas c502, Roche Diagnostic, Mannheim, Germany); the limit of detection was 0.03 g/L, with <3 and 1.8% imprecision, for apoA and apoB, respectively. The measurement accuracy of apolipoproteins has been ascertained by WHO/IFCC sp1-01 for apoA and sp3-07 for apoB. As an additional marker for dyslipidemia, we measured triglycerides. Triglycerides were measured by spectrophotometry (Cobas c701, Roche Diagnostic, Mannheim, Germany). The limit of detection was 0.1 mmol/L with imprecision of 2.1%. Analyses of all these markers were performed as routine analyses at Clinical Chemistry, Lund University Hospital, Sweden.

Hemoglobin was measured in blood from the K_2EDTA tube with a HemoCue (HemoCue, Hb 201+, Ängelholm, Sweden).

In our study, we analyzed a panel of TH1 and TH2 cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-15 and IL-21) in EDTA-plasma using Luminex XMAP technology on a Bio-plex 200 platform (Bio-Rad, Hercules, CA, USA), according to instructions from the manufacturer. The standard was diluted nine-fold and the standard points were fitted to a standard curve by a 5 parameter logistic model (Findlay and Dillard, 2007) in the Bio-plex software. IL-2 and IL-15 were excluded from further analysis due to low performance in the assay. All samples were analyzed in duplicate and the coefficient of variation was less than 30% for all duplicate samples. Plasma samples from three healthy individuals were included in each run as control samples.

2.4. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics, version 20 (IBM, Chicago, IL, USA). Statistical significance refers to $p<0.05$.

We used linear regression analyses to explore the associations between arsenic exposure (urinary arsenic concentrations) and blood pressure, hemoglobin, homocysteine, triglycerides and apolipoproteins. To account for the non-linear associations between arsenic and outcomes, total arsenic in urine, homocysteine and triglycerides were log2-transformed. Correlations between total inorganic arsenic in urine and cytokines were assessed with Spearman's rho coefficient, since for most cytokines, the distribution of residuals was markedly skewed, making parametric analysis not appropriate.

In the multivariable-adjusted linear regression analysis, we adjusted for age (model 1; Table 2), body mass index (BMI) and chewing of coca leaves (model 2), since age and BMI are established risk factors for cardiovascular disease and chewing of coca leaves is a common habit in this region of the Andes. We did not include other risk factors for impairment of the cardiovascular health, like alcohol consumption or smoking in the models, since very few women reported consumption of alcohol (one) or tobacco smoking (eight). However, we did perform a sensitivity analysis excluding smokers. Further sensitivity analyses were performed excluding participants that reported taking medication, having diabetes, high blood pressure, high cholesterol, or heart problems, or who reported being pregnant.

As there were co-exposures to lithium and boron, to some extent (urinary arsenic was correlated to urinary lithium, $r_S=0.60$ and with urinary boron, $r_S=0.67$; lithium and boron were correlated, $r_S=0.84$), the models were further adjusted for urinary lithium, but not for boron, since no experimental or epidemiological studies have indicated an association between boron and cardiovascular disease or inflammation.

3. Results

3.1. Characteristics of the study population

The general characteristics of the women, grouped by tertiles of arsenic metabolite concentrations in urine, are presented in Table 1 (data for all women are presented in Supplementary Table S1). The women had a mean age of 37 years. Mean BMI (25.8 ± 4.6 kg/m^2) was in the upper range of WHO's reference values (18.5–25 kg/m^2). However, the women were in general short (mean height = 152 cm, SD $\pm$ 5.4), which tends to give higher BMI values than for taller individuals. Mean values of systolic and diastolic blood pressures were 115 (SD $\pm$ 12) and 70 (SD $\pm$ 9.3), respectively. The hemoglobin concentrations (mean = 153 g/L, SD $\pm$ 16) were generally elevated, reflecting the fact that the participants were living at high altitude (Frisancho, 2013) and eating a diet containing a lot of meat. The mean homocysteine concentration in plasma was 6.6 μmol/L and only one woman had hyperhomocysteinemia (homocysteine > 15 μmol/L; reference values here and below were obtained from the main hospital in Salta, Argentina). The mean concentration of triglycerides was 1.4 g/L (SD $\pm$ 0.81) and 13 individuals (5.8%) had triglycerides above the cut-off (2.77 mmol/L). Mean values of apoA and apoB were 1.6 (SD $\pm$ 0.37) and 0.82 (SD $\pm$ 0.26) g/L respectively, and the mean apoB/apoA ratio was 0.53 (SD $\pm$ 0.16). Nine individuals (4.5%) had apoA above the cut-off (2.23 g/L) and 10 individuals (5%) had apoB lower than the cut-off (0.49 g/L). The cytokine concentrations did not show any sign of inflammation, the median concentrations (< LOD) were: IL-1β = 0.29 pg/mL (LOD < 0.15 pg/mL); IL-6 = 2.5 pg/mL (LOD < 0.23 pg/mL); IL-8 = 19 pg/mL (LOD < 0.92 pg/mL); IL-10 = 2.3 pg/mL (LOD < 0.28 pg/mL); IL-12 = 7.3 pg/mL (LOD < 1.5 pg/mL), and IL-21 = 2.2 pg/mL (LOD < 4.3 pg/mL). The median of urinary arsenic concentrations was 200 μg/L, and the median varied up to six-fold between the tertiles. The median for creatinine adjusted urinary arsenic was 288 μg/g, which is similar to the specific gravity adjusted urinary arsenic values. Moreover, the correlation between the creatinine adjusted and specific gravity adjusted urinary arsenic values was significant ($r_S = 0.84$, p value < 0.001).

The fraction of DMA in urine, concentrations of homocysteine, apoA, and ratio of apoB/apoA were significantly different (Jonckheere–Terpstra and Kruskal–Wallis tests) between the tertiles of arsenic in urine (Table 1).

3.2. Arsenic exposure, blood pressure and early risk markers of cardiovascular disease

Relationships between urinary arsenic concentrations (log2-transformed), blood pressure and risk markers of cardiovascular

Table 1
Characteristics of the study population stratified for tertiles of arsenic in urine (μg/L).

Variable	Median urinary arsenic 61 μg/L			Median urinary arsenic 200 μg/L			Median urinary arsenic 373 μg/L			*p*-Value*
	n	Median	5th–95th percentile	*n*	Median	5th–95th percentile	*n*	Median	5th–95th percentile	
Age (years)	75	35	21–56	75	35	20–62	75	34	21–62	0.95
BMI (kg/m^2)	75	25	19–34	75	27	20–35	75	24.6	19–35	0.79
Parity	75	3	0–11	75	3	0–11	75	4	1.0–10.0	0.17
Urinary arsenic (μg/L)	75	61	15–142	75	200	159–267	75	373	275–662	< 0.001
Inorganic arsenic in urine (%)	75	9.7	3.7–22	75	9.8	4.6–24	75	12	4.4–23	0.05
MMA in urine (%)	75	6.9	3.1–13	75	7.6	4.5–15	75	7.5	4.2–16	0.11
DMA in urine (%)	75	83	68–92	75	81	64–91	75	80	65–91	0.039
Systolic blood pressure	75	110	94–140	75	115	99–146	75	110	100–131	0.62
Diastolic blood pressure	75	70	55–85	75	70	55–90	75	65	55–85	0.19
Hemoglobin (g/L)	75	155	138–196	75	151	128–175	75	154	125–174	0.10
Homocysteine (μmol/L)	74	6.9	4–10	74	6.3	3.9–11	73	5.6	3.7–9.9	0.0090
Triglycerides (mmol/L)	66	1.2	0.60–3.3	69	1.3	0.75–2.7	65	1.1	0.70–2.7	0.38
apoA (g/L)	66	1.5	1.0–2.2	69	1.6	1.1–2.2	65	1.6	1.0–2.5	0.017
apoB (g/L)	66	0.82	0.46–1.4	69	0.81	0.5–1.4	65	0.72	0.43–1.1	0.072
apoB/apoA ratio	66	0.54	0.34–0.93	69	0.52	0.31–0.92	65	0.48	0.27–0.64	< 0.001

* The *p*-values were very similar with Kruskal–Wallis test and Jonckheere–Terpstra test for trend.

Table 2
Multivariable regression analysis of the effect of urinary arsenic concentrations on blood pressure and early risk markers of cardiovascular disease including hemoglobin. Beta-coefficient (β) for arsenic is presented for analysis of each outcome.

Outcome variables	[a]*n*	[b]Model 1		[c]Model 2	
		β (95% CI)	*p*-Val	β (95% CI)	*p*-Val
Systolic blood pressure	225	−0.94 (−2.0 to 0.12)	0.081	−0.93 (−2.0 to 0.13)	0.086
Diastolic blood pressure	225	−1.3 (−2.0 to −0.46)	0.0021	−1.3 (−2.04 to −0.47)	0.0019
Hemoglobin	225	−2.6 (−3.9 to −1.2)	< 0.001	−2.6 (−4.00 to −1.2)	< 0.001
Log2-homocysteine	221	−0.035 (−0.077 to 0.0061)	0.095	−0.035 (−0.076 to 0.0067)	0.099
Log2-triglycerides	200	−0.025 (−0.089 to 0.038)	0.43	−0.023 (−0.084 to 0.038)	0.45
apoA	200	0.041 (0.0045 to 0.078)	0.028	0.039 (0.0036 to 0.075)	0.031
apoB	200	−0.024 (−0.050 to 0.0016)	0.067	−0.024 (−0.050 to 0.0014)	0.064
Ratio apoB/apoA	200	−0.029 (−0.046 to −0.013)	< 0.001	−0.029 (−0.045 to −0.013)	< 0.001

[a] The number of women was the same for models 1 and 2.
[b] Outcome variable = $\alpha + \beta 1 \times \log 2 -$ arsenic in urine $+ \beta 2 \times$ age.
[c] Outcome variable = $\alpha + \beta 1 \times \log 2 -$ arsenic in urine $+ \beta 2 \times$ age $+ \beta 3 \times$ BMI $+ \beta 4 \times$ chewing of coca leaves.

disease (homocysteine and triglycerides were log2-transformed) were linear when considering the residuals. In linear regression analysis with adjustment for age (Table 2; Model 1), urinary arsenic concentrations were inversely associated with diastolic blood pressure (Fig. 1), hemoglobin (Supplementary Fig. S1), and apoB/A ratio; urinary arsenic concentrations were positively associated with apoA. In Model 2, the additional adjustment for BMI (or height Supplementary Table S4; Model 2) and coca-use did not change the parameter estimates or p-values substantially (Table 2).

Urinary arsenic concentrations were positively, but weakly, correlated with the anti-inflammatory cytokine IL-10 ($r_S=0.18$, $p=0.0077$) and the pro-inflammatory cytokine IL-12 ($r_S=0.15$, $p=0.026$). There were no significant correlations between arsenic and the other cytokines (data not shown).

Different sensitivity analyses were performed, excluding women who reported: (a) heart problems ($n=4$), (b) high blood pressure ($n=6$), (c) diabetes ($n=11$) (based on either questionnaire data or the presence of elevated glucose in urine), (d) pregnancy and (e) taking medicine; these exclusions affected the effect estimates to different extent, for most associations only marginally (Supplementary Tables S2 and S3). Excluding the smokers ($n=8$; Supplementary Table S3) the effect estimate of all outcomes did not change more than 12%.

Including lithium in the model resulted in larger inverse effect estimates for systolic and diastolic blood pressures, and hemoglobin, whereas the effect estimates for apoB and apoB/apoA decreased and became non-significant (Supplementary Table S4). Urinary lithium concentration was inversely associated with the pro-inflammatory cytokine IL-2 ($r_S=0.16$, $p=0.017$) by Spearman's rank correlations.

4. Discussion

This study on Andean women showed no indication of adverse effects of arsenic related to blood pressure or on risk markers of cardiovascular disease. Despite the fact that some women were at high age and chronically exposed to high levels of arsenic, the women had low blood pressure, low levels of triglycerides, and no signs of inflammation, as well as low levels of glucose and protein in urine. If anything, the women with high arsenic exposure showed lower blood pressure, lower homocysteine, and lower apoA concentrations. This is hard to explain and we cannot exclude that other underlying factors influenced these observations; for example, ethnicity could be important, since living at high altitude stresses the cardiovascular system and indigenous populations have adapted to the low oxygen pressure over time (Frisancho, 2013). Still, our results are in clear contrast to other studies that reported higher blood pressure, aberrant blood lipoprotein profiles, and hyperhomocysteinemia in relation to increasing arsenic exposure (Karim et al., 2013; Abhyankar et al., 2012; Pilsner et al., 2009). Moreover, there were no clear signs of inflammation in the women in our study whereas in vitro studies in mice and human cells have found that arsenic exposure reduced secretion of IL-2, IL-6, IL-10, and IL-12 (Cho et al., 2012; Soto-Pena and Vega, 2008). Thus, our study clearly shows that there are population differences with regard to susceptibility to arsenic-related cardiovascular toxicity.

However, our data suggested that urinary arsenic was associated with lower hemoglobin concentrations, a finding that is in accordance with studies on men and women in Bangladesh (Heck et al., 2008) and pregnant women in Chile (Hopenhayn et al., 2006). It is difficult to interpret this effect of arsenic on hemoglobin levels, as the populations living at high altitude have different regulation of hemoglobin to adjust to the low oxygen pressure compared with populations living at low altitude. Our finding therefore warrants further studies on the effects of arsenic on hemoglobin levels and whether this is associated with impaired iron status.

One drawback of the study is that the women were not fasting during blood sampling. However, we do not think this introduced a major bias in relation to the effects of arsenic, since the sampling was performed throughout the day in the same way both in areas with low and high arsenic exposure. The blood pressure of the Andean women was measured once and we could not determine hypertension. Elevated concentrations of glucose and protein in

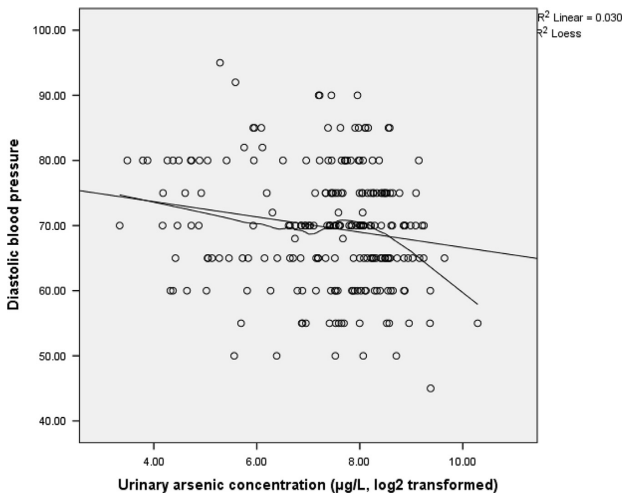

Fig. 1. Scatter plot depicting the association between log2-transformed urinary arsenic and diastolic blood pressure.

the urine might influence the specific gravity. However, very few individuals had elevated glucose in the urine and when excluded, including individuals with reported diabetes, we did not see any major changes in the effect estimates for the cardiovascular markers. Further, the women were exposed to lithium to a varying extent. Thus exposure to lithium might have masked some of the effects of arsenic. We handled this by taking lithium into account in the statistical analysis and the effect estimates became even more strongly inversely associated with arsenic, i.e., blood pressure and hemoglobin, but we observed no effect on homocysteine, triglycerides, and apoA. Lithium exposure can have an anti-inflammatory effect in humans (Nassar and Azab, 2014), but we did not find strong evidence that lithium influenced cytokine levels in our study.

The potential use of the results of this study in relation to public health will be explored in future studies.

5. Conclusion

In conclusion, our study shows that the effects of arsenic on blood pressure and early risk markers of cardiovascular disease differ between populations and this needs to be taken into account in risk assessment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2015.03.010.

References

Abhyankar, L.N., Jones, M.R., Gullar, E., Navas-Acien, A., 2012. Arsenic exposure and hypertension: a systemic review. Environ. Health Perspect. 120, 494–500.
Balakumar, P., Kaur, J., 2009. Arsenic exposure and cardiovascular disorders: an overview. Cardiovasc. Toxicol. 9 (4), 169–176.
Cho, Y., Ahn, K.H., Back, M.J., Choi, J.M., Ji, J.E., Won, J.H., et al., 2012. Age-related effects of sodium arsenite on splenocyte proliferation and TH1/TH2 cytokine production. Arch. Pharm. Res. 35, 375–382.
Concha, G., Nermell, B., Vahter, M., 2006. Spatial and temporal variations in arsenic exposure via drinking-water in northern Argentina. J. Health Popul. Nutr. 24, 317–327.
Concha, G., Broberg, K., Grander, M., Cardozo, A., Palm, B., Vahter, M., 2010. High-level exposure to lithium, boron, cesium, and arsenic via drinking water in the andes of northern Argentina. Environ. Sci. Technol. 44, 6875–6880.
Fangstrom, B., Hamadani, J., Nermell, B., Grander, M., Palm, B., Vahter, M., 2009. Impaired arsenic metabolism in children during weaning. Toxicol. Appl. Pharmacol. 239, 208–214.
Findlay, J.W., Dillard, R.F., 2007. Appropriate calibration curve fitting in ligand binding assays. AAPS J. 9, E260–E267.
Frisancho, A.R., 2013. Developmental functional adaptation to high altitude: review. Am. J. Hum. Biol. 25, 151–168.
Heck, J.E., Chen, Y., Grann, V.R., Slavkovich, V., Parvez, F., Ahsan, H., 2008. Arsenic exposure and anemia in Bangladesh: a population-based study. J. Occup. Environ. Med. 50, 80–87.
Hopenhayn-Rich, C., Biggs, M.L., Smith, A.H., Kalman, D.A., Moore, L.E., 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. Environ. Health Perspect. 104, 620–628.
Hopenhayn, C., Bush, H.M., Bingcang, A., Hertz-Picciotto, I., 2006. Association between arsenic exposure from drinking water and anemia during pregnancy. J. Occup. Environ. Med. 48, 635–643.
Hossain, M.B., Vahter, M., Concha, G., Broberg, K., 2012. Low-level environmental cadmium exposure is associated with DNA hypomethylation in Argentinean women. Environ. Health Perspect. 120, 879–884.
Joseph, J., Loscalzo, J., 2013. Methoxistasis: integrating the roles of homocysteine and folic acid in cardiovascular pathobiology. Nutrients 5, 3235–3256.
Karim, M.R., Rahman, M., Islam, K., Mamun, A.A., Hossain, S., Hossain, E., et al., 2013. Increases in oxidized low-density lipoproteins and other inflammatory and adhesion molecules with concomitant decrease in high-density lipoproteins in the individuals exposed to arsenic in Bangladesh. Toxicol. Sci. 135, 17–25.
Moon, K., Guallar, E., Navas-Acien, A., 2012. Arsenic exposure and cardiovascular disease: An updated systematic review. Curr. Atheroscler. Rep. 14, 542–555.
Moon, K., Guallar, E., Umans, J., Devereux, R., et al., 2013. Association between exposure to low to moderate arsenic levels and incident cardiovascular disease. Ann. Intern. Med. 159, 649–659.
Nassar, A., Azab, A.N., 2014. Effects of lithium on inflammation. ASC Chem. Neurosci., Epub ahead of print.
Nermell, B., Lindberg, A.L., Rahman, M., Berglund, M., Persson, L.A., El Arifeen, S., et al., 2008. Urinary arsenic concentration adjustment factors and malnutrition. Environ. Res. 106, 212–218.
Pilsner, J.R., Liu, X., Ahsan, H., Ilievski, V., Slavkovich, V., Levy, D., Factor-Litvak, P., Grazino, J.H., Gamble, M.V., 2009. Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. Environ. Health Perspect. 117, 254–260.
Shiue, I., Hristova, K., 2014. Higher urinary heavy metal, phthalate and arsenic concentrations accounted for 3–19% of the population attributable risk for high blood pressure: US NHANES 2009–2012. Hypertens. Res. 37, 1075–1081, Epub ahead of print.
Soto-Pena, G.A., Vega, L., 2008. Arsenic interferes with the signaling transduction pathway of t cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn in spleen cells. Toxicol. Appl. Pharmacol. 230, 216–226.
States, J.C., Srivastava, S., Chen, Y., Barchowsky, A., 2009. Arsenic and cardiovascular disease. Toxicol. Sci. 107 (2), 312–323.
Tognon, G., Berg, C., Mehlig, K., Thelle, D., Strandhagen, E., Gustavsson, J., et al., 2012. Comparison of apolipoprotein (ApoB/ApoA-1) and lipoprotein (total cholesterol/HDL) ratio determinants. Focus on obesity, diet and alcohol intake. PloS One 7, e40878.
Vahter, M., Concha, G., Nermell, B., Nilsson, R., Dulout, F., Natarajan, A.T., 1995. A unique metabolism of inorganic arsenic in native Andean women. Eur. J. Pharmacol. 293, 455–462.
Vahter, M., 2002. Mechanisms of arsenic biotransformation. Toxicology 181–182, 211–217.

The effects of arsenic exposure on blood pressure and markers of the cardiovascular system: evidence for population differences

Syeda Shegufta Ameer[a*], Karin Engström[a], Florencia Harari[b], Gabriela Concha[c], Marie Vahter[b], Karin Broberg[b]

a) Department of Laboratory Medicine, Section of Occupational and Environmental Medicine, Lund University, Lund, Sweden

b) Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden

c) Science Department, Risk Benefit Assessment Unit, National Food Agency, Uppsala, Sweden

Supplementary data

Supplementary data Table S1: General characteristics of the study population

Variable	**All**		
	n	**Median**	**Range**
Age (years)	225	34	18-65
Height (cm)	225	152	137-169
Weight (kg)	225	58	37-101
BMI	225	25	16-40.0
Parity	222	3	0-14
Arsenic in urine, specific gravity adjusted (µg/L)	225	200	10-1251
Arsenic in urine, creatinine adjusted (µg/g)	217	288	14-2661
Urinary creatinine (g/L)	217	0.99	0.020-2.7
Inorganic arsenic in urine (%)	225	10.9	2.3-34
MMA in urine (%)	225	7.5	2.4-22
DMA in urine (%)	225	81	57.9-95.1
Systolic blood pressure	225	115	90-155
Diastolic blood pressure	225	70	45-95
Hemoglobin (g/L)	225	154	77-202
Homocysteine (µmol/L)	221	6.0	2.6-17
Triglycerides (mmol/L)	200	1.2	0.40-7.3
apoA (g/L)	200	1.5	0.87-3.0
apoB (g/L)	200	0.8	0-1.83
Ratio apoB/apoA	200	0.5	0-1.2

Supplementary Material Table S2: Multivariable regression analysis between urinary arsenic concentrations and blood pressure and early risk markers of the cardiovascular disease, including hemoglobin, beta-coefficient (β) for arsenic is presented for each outcome variable.

Outcome variable	**Excluding women with heart disease (n =4)**			**Excluding women with high blood pressure (n = 6)**			**Excluding women with diabetes (n = 11)**		
	n	***β* (95% CI)**	***p*-value**	***n***	***β* (95% CI)**	***p*-value**	***n***	***β* (95% CI)**	***p*-value**
Systolic blood pressure	221	-0.80 (-1.9 to 0.27)	0.14	219	-0.98 (-2.1 to 0.97)	0.074	214	-0.84 (-1.9 to 0.25)	0.13
Diastolic blood pressure	221	-1.2 (-2.0 to -0.37)	0.0043	219	-1.2 (-1.9 to -0.41)	0.0029	214	-1.2 (-2.0 to -0.37)	0.0045
Hemoglobin	221	-2.6 (-4.1 to -1.2)	<0.001	219	-2.6 (-4.0 to -1.1)	<0.001	214	-2.5 (-3.9 to -1.1)	<0.001
Log2-Homocysteine	217	-0.034 (-0.077 to 0.0087)	0.12	215	-0.034 (-0.076 to 0.0076)	0.11	210	-0.032 (-0.074 to 0.011)	0.14
Log2-Triglycerides	197	-0.037 (-0.10 to 0.027)	0.26	195	-0.023 (-0.085 to 0.040)	0.48	191	-0.036 (-0.101 to 0.028)	0.27
apoA	197	0.045 (0.0075 to 0.082)	0.019	195	0.040 (0.0026 to 0.077)	0.036	191	0.036 (-0.0015 to 0.074)	0.060
apoB	197	-0.027 (-0.054 to 0.0012)	0.040	195	-0.023 (-0.048 to 0.0014)	0.064	191	-0.027 (-0.053 to -0.0016)	0.037
Ratio apoB/apoA	197	-0.033 (-0.049 to -0.016)	<0.001	195	-0.029 (-0.045 to -0.013)	<0.001	191	-0.030 (-0.047 to -0.014)	<0.001

Outcome variables = $\alpha + \beta 1 \times$ Log2 arsenic in urine $+ \beta 2 \times$ age

Supplementary Material Table S3: Multivariable regression analysis between urinary arsenic concentrations and blood pressure and early risk markers of the cardiovascular disease, including hemoglobin, beta-coefficient (β) for arsenic is presented for each outcome variables.

Outcome variables	**Excluding pregnant women (n =12)**			**Excluding women taking medicine (n = 15)**			**Excluding smokers (n = 8)**		
	n	***β* (95% CI)**	***p*-value**	***n***	***β* (95% CI)**	***p*-value**	***n***	***β* (95% CI)**	***p*-value**
Systolic blood pressure	195	-0.50 (-1.9 to 0.86)	0.47	210	-0.76 (-1.8 to 0.33)	0.17	217	-0.87 (-2.0 to 0.22)	0.12
Diastolic blood pressure	195	-1.2 (-2.1 to -0.18)	0.020	210	-1.1 (-1.9 to -0.24)	0.011	217	-1.3 (-2.1 to -0.49)	0.002
Hemoglobin	195	-3.1 (-4.9 to -1.2)	<0.001	210	-2.3 (-3.8 to -0.901)	0.0016	217	-2.5 (-3.9 to -1.1)	0.00082
Log2-Homocysteine	191	-0.010 (-0.063 to 0.042)	0.70	206	-0.035 (-0.077 to 0.0076)	0.11	213	-0.034 (-0.077 to 0.0093)	0.12
Log2-Triglycerides	170	-0.082 (-0.16 to 0.0068)	0.033	186	-0.028 (-0.094 to 0.038)	0.40	192	-0.037 (-0.101 to 0.028)	0.27
apoA	170	0.010 (0.035 to 0.055)	0.66	186	0.032 (-0.0053 to 0.070	0.092	192	0.036 (-0.0016 to 0.074)	0.060
apoB	170	-0.072 (-0.10 to -0.043)	<0.001	186	-0.026 (-0.052 to 0.00069)	0.056	192	-0.026 (-0.053 to -0.00027)	0.048
Ratio apoB/apoA	170	-0.051 (-0.071 to -0.031)	<0.001	186	-0.027 (-0.043 to -0.011)	0.0012	192	-0.028 (-0.045 to -0.012)	0.00082

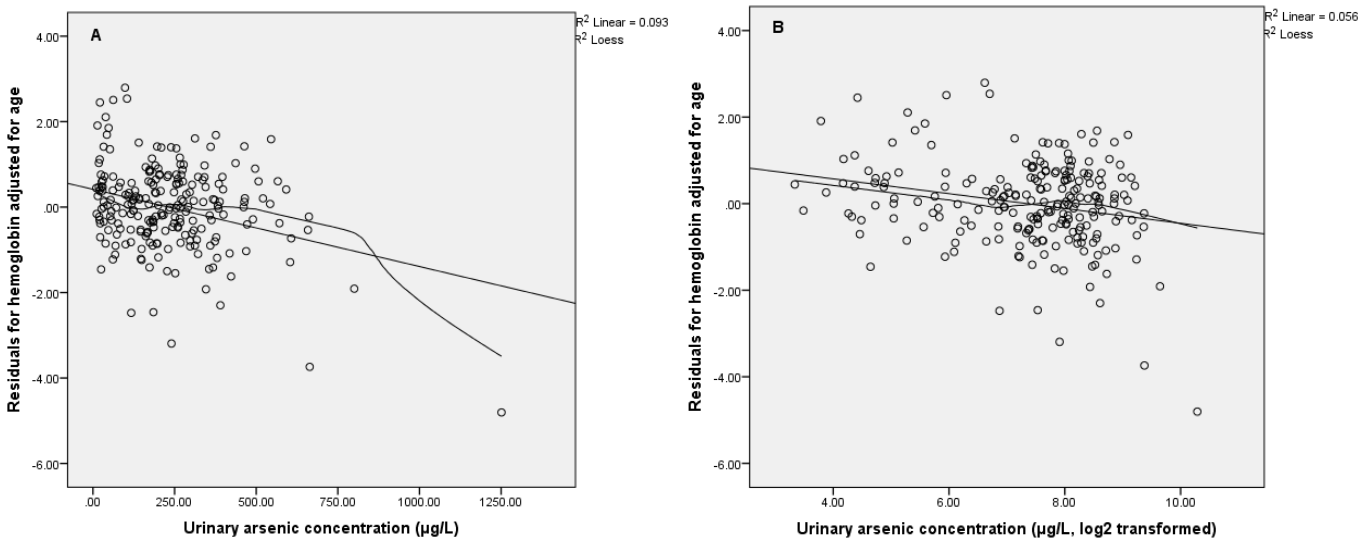
Outcome variables = $\alpha + \beta 1 \times$ Log2 arsenic in urine $+ \beta 2 \times$ age

Supplementary Material Table S4: Multivariable regression analysis of the association of urinary arsenic concentrations with blood pressure and early risk markers of the cardiovascular disease, including hemoglobin, taking lithium (Model 1) and height (Model 2) into account. Beta-coefficient (β) for arsenic is presented for each outcome variable.

Outcome variable	**[a]Model 1**			**[b]Model 2**		
	n	***β* (95% CI)**	***p*-Val**	***n***	***β* (95% CI)**	***p*-Val**
Systolic blood pressure	217	-1.7 (-3.5 to 0.04)	0.056	225	-0.90 (-2.0 to 0.17)	0.099
Diastolic blood pressure	217	-1.9 (-3.2 to -0.58)	0.005	225	-1.2 (-2.0 to -0.44)	0.0023
Hemoglobin	217	-4.9 (-7.2 to -2.6)	<0.001	225	-2.6 (-4.0 to -1.2)	<0.001
Log2-homocysteine	213	-0.04(-0.09 to 0.006)	0.09	221	-0.034 (-0.075 to 0.0077)	0.11
Log2-triglycerides	194	0.03 (-0.04 to 0.10)	0.43	200	-0.025 (-0.089 to 0.039)	0.44
apoA	194	0.072 (0.009 to 0.13)	0.025	200	0.041 (0.0040 to 0.078)	0.030
apoB	194	<0.001 (-0.04 to 0.04)	0.99	200	-0.025 (-0.051 to 0.00061)	0.056
Ratio apoB/apoA	194	-0.023 (-0.051 to 0.005)	0.10	200	-0.030 (-0.046 to -0.014)	<0.001

[a]Outcome variable = $\alpha + \beta 1$ x Log2 arsenic in urine + $\beta 2$ x age + $\beta 3$ x urinary lithium.

[b]Outcome variable = $\alpha + \beta 1$ x Log2 arsenic in urine + $\beta 2$ x age + $\beta 3$ x height + $\beta 4$ x chewing of coca leaves.



Supplementary Figure 1. Scatter plots for (A) arsenic concentrations and (B) log2-transformed arsenic concentrations versus age-adjusted residuals for hemoglobin concentrations.

Paper II

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Exposure to Inorganic Arsenic Is Associated with Increased Mitochondrial DNA Copy Number and Longer Telomere Length in Peripheral Blood

*Syeda S. Ameer[1], YiYi Xu[1], Karin Engström[1,2], Huiqi Li[1], Pia Tallving[1], Barbro Nermell[2], Analia Boemo[3], Luis A. Parada[4], Lidia G. Peñaloza[3], Gabriela Concha[5], Florencia Harari[2], Marie Vahter[2] and Karin Broberg[2]**

[1] Department of Laboratory Medicine, Division of Occupational and Environmental Medicine, Lund University, Lund, Sweden, [2] Unit of Metals and Health, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, [3] Facultad de Ciencias Exactas and Consejo de Investigación, Universidad Nacional de Salta, Salta, Argentina, [4] Institute of Experimental Pathology – UNSa – CONICET, Salta, Argentina, [5] Risk Benefit Assessment Unit, Science Department, National Food Agency, Uppsala, Sweden

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Edited by:
Gerd Patrick Bienert, Institute of Plant Genetics and Crop Plant Research (LG), Germany

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Eleonora Napoli, University of California, Davis, USA
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***Correspondence:**
Karin Broberg
karin.broberg@ki.se

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Background: Exposure to inorganic arsenic (iAs) through drinking water causes cancer. Alterations in mitochondrial DNA copy number (mtDNAcn) and telomere length in blood have been associated with cancer risk. We elucidated if arsenic exposure alters mtDNAcn and telomere length in individuals with different arsenic metabolizing capacity.

Methods: We studied two groups in the Salta province, Argentina, one in the Puna area of the Andes ($N = 264$, 89% females) and one in Chaco ($N = 169$, 75% females). We assessed arsenic exposure as the sum of arsenic metabolites [iAs, methylarsonic acid (MMA), dimethylarsinic acid (DMA)] in urine (U-As) using high-performance liquid chromatography coupled with hydride generation and inductively coupled plasma mass spectrometry. Efficiency of arsenic metabolism was expressed as percentage of urinary metabolites. MtDNAcn and telomere length were determined in blood by real-time PCR.

Results: Median U-As was 196 (5–95 percentile: 21–537) μg/L in Andes and 80 (5–95 percentile: 15–1637) μg/L in Chaco. The latter study group had less-efficient metabolism, with higher %iAs and %MMA in urine compared with the Andean group. U-As was significantly associated with increased mtDNAcn (log2 transformed to improve linearity) in Chaco ($\beta = 0.027$ per 100 μg/L, $p = 0.0085$; adjusted for age and sex), but not in Andes ($\beta = 0.025$, $p = 0.24$). U-As was also associated with longer telomere length in Chaco ($\beta = 0.016$, $p = 0.0066$) and Andes ($\beta = 0.0075$, $p = 0.029$). In both populations, individuals with above median %iAs showed significantly higher mtDNAcn and telomere length compared with individuals with below median %iAs.

Conclusions: Arsenic was associated with increased mtDNAcn and telomere length, particularly in individuals with less-efficient arsenic metabolism, a group who may have increased risk for arsenic-related cancer.

Keywords: MMA, energy, oxidative phosphorylation, cancer, AS3MT

INTRODUCTION

Elevated arsenic concentrations in drinking water occur in many countries, e.g., in Argentina, Bangladesh, India, and some parts of the U.S.A. Arsenic exposure is a known risk factor for different types of malignancies, mainly of epithelial origin, such as cancer of the lung, bladder, kidney, skin, liver, and prostate (International Agency for Researsch on Cancer, 2012). However, the mechanisms behind the carcinogenic effect of arsenic have not been completely clarified.

The susceptibility to arsenic differs between individuals and a key factor for individual susceptibility is the capacity to metabolize arsenic and excrete it efficiently. Inorganic arsenic (iAs) is metabolized to methylarsonic acid (MMA), and further, to dimethylarsinic acid (DMA). In humans, like most mammals, efficient methylation from iAs to DMA is associated with decreased reactivity and increased rate of urinary arsenic excretion (Vahter, 2002; Gardner et al., 2011). The degree of methylation to DMA varies within and between individuals and populations suggesting a genetic component for the arsenic metabolism (Vahter, 2002). Incomplete arsenic metabolism seems to be a marker of increased susceptibility to arsenic-related diseases: individuals with high fraction of inorganic arsenic and/or MMA in urine have higher risk for skin lesions and skin cancer (Lindberg et al., 2008; Pilsner et al., 2009; Antonelli et al., 2014; Engström et al., 2015) and cardiovascular disease (Chen et al., 2013; Wu et al., 2015) compared to individuals with low fraction of inorganic arsenic and/or MMA in urine. Genetic variation in arsenite methyltransferase (*AS3MT*), which encodes the major arsenic-methylating enzyme, significantly contributes to arsenic metabolism efficiency (Engström et al., 2011; Schlebusch et al., 2013, 2015) and is likely an underlying factor partly determining variation in susceptibility to arsenic between individuals and populations (Hossain et al., 2012; Pierce et al., 2013; Engström et al., 2015).

Telomeres, the tandem repeats (TTAGGG) at the ends of the chromosomes, protect the chromosomes from recombination and degradation (Blasco, 2005), which could lead to genome instability. In cross-sectional and prospective studies, alterations in telomere length in peripheral blood have been associated with increased risk for several types of cancer (Ma et al., 2011), such as cancers in the head and neck, bladder, breast, skin and lung (Wu et al., 2003; McGrath et al., 2007; Shen et al., 2007, 2011; Han et al., 2009; Seow et al., 2014). Previously, we (Li et al., 2012) and others (Zhang et al., 2013) reported that arsenic in urine was associated with longer telomeres in peripheral blood suggesting that arsenic modifies the individual telomere length.

Another cancer-related genetic component is the mitochondrial DNA (mtDNA). The mitochondrion is the key organelle for energy metabolism, but it also has important functions in the regulation of apoptosis (Lee and Wei, 2000) and cell proliferation (Martinez-Outschoorn et al., 2011). Recent findings have suggested that mitochondrial metabolic disease can lead to cancer (Seyfried, 2015). Mitochondria possess their own extra-nuclear circular DNA that exists in different copy numbers (mtDNAcn) in each mitochondrion. Mitochondria generate high levels of reactive oxygen species (ROS) during energy production. Lack of introns and protective histones and its proximity to the electron transport chain make the mtDNA highly susceptible to oxidative damage (Van Houten et al., 2006). Alterations in mtDNAcn may be associated with cancer risk as indicated by case-control and prospective cohort studies which have shown that mtDNAcn is associated with increased risk of cancer in the breast, head and neck, prostate, kidney and colon/rectum in women (Thyagarajan et al., 2013; Cheau-Feng Lin et al., 2014; Huang et al., 2014; Zhou et al., 2014; Lemnrau et al., 2015; Melkonian et al., 2015).

We have previously reported that the indigenous people living in the Andean part of the Salta province in the northern Argentina, the so called Puna area, have a unique capacity of metabolizing inorganic arsenic, mainly due to presence of genetic polymorphisms in *AS3MT*, which is one of the main genes regulating arsenic methylation (Vahter et al., 1995; Engström et al., 2011; Schlebusch et al., 2013, 2015). Other areas in Salta province, such as Chaco, situated in the eastern part of the Salta province, also have arsenic-contaminated drinking water (Concha et al., 1998). People living in Chaco are mainly of mixed ethnicity (descendants of Spanish/European). The local health authority in Salta invited our group to conduct a study there with the aim to compare the populations from the Andes and the Chaco regions for arsenic metabolism and toxicity. Thus, the aim of the present study was to evaluate the effect of arsenic, including modifying effects of the arsenic metabolizing capacity, on the cancer-related markers telomere length and mtDNAcn in two arsenic-exposed study groups with different genetic background in northern Argentina.

MATERIALS AND METHODS

Study Areas and Study Participants

Andes

Two hundred and sixty four individuals, including men (n = 28) and women (n = 236), were recruited in 2008 (n = 218) and 2011 (n = 46) in the Puna area of the Andes Mountains (about 3800 m above sea level) in the province of Salta, northern Argentina. The recruitments in 2008 and 2011 were performed in a similar way and individuals who participated in 2008 were not recruited in 2011. Most of the individuals were living in the village of San Antonio de los Cobres (n = 192), which has elevated arsenic concentrations in the drinking water (∼200 μg/L), and 72 individuals were living in small surrounding villages with lower water arsenic concentrations (3.5–70 μg/L) (Concha et al., 2010). The source of drinking water in San Antonio de los Cobres is a natural spring, Agua de Castilla, located about 1 km outside the village, and the water is chlorinated before being distributed through pipes to the village (Concha et al., 2010). The total population of San Antonio de los Cobres and surrounding villages is about 8100 inhabitants, and are mainly of indigenous origin (Kolla ethnicity).

Details of the recruitments have been described elsewhere (Engström et al., 2011). Briefly, individuals were interviewed about their age, ancestry, time of residence, drinking water sources, dietary habits, medical history, smoking, alcohol

consumption, coca chewing, as well as parity (for women). Also, weight and height were measured and used for calculation of body mass index (BMI). We have previously reported a positive association of arsenic exposure with telomere length in the women recruited from this study area in 2008 (Li et al., 2012).

Chaco

One hundred and sixty-nine individuals were recruited in the Chaco and Anta areas east of the city of Salta (up to 300 m above sea level); here referred to as participants from Chaco. Individuals in Chaco were informed about the study by radio announcements, by primary health care workers, and by contacting individuals in the lines at the post-office. In the Rivadavia hospital the patients' attendants were asked to participate and those who wanted to volunteer were included in the study. Individuals were living in different areas [El Rincón ($n = 25$), Los Rosales ($n = 22$) and other villages in rural Anta county ($n = 36$); Rivadavia ($n = 26$), La Unión ($n = 12$) and Nueva Población ($n = 6$) in Rivadavia county; and El Galpón ($n = 42$) in Metán county]. The recruitment was performed in 1999 (Anta) and 2013 (all other areas). The arsenic concentrations in drinking water (mainly well water) varied between 110 and 837 μg/L in Anta (several water sources), 4.7 μg/L in El Galpón (samples collected from several small villages), about 230 μg/L in Rivadavia, 72 μg/L in Los Rosales, 983 μg/L in Nueva Población and 23 μg/L in La Unión. The total population in Anta is ∼8000 inhabitants, in El Galpón ∼8400, and in Rivadavia, and La Unión 6400. The people are mostly "creoles," i.e., of Spanish/European descents. Sampling was done following the standard procedure developed by Unit of Metals and Health, Institute of Environmental Medicine at Karolinska Institutet, and the study participants in Chaco were asked similar questions as in the Andes.

Men and women, 16 years and older, were included in the study. The Health Ministry of Salta (Salta, Argentina) and the Regional Ethical Committee at the Karolinska Institutet (Stockholm, Sweden) approved the study. Oral and written informed consent was obtained from all study participants.

Urine and Blood Sampling

Spot urine and blood samples were collected from all study participants and the sample collection was performed throughout the day. Mid-stream urine samples were collected in plastic urine collection cups and then transferred to 24 mL polyethylene tubes and immediately frozen. Peripheral blood samples were collected in K_2EDTA tubes (Vacuette; Greiner Bio-One, Greiner, Germany) for DNA isolation.

Analysis of Arsenic Exposure

We measured total arsenic concentrations in water samples collected from the Andes and Chaco regions by inductively coupled plasma mass spectrometry (ICPMS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) as previously described (Concha et al., 2010). Water from rural Anta was analyzed by atomic absorption spectrophotometry (AAS) (Concha et al., 2006). We did not perform speciation of arsenic in the water. Most of the absorbed pentavalent arsenic is rapidly reduced in blood (Vahter, 2002), before the trivalent form (but not the pentavalent) is taken up by the hepatocytes where it is methylated (Lerman et al., 1983). Exposure to arsenic was determined based on the sum of concentrations of iAs and its metabolites (MMA + DMA) in urine (U-As). We assessed the efficiency of arsenic metabolism by the fractions (percentages) of iAs, MMA, and DMA in urine (Vahter, 2002). Arsenic metabolites in urine were measured by high-performance liquid chromatography (HPLC: Agilent 1100 series system, Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and ICPMS (HPLC-HG-ICPMS) as described previously (Gardner et al., 2011). Urine samples from rural Anta was analyzed for arsenic metabolites by HPLC-AAS, as previously described (Concha et al., 2006). To compensate for variation in dilution of urine, the arsenic concentrations were adjusted to the mean specific gravity, measured by a hand refractometer (Atago, Japan) or a digital refractometer (EUROMEX RD 712 clinical refractometer; EUROMEX, Arnhem, the Netherlands). We evaluated the data using overall specific gravity value of 1.020 for adjustment for all samples.

Analysis of Mitochondrial DNA Copy Number

Human mtDNA is a small, double-stranded DNA encoding 22 tRNAs, 2 rRNAs, and 13 genes that code for protein subunits of the electron transport chain complexes. The primers were selected from the mtDNA tRNA region, because other mtDNA regions are highly polymorphic and may contain common deletions. The mitochondrial $tRNA^{Leu\ (UUR)}$ gene region is rarely deleted and contains only a few rare single nucleotide polymorphisms (mtSNP) (Venegas and Halberg, 2012). The relative mtDNAcn was measured based on the method by Hou et al. (2010) by using real-time PCR (7900HT, Applied Biosystems, Foster City, CA, U.S.A.). Master mixes were prepared with KAPA SYBR FAST qPCR Kit Master Mix (2X) ABI Prism (Kapa Biosystems, Woburn, MA, U.S.A.), and primers (0.20 μM for each primer) for mtDNAcn were: forward 5′-CAC CCA AGA ACA GGG TTT GT-3′ and reverse 5′-TGG CCA TGG GTA TGT TGT TA-3′; and primers for the hemoglobin beta (*HBB*) gene were: forward 5′-TGT GCT GGC CCA TCA CTT TG-3′ and reverse 5′-ACC AGC CAC CAC TTT CTG ATA GG-3′. The mtDNA amplicon is 107 bp long and the mtDNA primers are specific to mtDNA as evaluated by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)(Gen bank accession number: Accession no: NC 012920.1). Each reaction (end volume 10 μl) consisted of 2.5 μl of DNA (4 ng/μl) and 7.5 μl master mix. The thermal cycle profile was 95°C for 3 min, followed by 95°C for 3 s and 60°C for 20 s for 25 cycles (mtDNAcn) or 35 cycles (*HBB*). A standard curve, one control sample, and a sample with no template were also included in each run. All samples and standard curve points were run in triplicate. For the standard curve, one reference DNA sample was diluted serially twofold to produce five concentrations of 1–16 ng/μl. R^2 for each standard curve was >0.99. Standard deviations of <0.1 were accepted for the C_t values of each triplicate. SDS 2.4.1 software (Life Technologies) calculated the relative quantity of mtDNAcn and

HBB for each sample, based on the standard curve. The relative mtDNAcn was the quotient of the quantity of mtDNAcn and *HBB*, and thus, it is an arbitrary value. The coefficient of variation (CV) of the control sample included in every run was 12%.

Analysis of Telomere Length

DNA was isolated from peripheral blood with the Qiagen DNA blood Midi kit (Qiagen, Hilden, Germany). The relative telomere length was measured using real-time PCR (7900HT), as described previously (Li et al., 2012). Briefly, the master mix for telomere length determination was prepared with telomere-specific primers (0.45 μM of each primer), 1 × PCR Buffer (all PCR reagents from Life Technologies) 1.75 mM $MgCl_2$, 0.8 mM dNTPs, 0.3 mM SYBR Green, 1 × Rox, and 0.5 U Platinium *Taq*. Master mix for *HBB* runs were prepared with *HBB* primers (0.40 μM for each primer) and KAPA SYBR FAST qPCR Kit Master Mix (2X) ABI Prism (Kapa Biosystems). Five microliters of sample DNA (4 ng/μl) was added to each reaction (final volume 20 μl). A standard curve, a reference DNA, and a negative control were also included in each run, all samples, standards, and controls were run in triplicate. For the standard curve, one calibrator DNA sample was diluted serially by twofold, to produce concentrations of 1–16 ng/μl. R^2 for each standard curve was >0.99. Standard deviations (for C_t values of each triplicate) were accepted at <0.1. The relative length of the telomeres was obtained through calculating the ratio (T/S) of the telomere repeat product to a single-copy gene product (S, here *HBB*) for each individual, by the formula $T/S = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{telomere} - Ct_{HBB}$. This ratio was then compared with the ratio of the reference DNA. The telomere length ratio is an arbitrary value. The CV of the reference DNA included in every run was 9.8%. The telomere length for subjects in the Andes and Chaco were measured at two different time-points and with different reference DNAs. To make the data comparable, we measured the ratio between the two different reference DNAs and adjusted the values of Andes by this ratio, so that all data were based on the same reference DNA.

Statistical Analyses

Statistical analyses were performed using SPSS software, version 22 (IBM, Chicago, IL, USA). Statistical significance refers to $p < 0.05$. Data from the study groups in the Andes and Chaco were analyzed separately.

Mann-Whitney U test was used to evaluate the differences in characteristics of the study participants between the study groups. In order to investigate how the characteristics differed with U-As, we stratified U-As into tertiles to evaluate potential non-linear differences in characteristics by Jonckheere-Terpstra trend test.

We evaluated the associations between U-As, mtDNAcn, telomere length and potentially confounders (age, gender, BMI, and chewing of coca leaves) using Spearman's rank correlation coefficient. The linearity between U-As and mtDNAcn as well as U-As and telomere length were inspected using scatter plots. In order to improve linearity between U-As and mtDNAcn, we log2-transformed mtDNAcn for both Andes and Chaco. Linear regression analyses were performed to evaluate the associations between U-As and log2-mtDNAcn, and U-As and telomere length. The crude model (model 1) included only U-As and the adjusted model (model 2) included age and gender as covariates (confounders). Age and gender were selected as covariates based on the knowledge from published literature. Other covariates such as BMI and coca chewing were considered for adjustments in sensitivity analyses (see below).

The influence of efficiency of arsenic metabolism on the associations between U-As and log2-mtDNAcn or U-As and telomere length were analyzed by including an interaction term between U-As and the fraction of each metabolite separately (defined as above and below median of %iAs, %MMA, or %DMA). We also performed linear regression analyses with individuals stratified by median values of the U-As metabolites.

Several sensitivity analyses were performed. The differences in telomere length or mtDNAcn could reflect alterations in cell composition. We therefore analyzed the associations between U-As and log2-mtDNAcn as well as U-As and telomere length in a subgroup ($n = 79$ Andean women) where we adjusted for estimated fractions of cell types (CD4 and CD8 T-cells, B-cells, natural killer cells, monocytes, and granulocytes) in blood. The cell composition was derived from epigenetic signatures of cells retrieved from whole-genome DNA methylation data using Infinium HumanMethylation 450 K BeadChip [lllumina, CA, U.S.A., Engström et al., 2013] and was estimated by the method by Houseman et al. (2012), which estimates the relative proportion of pure cell types within a sample, where the DNA methylation signature is a surrogate for the distribution of white blood cells. We also did sensitivity analysis for chewing of coca leaves in the Andes only, since none of the participants chewed coca-leaves in Chaco. We had information about smoking and BMI for a few individuals in Chaco and mainly from individuals with low exposure to arsenic; therefore sensitivity analyses with adjustments for smoking or BMI were not considered meaningful. Further, few individuals ($n = 6$) in Chaco reported hyperkeratosis, we also performed sensitivity analyses excluding those individuals to see the effect of arsenic on mtDNAcn and telomere length in rest of the Chaco population.

RESULTS

Characteristics of the Study Groups

The general characteristics of the two study groups are presented in **Table 1** (characteristics stratified by tertiles of U-As are presented in Supplementary Material Table S2) and the drinking water concentrations in the different villages are presented in Supplementary Material Table S1. The arsenic concentrations in water showed a large range both in Andes and Chaco and the corresponding U-As concentrations indicated that the major exposure source to arsenic was the drinking water (Andes: $r_S = 0.72$, $p < 0.001$; Chaco: $r_S = 0.87$, $p < 0.001$). The study groups differed in height and weight, where individuals in Andes were shorter and weighed less than in Chaco. Most participants were women; 11% in Andes and 25% in Chaco were men. We observed a trend of increase in BMI by increasing U-As concentrations in Chaco (Supplementary Material Table S2). The overall median U-As was 196 μg/L and 80 μg/L for the Andes

TABLE 1 | Characteristics of the Andes and Chaco study groups.

	Andes			Chaco			
Variable	***n***	**Median**	**5/95 percentile**	***n***	**Median**	**5/95 percentile**	***p*-value [a]**
Age (years)	264	35	20 to 65	169	39	18 to 69	0.078
Sex	264		89% female	169		75% female	<0.001
Height (cm)	264	153	144 to 167	127	158	148 to 174	<0.001
Weight (kg)	264	59	45 to 84	127	63	43 to 92	0.023
BMI (kg/m^2)	264	25	19 to 35	127	25	18 to 36	0.80
Parity	243	3	0 to 11			No data	
Arsenic in urine (μg/L)	264	196	21 to 537	169	80	15 to 1637	<0.001
iAs in urine (%)	264	11	4.3 to 23	169	17	5.6 to 34	<0.001
MMA in urine (%)	264	7.9	4.0 to 14	169	10	3.0 to 22	<0.001
DMA in urine (%)	264	81	66 to 91	169	71	49 to 86	<0.001
mtDNAcn[b]	244	0.57	0.33 to 1.0	159	0.84	0.44 to 1.5	<0.001
Telomere length[b]	208	0.43	0.30 to 0.58	159	1.10	0.73 to 1.7	<0.001

[a] *p-value is derived from the Mann-Whitney U-test.*
[b] *The relative mtDNAcn/telomere length was the quotient of the quantity of mtDNAcn/telomere and HBB, and thus, it is an arbitrary value.*

and Chaco study groups, respectively. The metabolite pattern differed significantly between the study groups: the median % iAs and %MMA were higher and the median %DMA was lower in Chaco compared with the Andes (**Table 1**). The %iAs increased and %DMA decreased significantly with increasing U-As tertiles in the Andes, and %MMA increased and %DMA decreased significantly with increasing U-As tertiles in Chaco (Supplementary Material Table S2).

The median values of relative mtDNAcn and the relative telomere length differed significantly between the study groups, where participants in Chaco had higher mtDNAcn and telomere length than participants in Andes ($p < 0.001$, **Table 1**). The median values of mtDNAcn and telomere length differed significantly between the U-As tertiles in Chaco (Supplementary Material Table S2). MtDNAcn and telomere length were positively correlated (Andes, $r_S = 0.14$, $p = 0.054$; Chaco, $r_S = 0.29$, $p < 0.001$). Telomere length was negatively correlated with age in both study groups (Andes $r_S = -0.31$, $p < 0.001$, Chaco $r_S = -0.36$, $p < 0.001$), while mtDNAcn was not correlated with age in any of the groups. The median mtDNAcn differed between the sexes; it was lower for men in Andes but lower for women in Chaco (Andes: women = 0.58, men = 0.54, $p = 0.15$; Chaco: women = 0.77, men = 0.98, $p = 0.001$). The median telomere length was higher for men in Chaco, but it did not differ between the sexes in the Andes (Andes: women = 0.43, men = 0.43, $p = 0.29$; Chaco: women = 1.06, men = 1.21, $p = 0.031$).

The study group from Chaco included six study participants in a family clinically diagnosed with arsenic related hyperkeratosis. These six participants had very high U-As concentrations (median 1943 μg/L; range 623–2258 μg/L) and also high %MMA in urine (average 21%; range 18–24%) compared with the other study participants in Chaco (Supplementary Material Table S3). Their telomere length did not differ from the rest of the participants in Chaco, but their mtDNAcn was higher (median relative values 1.1 vs. 0.83).

Arsenic Exposure, Mitochondrial DNA Copy Number, and Telomere Length

We observed linear relationships between U-As and log2-mtDNAcn, as well as U-As and telomere length in blood in both study groups (**Figure 1**). Increasing U-As was significantly associated with both higher log2-mtDNAcn and with longer telomere length in Chaco, and to a lesser extent for both outcomes in Andes (models 1 and 2; **Table 2**). Among the Andean women, we made further adjustments for estimated cell proportions of white blood cells (CD4 and CD8 T-cells, B-cells, natural killer cells, monocytes, and granulocytes). MtDNAcn and telomere length were not significantly correlated with the estimated cell proportion for any cell type. When adjusting the analyses for estimated cell proportions, the effect estimates for U-As decreased between 9 and 10% for log2-mtDNAcn and about 2% for telomere length, but the associations still remained with $p < 0.05$. We also adjusted the analyses for chewing of coca-leaves in the Andes: the effect estimates for U-As did not change for mtDNAcn, but increased 3% for telomere length. Finally, we excluded people with hyperkeratosis in Chaco, and we found that the association between U-As and mtDNAcn or U-As and telomere length were not driven by these individuals, and the associations still remained below $p = 0.05$.

Modification by Arsenic Metabolism Efficiency

Because the arsenic metabolism efficiency has been shown to be a susceptibility factor for arsenic-related disease (Antonelli et al., 2014), we further evaluated the role of the arsenic metabolism for effects of arsenic on telomere length and mtDNAcn. We found statistically significant interactions between %iAs and U-As, as well as between %DMA and U-As on the effect of log2-mtDNAcn in the Andes (adjusted models, $p = 0.013$ for %iAs, $p = 0.0083$ for %DMA), but not in Chaco (**Table 3**). After stratifying the data sets at the median values of %iAs and %DMA, there were, both

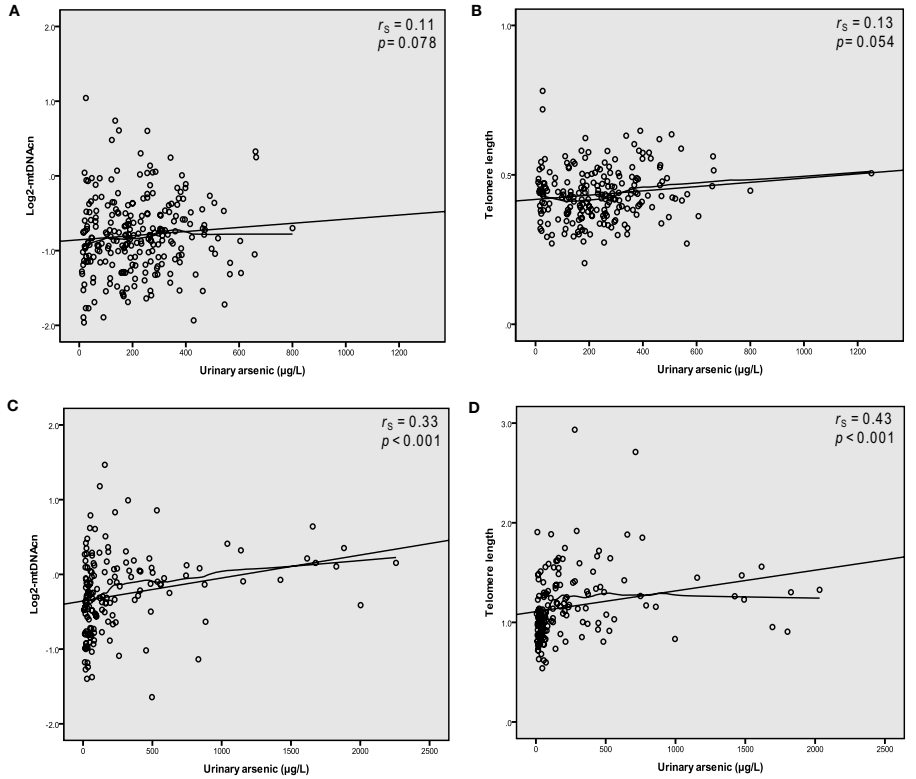

FIGURE 1 | Arsenic in urine is associated with higher copy number of mitochondrial DNA (log2-transformed, abbreviation mtDNAcn) and longer telomeres in blood. Scatter plots depicting relations between urinary arsenic (sum of inorganic arsenic metabolites) concentrations and log2-mtDNAcn and telomere length in Andes **(A,B)** and Chaco **(C,D)**.

TABLE 2 | Associations of U-As[a] with log2-mtDNAcn and telomere length in peripheral blood.

Marker/study group		Model 1[b]		Model 2[c]	
	n	β (95% CI)	*p*-value	β (95% CI)	*p*-value
mtDNAcn					
Andes	244	0.028 (−0.014 to 0.069)	0.19	0.025 (−0.017 to 0.066)	0.24
Chaco	159	0.031 (0.011 to 0.050)	0.0024	0.027 (0.0069 to 0.046)	0.0085
TELOMERE LENGTH					
Andes	208	0.0071 (0.00012 to 0.014)	0.046	0.0075 (0.00078 to 0.014)	0.029
Chaco	159	0.019 (0.0070 to 0.032)	0.0024	0.016 (0.0045 to 0.028)	0.0066

[a] *U-As referred to as sum of urinary arsenic metabolites.*

[b] *Log2-mtDNAcn/telomere length* $= \alpha + \beta 1 \times$ *U-As (per 100* μ*g/L).*

[c] *Log2-mtDNAcn/telomere length* $= \alpha + \beta 1 \times$ *U-As (per 100* μ*g/L)* $+ \beta 2 \times$ *age* $+ \beta 3 \times$ *gender.*

TABLE 3 | Multivariable regression analyses of the associations between U-As and log2-mtDNAcn stratified for arsenic metabolism efficiency (above and below median of fraction of each metabolite)[a].

Study group	mtDNAcn[b]	% iAs	*n*	β_1 (95% CI)[c]	*p*-value[c]	*p*-value[d]
Andes	0.56	<11	122	−0.018 (−0.076 to 0.039)	0.53	0.013
	0.58	≥11	122	0.084 (0.022 to 0.15)	0.0080	
Chaco	0.76	<17	80	0.012 (−0.020 to 0.044)	0.45	0.82
	0.93	>17	79	0.028 (0.0019 to 0.054)	0.036	
		% DMA				
Andes	0.58	<80.9	122	0.076 (0.017 to 0.13)	0.011	0.0083
	0.56	≥80.9	122	−0.030 (−0.092 to 0.031)	0.33	
Chaco	0.94	<71.1	79	0.027 (0.0038 to 0.049)	0.023	0.83
	0.77	≥71.1	80	0.020 (−0.038 to 0.079)	0.49	

[a]*U-As referred to as sum of urinary arsenic metabolites.*
[b]*Presented as median relative values of mtDNAcn.*
[c]*Log2-mtDNAcn = α + β1 × U-As (per 100 μg/L) + β2 × age + β3 × gender.*
[d]*p-value for interaction (β4) from the equation: Log2-mtDNAcn = α + β1 × U-As (per 100 μg/L) + β2 × age + β3 × gender + β4 × (U-As × < and > median %iAs/%MMA/%DMA).*

TABLE 4 | Multivariable regression analyses of the associations between U-As and telomere length stratified for arsenic metabolism efficiency (above and below median of fraction of each metabolite)[a].

Study group	TL[b]	% iAs	*n*	β_1 (95% CI)[c]	*p*-value[c]	*p*-value[d]
Andes	0.41	<11	94	0.0024 (−0.0089 to 0.014)	0.67	0.35
	0.45	≥11	114	0.0089 (0.00036 to 0.017)	0.041	
Chaco	1.01	<17	80	0.011 (−0.0059 to 0.028)	0.20	0.35
	1.19	>17	79	0.018 (0.0019 to 0.034)	0.029	
		% DMA				
Andes	0.45	<80.9	114	0.0069 (−0.0012 to 0.015)	0.093	0.73
	0.41	≥80.9	94	0.0051 (−0.0076 to 0.018)	0.43	
Chaco	1.23	<71.1	79	0.012 (−0.0028 to 0.026)	0.11	0.61
	1.001	≥71.1	80	0.024 (−0.0053 to 0.054)	0.11	

[a]*U-As referred to as sum of urinary arsenic metabolites. The number of individuals in Andes in each < and ≥ median split groups varied for telomere length as telomere length measurements were only available for individuals sampled in 2008.*
[b]*Presented as median relative values of telomere length.*
[c]*Telomere length = α + β1 × U-As (per 100 μg/L) + β2 × age + β3 × gender.*
[d]*p-value for interaction (β4) from the equation: telomere length = α + β1 × U-As (per 100 μg/L) + β2 × age + β3 × gender + β4 × (U-As × < and > median %iAs/%MMA/%DMA).*

in Andes and Chaco, significant positive associations between U-As and log2-mtDNAcn among individuals with high %iAs and low %DMA, but not among individuals with low %iAs and high %DMA.

There were no significant interactions between fractions of arsenic metabolites and U-As on telomere length (**Table 4**). However, in the stratified analyses, statistically significant positive associations between U-As and telomere length were only found among individuals with high %iAs in both the study groups. There was less of a clear pattern for modification by %DMA or by %MMA (Supplementary Material Table S4).

DISCUSSION

In this study, we explored associations of arsenic exposure through drinking water, assessed by the concentration of arsenic metabolites in urine, with carcinogenic markers (telomere length and mtDNAcn) in two study groups with different ethnic backgrounds. We found that the effect of arsenic exposure varied between the study groups. In Chaco, arsenic exposure showed persuasive associations with both telomere length and mtDNAcn, but this was not so clear in the Andes, where only association was found with telomere length. This discordance in effects likely relates to variation in the efficiency of arsenic metabolism: the Chaco individuals had, compared with the Andes, in general less-efficient metabolism, reflected by higher fractions of iAs and MMA and lower fractions of DMA in urine. Associations between U-As and the DNA markers were observed also in the Andean study group, but only in the individuals with less efficient metabolism, i.e., with highest fraction of iAs in urine. These findings stress the role of arsenic metabolism in the toxicity of arsenic, and that some individuals and population groups, due to their lower capacity for arsenic biotransformation, might be more susceptible to arsenic toxicity than others. We recently reported a signal for genetic selection in the *AS3MT* gene in people in the Andes (Schlebusch et al., 2013, 2015), partly overlapping with the

present study group, and our data here support our hypothesis that the indigenous people in the Andes show fewer adverse effects of arsenic exposure due to a long-term adaptation to an arsenic-rich environment, compared with a study group like the Chaco, where people have lived in an arsenic-contaminated area for much shorter time.

Similar to our study, others have found that blood mtDNAcn increased in response to exposure to different carcinogenic hazards, such as exposure to low levels of benzene, maternal smoking, and sun exposure (Carugno et al., 2012; Gebhard et al., 2014; Stangenberg et al., 2015), but there are only few studies addressing the influence of arsenic on mtDNAcn. One study reported increased mitochondrial biogenesis and function in arsenic-induced skin cancer patients compared with healthy subjects (Lee et al., 2011). In contrast, human-hamster AL cells treated with high concentrations of sodium arsenite (0.5–1 mg/L), showed decreased mtDNAcn along with increased large heteroplasmic mtDNA deletions (Partridge et al., 2007). It has been suggested that the increased oxidative stress from exposure to toxic compounds has a dual influence on the mtDNA (Hou et al., 2010). Mild stress might stimulate mtDNA synthesis and increase the number of mitochondria to fulfill the need for cellular respiratory capacity for cell survival. However, excessive oxidative stress may cause decreased mtDNA synthesis, possibly due to increasing defects in mitochondria resulting in apoptosis and cell death (Hou et al., 2010). This duality in response to mild vs. excessive oxidative stress might also explain previous findings on the effects of arsenic on telomere length. In human cord blood cells, sub-nM arsenite increased *TERT* (the main telomere-maintaining enzyme) gene and protein expression *in vitro*, resulting in maintained telomere length, while at 1 μM arsenite, *TERT* expression and telomere length decreased (Ferrario et al., 2009). In people exposed to arsenic via drinking water (1–1000 μg/L) in Inner Mongolia, *TERT* expression was positively associated with arsenic concentrations in water and in fingernails, and with the severity of arsenic related hyperkeratosis (Mo et al., 2009). The latter should be noted as we found a family in Chaco demonstrating hyperkeratosis. This family did not have longer telomeres, but did have more mtDNAcn compared with the individuals without hyperkeratosis in Chaco. These findings, however, should be very cautiously interpreted as they were based on few study subjects.

We found a significant correlation between telomere length and mtDNAcn in both study groups. *TERT* maintains telomere length under oxidative stress, but in a time- and dose-dependent manner, *TERT* gets excluded from the nucleus and localizes in the mitochondria, giving protection by decreasing mitochondrial superoxide production and cell peroxide levels (Ahmed et al., 2008). One can hypothesize that in our study with low to moderate exposure to arsenic, arsenic stimulates *TERT* expression, which in turn results in telomere lengthening as well as stimulation of mitochondrial DNA production.

However, in prospective studies, longer telomeres in blood have been associated with increased risk for lung (Shen et al., 2011; Seow et al., 2014) and pancreatic cancers (Lynch et al., 2013). We found in both study groups that arsenic was associated with longer telomeres in blood, even after taking cell profiles in blood into account. This confirms earlier findings (Ferrario et al., 2009) and provides strong evidence that arsenic has a true positive effect on telomeres. Arsenic causes lung cancer (International Agency for Reseasrch on Cancer, 2012), and our findings here suggests a mechanistic link that this occurs by lengthening of the telomeres. Higher mtDNAcn in blood has in one prospective study been associated with increased risk of breast cancer (Thyagarajan et al., 2013; Lemnrau et al., 2015), and in case-control studies been linked to head and neck cancer (Cheau-Feng Lin et al., 2014), prostate cancer (Zhou et al., 2014), and chronic lymphocytic lymphoma (Hosnijeh et al., 2014). Of these cancer forms, arsenic has to some extent been associated with breast and prostate cancer (López-Carrillo et al., 2014; Bardach et al., 2015). MtDNAcn seems to be a promising marker for future cancer risk, but these women need to be followed up in order to evaluate the actual link between arsenic, mtDNAcn, telomere length and cancer.

Telomere length and mtDNAcn levels differed between the study groups, where the Andean study group showed much lower median values of mtDNAcn and telomere length compared with the Chaco study group. We speculate that this disparity is related to their ethnic backgrounds and living conditions, particularly the differences in altitude. High altitude is correlated with high hemoglobin (Ameer et al., 2015), and in turn high iron levels in blood. Iron concentrations is associated with increased oxidative stress (Tuomainen et al., 2007), which could shorten the telomeres and affect the mtDNA contents.

In conclusion, we here provide evidence that arsenic exposure through drinking water increases the telomere length and mtDNAcn, two pre-carcinogenic markers, and that the arsenic methylating capacity modifies the degree of DNA changes.

AUTHOR CONTRIBUTIONS

KB and MV conceived the study. KE, PT, AB, LAP, LGP, GC, FH, BN, MV, and KB did the sampling and data collection. GC, BN, FH, and MV performed the arsenic measurements. SSA, YX, and HL designed the experiments for mtDNA and TL and performed them. SSA performed statistical analysis, interpreted the data. SSA and KB wrote the manuscript, which was reviewed by the coauthors. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00087

REFERENCES

Ahmed, S., Passos, J. F., Birket, M. J., Beckmann, T., Brings, S., Peters, H., et al. (2008). Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. *J. Cell Sci.* 121, 1046–1053. doi: 10.1242/jcs.019372

Ameer, S. S., Engström, K., Harari, F., Concha, G., Vahter, M., and Broberg, K. (2015). The effects of arsenic exposure on blood pressure and early risk markers of cardiovascular disease: evidence for population differences. *Environ. Res.* 140, 32–36. doi: 10.1016/j.envres.2015.03.010

Antonelli, R., Shao, K., Thomas, D. J., Sams, R. II, and Cowden, J. (2014). *AS3MT, GSTO,* and *PNP* polymorphisms: impact on arsenic methylation and implications for disease susceptibility. *Environ. Res.* 132, 156–167. doi: 10.1016/j.envres.2014.03.012

Bardach, A. E., Ciapponi, A., Soto, N., Chaparro, M. R., Calderon, M., Briatore, A., et al. (2015). Epidemiology of chronic disease related to arsenic in Argentina: a systematic review. *Sci. Total Environ.* 538, 802–816. doi: 10.1016/j.scitotenv.2015.08.070

Blasco, M. A. (2005). Telomeres and human disease: ageing, cancer and beyond. *Nat. Rev. Genet.* 6, 611–622. doi: 10.1038/nrg1656

Carugno, M., Pesatori, A. C., Dioni, L., Hoxha, M., Bollati, V., Albetti, B., et al. (2012). Increased mitochondrial DNA copy number in occupations associated with low-dose benzene exposure. *Environ. Health Perspect.* 120, 210–215. doi: 10.1289/ehp.1103979

Cheau-Feng Lin, F., Jeng, Y. C., Huang, T. Y., Chi, C. S., Chou, M. C., and Chin-Shaw Tsai, S. (2014). Mitochondrial DNA copy number is associated with diagnosis and prognosis of head and neck cancer. *Biomarkers* 19, 269–274. doi: 10.3109/1354750X.2014.902101

Chen, Y., Wu, F., Liu, M., Parvez, F., Slavkovich, V., Eunus, M., et al. (2013). A prospective study of arsenic exposure, arsenic methylation capacity, and risk of cardiovascular disease in Bangladesh. *Environ. Health Perspect.* 121, 832–838. doi: 10.1289/ehp.1205797

Concha, G., Broberg, K., Grandér, M., Cardozo, A., Palm, B., and Vahter, M. (2010). High-level exposure to lithium, boron, cesium, and arsenic via drinking water in the Andes of northern Argentina. *Environ. Sci. Technol.* 44, 6875–6880. doi: 10.1021/es1010384

Concha, G., Nermell, B., and Vahter, M. (2006). Spatial and temporal variations in aresnic exposure via drinking-water in northern Argentina. *J. Health Popul. Nutr.* 24, 317–326.

Concha, G., Nermell, B., and Vahter, M. V. (1998). Metabolism of inorganic arsenic in children with chronic high arsenic exposure in northern Argentina. *Environ. Health Perspect.* 106, 355–359. doi: 10.1289/ehp.98106355

Engström, K. S., Hossain, M. B., Lauss, M., Ahmed, S., Raqib, R., Vahter, M., et al. (2013). Efficient arsenic metabolism–the *AS3MT* haplotype is associated with DNA methylation and expression of multiple genes around *AS3MT*. *PLoS ONE* 8:e53732. doi: 10.1371/journal.pone.0053732

Engström, K. S., Vahter, M., Fletcher, T., Leonardi, G., Goessler, W., Gurzau, E., et al. (2015). Genetic variation in arsenic (+3 oxidation state) methyltransferase (*AS3MT*), arsenic metabolism and risk of basal cell carcinoma in a European population. *Environ. Mol. Mutagen.* 56, 60–69. doi: 10.1002/em.21896

Engström, K., Vahter, M., Mlakar, S. J., Concha, G., Nermell, B., Raqib, R., et al. (2011). Polymorphisms in Arsenic(+III Oxidation State) Methyltransferase (*AS3MT*) predict gene expression of *AS3MT* as well as arsenic metabolism. *Environ. Health Perspect.* 119, 182–188. doi: 10.1289/ehp.1002471

Ferrario, D., Collotta, A., Carfì, M., Bowe, G., Vahter, M., Hartung, T., et al. (2009). Arsenic induces telomerase expression and maintains telomere length in human cord blood cells. *Toxicology* 260, 132–141. doi: 10.1016/j.tox.2009.03.019

Gardner, R. M., Nermell, B., Kippler, M., Grander, M., Li, L., Ekström, E. C., et al. (2011). Arsenic methylation efficiency increases during the first trimester of pregnancy independent of folate status. *Reprod. Toxicol.* 31, 210–218. doi: 10.1016/j.reprotox.2010.11.002

Gebhard, D., Mahler, B., Matt, K., Burger, K., and Bergemann, J. (2014). Mitochondrial DNA copy number - but not a mitochondrial tandem CC to TT transition - is increased in sun-exposed skin. *Exp. Dermatol.* 23, 209–211. doi: 10.1111/exd.12327

Han, J., Qureshi, A. A., Prescott, J., Guo, Q., Ye, L., Hunter, D. J., et al. (2009). A prospective study of telomere length and the risk of skin cancer. *J. Invest. Dermatol.* 129, 415–421. doi: 10.1038/jid.2008.238

Hosnijeh, F. S., Lan, Q., Rothman, N., San Liu, C., Cheng, W. L., Nieters, A., et al. (2014). Mitochondrial DNA copy number and future risk of B-cell lymphoma in a nested case-control study in the prospective EPIC cohort. *Blood* 124, 530–535. doi: 10.1182/blood-2013-10-532085

Hossain, M. B., Vahter, M., Concha, G., and Broberg, K. (2012). Environmental arsenic exposure and DNA methylation of the tumor suppressor gene *p16* and the DNA repair gene *MLH1*: effect of arsenic metabolism and genotype. *Metallomics* 4, 1167–1175. doi: 10.1039/c2mt20120h

Hou, L., Zhu, Z. Z., Zhang, X., Nordio, F., Bonzini, M., Schwartz, J., et al. (2010). Airborne particulate matter and mitochondrial damage: a cross-sectional study. *Environ. Health* 9:48. doi: 10.1186/1476-069X-9-48

Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., et al. (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* 13:86. doi: 10.1186/1471-2105-13-86

Huang, B., Gao, Y. T., Shu, X. O., Wen, W., Yang, G., Li, G., et al. (2014). Association of leukocyte mitochondrial DNA copy number with colorectal cancer risk: results from the Shanghai women's health study. *Cancer Epidemiol. Biomarkers Prev.* 23, 2357–2365. doi: 10.1158/1055-9965.EPI-14-0297

IARC (International Agency for Reseasrch on Cancer) (2012). Arsenic, metals, fibres, and dusts. *IARC Monogr. Eval. Carcinog. Risks Hum.* 100(Pt C):11–465.

Lee, C. H., Wu, S. B., Hong, C. H., Liao, W. T., Wu, C. Y., Chen, G. S., et al. (2011). Aberrant cell proliferation by enhanced mitochondrial biogenesis via mtTFA in arsenical skin cancers. *Am. J. Pathol.* 178, 2066–2076. doi: 10.1016/j.ajpath.2011.01.056

Lee, H. C., and Wei, Y. H. (2000). Mitochondrial role in life and death of the cell. *J. Biomed. Sci.* 7, 2–15. doi: 10.1007/BF02255913

Lemnrau, A., Brook, M. N., Fletcher, O., Coulson, P., Jones, M., Tomczyk, K., et al. (2015). Mitochondrial DNA copy number in peripheral blood cells and risk of developing breast cancer. *Cancer Res.* 75, 2844–2850. doi: 10.1158/0008-5472.CAN-14-1692

Lerman, S. A., Clarkson, T. W., and Gerson, R. J. (1983). Arsenic uptake and metabolism by liver cells is dependent on arsenic oxidation state. *Chem. Biol. Interact.* 45, 401–406. doi: 10.1016/0009-2797(83)90087-X

Li, H., Engström, K., Vahter, M., and Broberg, K. (2012). Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. *Chem. Res. Toxicol.* 25, 2333–2339. doi: 10.1021/tx300222t

Lindberg, A. L., Rahman, M., Persson, L. A., and Vahter, M. (2008). The risk of arsenic induced skin lesions in Bangladeshi men and women is affected by arsenic metabolism and the age at first exposure. *Toxicol. Appl. Pharmacol.* 230, 9–16. doi: 10.1016/j.taap.2008.02.001

López-Carrillo, L., Hernández-Ramírez, R. U., Gandolfi, A. J., Ornelas-Aguirre, J. M., Torres-Sánchez, L., and Cebrian, M. E. (2014). Arsenic methylation capacity is associated with breast cancer in northern Mexico. *Toxicol. Appl. Pharmacol.* 280, 53–59. doi: 10.1016/j.taap.2014.07.013

Lynch, S. M., Major, J. M., Cawthon, R., Weinstein, S. J., Virtamo, J., Lan, Q., et al. (2013). A prospective analysis of telomere length and pancreatic cancer in the alpha-tocopherol beta-carotene cancer (ATBC) prevention study. *Int. J. Cancer* 133, 2672–2680. doi: 10.1002/ijc.28272

Ma, H., Zhou, Z., Wei, S., Liu, Z., Pooley, K. A., Dunning, A. M., et al. (2011). Shortened telomere length is associated with increased risk of cancer: a meta-analysis. *PLoS ONE* 6:e20466. doi: 10.1371/journal.pone.0020466

Martinez-Outschoorn, U. E., Pavlides, S., Sotgia, F., and Lisanti, M. P. (2011). Mitochondrial biogenesis drives tumor cell proliferation. *Am. J. Pathol.* 178, 1949–1952. doi: 10.1016/j.ajpath.2011.03.002

McGrath, M., Wong, J. Y., Michaud, D., Hunter, D. J., and De Vivo, I. (2007). Telomere length, cigarette smoking, and bladder cancer risk in men and women. *Cancer Epidemiol. Biomarkers Prev.* 16, 815–819. doi: 10.1158/1055-9965.EPI-06-0961

Melkonian, S. C., Wang, X., Gu, J., Matin, S. F., Tannir, N. M., Wood, C. G., et al. (2015). Mitochondrial DNA copy number in peripheral blood leukocytes and the risk of clear cell renal cell carcinoma. *Carcinogenesis* 36, 249–255. doi: 10.1093/carcin/bgu248

Mo, J., Xia, Y., Ning, Z., Wade, T. J., and Mumford, J. L. (2009). Elevated human telomerase reverse transcriptase gene expression in blood cells associated with

chronic arsenic exposure in Inner Mongolia, China. *Environ. Health Perspect.* 117, 354–360. doi: 10.1289/ehp.11532

Partridge, M. A., Huang, S. X., Hernandez-Rosa, E., Davidson, M. M., and Hei, T. K. (2007). Arsenic induced mitochondrial DNA damage and altered mitochondrial oxidative function: implications for genotoxic mechanisms in mammalian cells. *Cancer Res.* 67, 5239–5247. doi: 10.1158/0008-5472.CAN-07-0074

Pierce, B. L., Tong, L., Argos, M., Gao, J., Farzana, J., Roy, S., et al. (2013). Arsenic metabolism efficiency has a causal role in arsenic toxicity: mendelian randomization and gene-environment interaction. *Int. J. Epidemiol.* 42, 1862–1871. doi: 10.1093/ije/dyt182

Pilsner, J. R., Liu, X., Ahsan, H., Ilievski, V., Slavkovich, V., Levy, D., et al. (2009). Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. *Environ. Health Perspect.* 117, 254–260. doi: 10.1289/ehp.11872

Schlebusch, C. M., Gattepaille, L. M., Engström, K., Vahter, M., Jakobsson, M., and Broberg, K. (2015). Human adaptation to arsenic-rich environments. *Mol. Biol. Evol.* 32, 1544–1555. doi: 10.1093/molbev/msv046

Schlebusch, C. M., Lewis, C. M. Jr., Vahter, M., Engström, K., Tito, R. Y., Obregón-Tito, A. J., et al. (2013). Possible positive selection for an arsenic-protective haplotype in humans. *Environ. Health Perspect.* 121, 53–58. doi: 10.1289/ehp.1205504

Seow, W. J., Cawthon, R. M., Purdue, M. P., Hu, W., Gao, Y. T., Huang, W. Y., et al. (2014). Telomere length in white blood cell DNA and lung cancer: a pooled analysis of three prospective cohorts. *Cancer Res.* 74, 4090–4098. doi: 10.1158/0008-5472.CAN-14-0459

Seyfried, T. N. (2015). Cancer as a mitochondrial metabolic disease. *Front. Cell Dev. Biol.* 3:43. doi: 10.3389/fcell.2015.00043

Shen, J., Terry, M. B., Gurvich, I., Liao, Y., Senie, R. T., and Santella, R. M. (2007). Short telomere length and breast cancer risk: a study in sister sets. *Cancer Res.* 67, 5538–5544. doi: 10.1158/0008-5472.CAN-06-3490

Shen, M., Cawthon, R., Rothman, N., Weinstein, S. J., Virtamo, J., Hosgood, H. D. III, et al. (2011). A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of lung cancer. *Lung Cancer* 73, 133–137. doi: 10.1016/j.lungcan.2010.11.009

Stangenberg, S., Nguyen, L. T., Chen, H., Al-Odat, I., Killingsworth, M. C., Gosnell, M. E., et al. (2015). Oxidative stress, mitochondrial perturbations and fetal programming of renal disease induced by maternal smoking. *Int. J. Biochem. Cell Biol.* 64, 81–90. doi: 10.1016/j.biocel.2015.03.017

Thyagarajan, B., Wang, R., Nelson, H., Barcelo, H., Koh, W. P., and Yuan, J. M. (2013). Mitochondrial DNA copy number is associated with breast cancer risk. *PLoS ONE* 8:e65968. doi: 10.1371/journal.pone.0065968

Tuomainen, T. P., Loft, S., Nyyssonen, K., Punnonen, K., Salonen, J. T., and Poulsen, H. E. (2007). Body iron is a contributor to oxidative damage of DNA. *Free Radic. Res.* 41, 324–328. doi: 10.1080/10715760601091642

Vahter, M. (2002). Mechanisms of arsenic biotransformation. *Toxicology* 181–182, 211–217. doi: 10.1016/S0300-483X(02)00285-8

Vahter, M., Concha, G., Nermell, B., Nilsson, R., Dulout, F., and Natarajan, A. T. (1995). A unique metabolism of inorganic arsenic in native Andean women. *Eur. J. Pharmacol.* 293, 455–462. doi: 10.1016/0926-6917(95)90066-7

Van Houten, B., Woshner, V., and Santos, J. H. (2006). Role of mitochondrial DNA in toxic responses to oxidative stress. *DNA Repair (Amst).* 5, 145–152. doi: 10.1016/j.dnarep.2005.03.002

Venegas, V., and Halberg, M. C. (2012). Measurement of mitochondrial DNA copy number. *Methods Mol. Biol.* 837, 327–335. doi: 10.1007/978-1-61779-504-6_22

Wu, F., Jasmine, F., Kibriya, M. G., Liu, M., Cheng, X., Parvez, F., et al. (2015). Interaction between arsenic exposure from drinking water and genetic polymorphisms on cardiovascular disease in Bangladesh: a prospective case-cohort study. *Environ. Health Perspect.* 123, 451–457. doi: 10.1289/ehp.1307883

Wu, X., Amos, C. I., Zhu, Y., Zhao, H., Grossman, B. H., Shay, J. W., et al. (2003). Telomere dysfunction: a potential cancer predisposition factor. *J. Natl. Cancer Inst.* 95, 1211–1218. doi: 10.1093/jnci/djg011

Zhang, X., Lin, S., Funk, W. E., and Hou, L. (2013). Environmental and occupational exposure to chemicals and telomere length in human studies. *Occup. Environ. Med.* 70, 743–749. doi: 10.1136/oemed-2012-101350

Zhou, W., Zhu, M., Gui, M., Huang, L., Long, Z., Wang, L., et al. (2014). Peripheral blood mitochondrial DNA copy number is associated with prostate cancer risk and tumor burden. *PLoS ONE* 9:e109470. doi: 10.1371/journal.pone.0109470

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Article Title

Exposure to inorganic arsenic is associated with increased mitochondrial DNA copy number and longer telomere length in peripheral blood

Syeda Shegufta Ameer[1], YiYi Xu[1], Karin Engström[2], Huiqi Li[1], Pia Tallving[1], Barbro Nermell[2], Analia Boemo[3], Luis A. Parada[4], Lidia Guadalupe Peñaloza[3], Gabriela Concha[5], Florencia Harari[2], Marie Vahter[2], Karin Broberg[2*]

1) Department of Laboratory Medicine, Division of Occupational and Environmental Medicine, Lund University, Lund, Sweden

2) Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden

3) Facultad de Ciencias Exactas & Consejo de Investiga ción, Universidad Nacional de Salta, Salta, Argentina

4) Institute of Experimental Pathology – UNSa – CONICET, Salta, Argentina

5) Science Department, Risk Benefit Assessment Unit, National Food Agency, Uppsala, Sweden

[*] Corresponding author

Address correspondence to Karin Broberg, Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden. Telephone: +46737823750. Fax: +46 08336981; E-mail: karin.broberg@ki.se

Supplementary Material Table S1: Concentrations of arsenic in drinking water and urine of women in Andes and Chaco.

Study groups	*n*	Arsenic in water (µg/L)	*n*	Urinary arsenic (µg/L)
Andes				
San Antonio de los Cobres	2	208	192	260 (20 – 1251)
Tolar Grande	2	3.5	26	25 (10 – 121)
Salar de Pocitos	2	72	5	73 (27 – 117)
Olacapato	2	12	10	31 (14 – 56)
Santa Rosa de los Pastos Grandes	1	31	15	71 (33 – 118)
Cobres	1	3.5	13	47 (22 – 149)
Rosario de Lerma	3	0.67	3	68 (64 – 147)
Chaco				
Anta	7	212	36	315 (70 – 1843)
El Rincón	2	13	25	43 (17 – 97)
Los Rosales	1	72	22	150 (60 – 877)
El Galpón	7	4.7	42	20 (6.5 – 236)
Rivadavia	2	243	26	346 (76 – 585)
La Unión	1	23	12	53 (27 – 71)
Nueva Población	1	983	6	1943 (623 – 2258)

*The arsenic concentrations in water and urine were analyzed in our laboratory at Karolinska Institutet. The arsenic concentrations in water and urine for Andes population were previously reported in Concha et al. (Concha et al., 2010).

Supplementary Material Table S2: Characteristics of the Andes and Chaco study groups stratified for tertiles of U-As [a] (µg/L).

		Tertile 1			*Tertile 2*			*Tertile 3*		
Variables	***n***	**Median**	**5th-95th %**	***n***	**Median**	**5th-95th %**	***n***	**Median**	**5th-95th %**	***p**- value**
Age (years)										
Andes	88	35	19-63	88	36	19-65	88	36	21-66	0.36
Chaco	57	43	20-64	56	37	18-74	56	41	16-64	0.83
Sex (% women)										
Andes	88		86%	88		92%	88		90%	0.46
Chaco	57		93%	56		75%	56		57%	<0.001
BMI (kg/m^2)										
Andes	88	25	19-35	88	26	21-35	88	25	19-35	0.96
Chaco	57	24	19-36	42	25	17-36	28	29	18-35	0.044
Urinary arsenic (µg/L) [b]										
Andes	88	53	15-124	88	196	142-266	88	368	275-660	<0.001
Chaco	57	22	12-42	56	80	49-184	56	491	215-2016	<0.001
iAs in urine (%)										
Andes	88	9.1	3.7-23	88	11	4.6-24	88	12	4.6-24	0.026
Chaco	57	19	4.2-37	56	15	5.2-38	56	18	7.8-37	0.060
MMA in urine (%)										
Andes	88	7.2	3.1-13	88	8.2	4.6-15	88	7.9	4.2-16	0.098
Chaco	57	9.1	2.5-16	56	9.1	3.1-18	56	15	6.4-24	<0.001
DMA in urine (%)										
Andes	88	83	68-92	88	80	64-90	88	79	65-90	0.017
Chaco	57	73	49-84	56	75	55-88	56	65	45-84	0.010
mtDNAcn										
Andes	81	0.54	0.29-0.98	81	0.58	0.33-1.2	82	0.59	0.34-1.04	0.22
Chaco	50	0.74	0.42-1.30	55	0.80	0.52-1.8	54	0.97	0.42-1.8	<0.001
Telomere length										
Andes	69	0.43	0.30-0.56	70	0.42	0.29-0.58	69	0.44	0.31-0.63	0.079
Chaco	50	0.98	0.70-1.5	55	1.10	0.61-1.6	54	1.26	0.83-2.1	<0.001

**p*-value is derived from Jonckheere-Terpstra trend test between the tertiles. [a]U-As referred to as sum of urinary arsenic metabolites. [b]adjusted to a mean specific gravity of 1.020.

Supplementary Material Table S3: General characteristics of the study participants with hyperkeratosis and remaining participants in Chaco.

	Hyperkeratosis		**Rest of Chaco**	
Variables	**Median (*n*=6)**	**Min to max**	**Median (*n*=153)**	**Min to max**
Urinary arsenic (µg/L)	1943	623 to 2258	78	6.52 to 1843
%iAs	14	8.2 to 27	17	2.3 to 56
%MMA	21	18 to 24	10.2	0.75 to 27
%DMA	65	52 to 72	71	36 to 97
mtDNAcn	1.11	0.75 to 1.6	0.83	0.21 to 2.8
Telomere length	1.03	0.91 to 1.3	1.09	0.54 to 2.9

Supplementary Material Table S4. Multivariable regression analyses of the associations between U-As and log2-mtDNAcn stratified for arsenic metabolism efficiency (above and below median of fraction of each metabolite)[a].

Study group	Median	% MMA	*n*	β_1 (95% CI)[c]	*p*-value[c]	*p*-value[d]
	mtDNAcn[b]					
Andes	0.56	<7.9	122	0.017 (-0.034 to 0.069)	0.50	0.48
	0.58	>7.9	122	0.030 (-0.035 to 0.095)	0.37	
Chaco	0.77	<10	79	0.066 (-0.023 to 0.15)	0.14	0.31
	0.92	>10	80	0.024 (0.0030 to 0.045)	0.026	
	Telomere Length[b]					
Andes	0.43	<7.9	106	0.0048 (-0.0068 to 0.016)	0.42	0.37
	0.43	>7.9	102	0.010 (0.0019 to 0.018)	0.017	
Chaco	1.06	<10	79	0.050 (0.010 to 0.090)	0.015	0.15
	1.15	>10	80	0.013 (-0.0024 to 0.028)	0.098	

[a]U-As referred to as sum of urinary arsenic metabolites. The number of individuals in Andes in each < and ≥ median split groups varied for telomere length as telomere length measurements were only available for individuals sampled in 2008.
[b]Presented as median relative values of mtDNAcn and telomere lenght.
[c]Log2-mtDNAcn / telomere length = $\alpha + \beta1 \times$ U-As (per 100 µg/L) + $\beta2 \times$ age + $\beta3 \times$ gender
[d]*p*-value for interaction ($\beta4$) from the equation: Log2-mtDNAcn / telomere length = $\alpha + \beta1 \times$ U-As (per 100 µg/L) + $\beta2 \times$ age + $\beta3 \times$ gender + $\beta4 \times$ (U-As × < and > median %iAs/%MMA/%DMA).

Paper III

Arsenic exposure from drinking water is associated with decreased gene expression in peripheral blood, possibly via increased DNA methylation

Syeda Shegufta Ameer[1], Karin Engström[1,2], Mohammad Bakhtiar Hossain[1], Gabriela Concha[3], Marie Vahter[2], Karin Broberg[2*]

1) Department of Laboratory Medicine, Division of Occupational and Environmental Medicine, Lund University, Lund, Sweden

2) Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden

3) Science Department, Risk Benefit Assessment Unit, National Food Agency, Uppsala, Sweden

[*] Corresponding author

Address correspondence to K. Broberg, Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, SE-17177 Stockholm, Sweden. Telephone: +46737823750. Fax: +46-8-336981; E-mail: Karin.broberg@ki.se

Abbreviations

DMA = dimethylarsinic acid; MMA = monomethylarsonic acid; ICP-MS = inductively coupled plasma mass spectrometry; iAs = inorganic arsenic; U-As = sum of inorganic arsenic metabolites.

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Abstract

Background: Exposure to inorganic arsenic increases the risk of cancer and non-malignant diseases. Inefficient arsenic metabolism is a marker for susceptibility to arsenic toxicity. Arsenic may alter gene expression, possibly by altering DNA methylation.

Objectives: To elucidate the associations between arsenic exposure, gene expression, and DNA methylation in peripheral blood, and the modifying effects of arsenic metabolism.

Methods: The study participants, women from the Andes, Argentina, were exposed to arsenic via drinking water. Arsenic exposure was assessed as the sum of arsenic metabolites (inorganic arsenic, methylarsonic acid, and dimethylarsinic acid) in urine (U-As), using high performance liquid-chromatography hydride-generation inductively-coupled-plasma-mass-spectrometry, and the arsenic metabolism efficiency was assessed by the urinary fractions (%) of the individual metabolites. Genome-wide gene expression (N=80 women) and DNA methylation (N=93; 80 overlapping with gene expression) in peripheral blood were measured using Illumina DirectHyb HumanHT-12 v4.0 and Infinium Human-Methylation 450K BeadChip, respectively.

Results: U-As concentrations, ranging 10-1251 µg/L, was associated with decreased gene expression in blood: 64% of the top 1000 differentially expressed genes were down-regulated with increasing U-As. U-As was also associated with hypermethylation: 87% of the top 1000 CpGs associated with U-As were hypermethylated with increasing U-As. The expression of six genes and six individual CpG sites were significantly associated with increased U-As concentration. Pathway analyses revealed enrichment of genes related to cell death and cancer. The pathways differed somewhat depending on arsenic metabolism efficiency. We found no evident overlap between arsenic-related gene expression and DNA methylation for individual genes.

Conclusions: Increased arsenic exposure was associated with lower gene expression in peripheral blood, possibly by perturbing DNA methylation.

Introduction

Millions of people world-wide consume inorganic arsenic in their drinking water. Large regions in Bangladesh, India, Taiwan, Argentina, United States, Vietnam, Mexico, and Chile have drinking water that exceeds the WHO recommended limit of 10 µg/ml of arsenic. Arsenic, an established carcinogen (IARC. 2012), has also been associated with non-malignant diseases such as diabetes and cardiovascular diseases, including hypertension (Abhyankar et al. 2012; Kuo et al. 2013; Moon et al. 2012; Moon et al. 2013). Humans metabolize inorganic arsenic (iAs) to methylarsonic acid (MMA), and dimethylarsinic acid (DMA), which are excreted in urine. Multiple studies indicate that incomplete arsenic metabolism, with higher fractions of iAs and MMA in the urine, is a marker for increased susceptibility to arsenic-related diseases, as shown for skin lesions and skin cancer (Antonelli et al. 2014; Engstrom et al. 2015; Pilsner et al. 2009), cardiovascular disease (Chen et al. 2013a), and genotoxicity (Ameer et al. 2016; Hossain et al. 2012; Li et al. 2012).

Several mechanisms have been suggested for the arsenic-related toxicity (Dangleben et al. 2013; Farzan et al. 2013; Flora 2011; IARC 2004; Vahter 2009). Recently, studies have indicated that arsenic toxicity may involve dysregulation of gene expression through interference with epigenetic modifications, possibly through DNA methylation or the proteins that regulate gene expression (Argos 2015; Rojas et al. 2015). Animal studies have shown that mice that were prenatally exposed (from gestation day 8 to 18) to high levels of arsenic (85 mg/L in drinking water) had altered expressions of oncogenes, tumor suppressor genes, and stress-related genes in their livers as adults (Liu et al. 2006; Liu et al. 2004). In humans, maternal toenail arsenic concentrations (0.1 to 68.63 µg/g) were associated with differentially expressed genes of inflammatory signalling pathways and transcriptional responses in cord blood from newborns in a mother-child cohort in Thailand (Fry et al. 2007). Mexican women who were exposed to arsenic from drinking water (mean water arsenic concentration 110

µg/L) and had arsenic-associated skin lesions showed CpG hypermethylation in 182 genes, compared with unexposed individuals (Smeester et al. 2011). Another study on the same individuals as in Smeester et al. (2011) (mean urinary arsenic concentration 10.7 µg/L) showed that subjects with different arsenic metabolite concentrations had different DNA methylation profiles, suggesting that the arsenic metabolism modifies how arsenic interferes with DNA methylation (Bailey et al. 2013). In a Bangladeshi mother-child cohort, higher arsenic exposure (89 µg/L in urine) during pregnancy was associated with lower methylation in CpG sites in cord blood, mainly in boys (Broberg et al. 2014).

So far, few *in vivo* studies have investigated whether changes in arsenic-related gene expression are linked to changes in epigenetic processes. We therefore conducted this study to elucidate how chronic arsenic exposure is associated with gene expression and DNA methylation in peripheral blood in arsenic-exposed women in the Argentinean Andes, using a genome-wide approach, and taking into account the modifying effects of the efficiency of arsenic metabolism.

Materials and methods

Study areas and study participants

The study participants were women living in the Puna area of the northern Argentinian Andes in the province of Salta, an area with varying concentrations of inorganic arsenic in the drinking water. Members of this population, and mainly of indigenous origin (Kolla ethnicity), generally have efficient metabolism of arsenic (Vahter et al. 1995) and this phenotype was recently linked to adaptation to tolerate arsenic (Schlebusch et al. 2013; Schlebusch et al. 2015). The total population of San Antonio de los Cobres and surrounding villages is about 8,000 inhabitants. The details of the recruitment procedure have been

presented before (Engstrom et al. 2011). In 2008, we recruited a total of 172 apparently healthy women living in the village San Antonio de los Cobres, with water arsenic levels of ~208 µg/L, and in small surrounding villages (Tolar Grande, Salara de Pocitos, and Olacapato) with lower water arsenic concentrations of 3.5-72 µg/L (Concha et al. 2010). Of these women, we collected peripheral blood in PAX tubes for 103 individuals. Among those based on sufficient high-quality RNA and DNA, 94 women were initially included in the DNA methylation analyses and 90 women were initially included in the gene expression analyses. However, one individual was later removed from the DNA methylation analysis, due to a strong distortion in the estimated cell type proportions (data not shown), which could indicate an ongoing disease. Thus, the DNA methylation analyses included 93 participants (Supplementary Figure 1). Peripheral blood contains different types of cells and the cell composition likely affects gene expression and DNA methylation. The method by Houseman et al. (Houseman et al. 2012) estimates the proportions of different cell types, based on the 450K data, and this method was used to estimate the blood cell types for all 93 samples in the analysis of DNA methylation. Further, 80 of the women had both RNA and DNA samples, which enabled us to take blood cell count into account in the analysis of gene expression.

Briefly, individuals were interviewed about their age, ancestry, drinking water sources, dietary habits, medical history, time of residence, smoking, alcohol consumption, coca chewing, as well as parity. Also, weight and height were measured and used for calculation of body mass index (BMI).

The Health Ministry of Salta (Salta, Argentina) and the Regional Ethical Committee at the Karolinska Institutet (Stockholm, Sweden) approved the study. Oral and written informed consent was obtained from all study participants.

Blood and urine collection

Non-fasting blood and spot urine samples were collected throughout the day at the local health clinics and at the hospital in San Antonio de los Cobres. Mid-stream urine samples were collected in plastic urine collection cups and then transferred to 24-mL polyethylene tubes and immediately frozen. Peripheral blood was collected in K_2EDTA tubes (Vacuette; Greiner, Germany), for DNA extraction, and in PAX tubes (Becton Dickinson, Franklin Lakes, NJ) for RNA extraction. Urine and blood samples were kept at -20°C, transported (with cooling blocks) to Sweden and stored at -80°C.

Assessment of arsenic exposure

Exposure to arsenic was determined based on the sum of concentrations of iAs and its metabolites (iAs + MMA + DMA) in urine (U-As). We assessed the efficiency of arsenic metabolism by the fractions (percentages) of iAs, MMA, and DMA in urine (Vahter 2002). Arsenic metabolites in urine were determined by high-performance liquid chromatography (HPLC: Agilent 1100 series system, Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and inductively coupled plasma mass spectrometry (ICPMS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) (HPLC-HG-ICPMS) as described previously (Fangstrom et al. 2008; Gardner et al. 2011). To compensate for the variation in dilution of urine, the arsenic concentrations were adjusted to the mean specific gravity (Nermell et al. 2008), measured by a hand refractometer (Atago, Japan). The mean specific gravity was 1.020 for the study participants.

RNA isolation and gene expression analysis

RNA in peripheral blood was isolated using the PAXgene blood RNA kit (PreAnalytiX, Qiagen). RNA concentration and purity were measured with a Nanodrop spectrophotometer

(Wilmington, DE, USA) and RNA integrity (RIN) was evaluated on a Bioanalyzer 2100 (Agilent, CA, USA). The RNA quality was good (RIN > 7.5) (Engstrom et al. 2013). DirectHyb HumanHT-12 v4.0 (Illumina, CA, USA) was used for gene expression analysis, at the SCIBLU facility of Lund University. Gene expression data was quantile normalized in the GenomeStudio software (Illumina). In total, the array data included 47,323 probes before filtering. Probes with a detection p-value above 0.1 (i.e., p-value for the difference between the array and the background noise) in more than 90% of the samples were removed, resulting in 14,290 (30%) probes that remained for further analysis.

DNA isolation and DNA methylation analysis

DNA was isolated using the Qiagen DNA Blood Midi kit (Qiagen, Hilden, Germany). DNA quality was evaluated with a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and a Bioanalyzer 2100, and showed good quality (260/280 nm >1.80). DNA was bisulfite treated using the EZ DNA Methylation kit (Zymo, CA, USA) (Engstrom et al. 2013). About 200 ng of bisulfite-treated DNA was used for hybridization to the Infinium Human-Methylation 450K BeadChip (Illumina, WI, USA) and the analysis was performed at the SCIBLU facility in Lund, Sweden. All BeadChips were from the same batch. Image processing, background correction, quality control, filtering, and normalization (by the SWAN procedure) were performed in the R package minfi. The 450K BeadChip included a total of 485,512 sites before filtering. All samples performed well, since all samples had at least >98% of the CpGs with a detection p-value below 0.01. We removed CpGs for which more than 20% of the samples had a detection p-value above 0.01 (N=871). Furthermore, the following probes were removed: rs probes [probes that measure single nucleotide polymorphisms (SNPs)] and CpH probes (representing non-CpG methylation) (N=3,091), probes with *in silico* nonspecific binding (N = 27,384) (Chen et al. 2013b), and

probes with common SNPs (according to the function dropLociWithSnps in minfi; N=17,217). In total, 436,949 probes remained for further analysis.

Pyrosequencing

We validated DNA methylation of 2 of the top 25 genes (*CACNA1A* and *MOG*) by pyrosequencing. One µg (50 ng/µL) DNA was bisulfite-treated using EPITect kit (Qiagen) and stored at -20°C until further use. For the bisulfite-treated DNA, 0.6 to 1.0 µl was used in the PCR. Pyrosequencing assays were designed using Assay design (version 2.0, Qiagen) software (Supplementary Table 1). The PCR was performed using the PyroMark PCR kit (Qiagen) and the pyrosequencing was performed as described previously (Hossain et al. 2015).

Statistical analyses

All statistical analyses were performed using R (v.3.1.3) and SPSS software, version 22 (IBM, Chicago, IL, USA). The method by Houseman et al. (Houseman et al. 2012) estimates the proportions of different cell types, based on the 450K data and here we used our 450K data to estimate the cell type proportions in each sample, using the minfi estimateCellCounts function. The estimated cell type proportions were then used to adjust the gene expression and DNA methylation analyses, in blood that was sampled at the same time and in the same individuals. Gene expression data were expressed as $\log_2$-transformed intensities as the gene expression was heavily skewed. DNA methylation was expressed as M-values, which is the $\log_2$ of Beta/(1-Beta). Beta values range between 0 (not methylated) and 1 (fully methylated).

Principal component analysis (PCA) was performed to evaluate the influence of technical and biological variables on gene expression and DNA methylation. Associations between variables and principal components were evaluated using linear regression analyses,

and a heatmap depicting the $\log_{10}$ of p-values obtained by the linear models by R package swamp developed by Lauss et al (Lauss et al. 2013) is shown in Supplementary Figure 2. For the PCA, we employed the universally applicable singular value decomposition. In the downstream analyses, we adjusted for cell type proportions with the strongest influence on gene expression or DNA methylation according to the PCA; we did not adjust for all cell types, since the cell type proportions showed strong correlations between the different cell types (data not shown).

In the analyses of the associations between U-As and gene expression or DNA methylation, the women were grouped into quartiles according to their concentrations of U-As, due to somewhat skewed distributions (also when U-As was natural log-transformed), as evident from inspection of the histograms. The quartiles of U-As were used as continuous variables (labeled 1 – 4). A trend of the association between U-As and gene expression or DNA methylation was evaluated by fitting a robust linear regression model, a model less sensitive to outliers than ordinary linear regression (Fox and Weisberg 2011; Joubert et al. 2012), to each array using the R package limma (with empirical Bayes smoothing applied to the standard errors) adjusting for age, coca usage, and estimated fractions of granulocytes, monocytes, and CD4+ cells (models for gene expression) or age, coca usage, granulocyte and natural killer cells (models for DNA methylation) based on the respective PCA plots. The influence of efficiency of arsenic metabolism on the associations between U-As quartiles and gene expression/DNA methylation was analyzed by stratifying the linear regression analyses for below- and above-median %MMA or %iAs. P-values were adjusted for multiple comparisons by the Benjamini-Hochberg false discovery rate (FDR) method (Benjamini and Hochberg 1995) to obtain q-values. A q-value of 0.05 or lower was considered statistically significant.

Pathway analysis

We evaluated both up- and downregulated genes with a q-value < 0.1 using Ingenuity Pathway Analysis (IPA, Qiagen, CA, USA) to identify relevant pathways, diseases, and networks related to the genes that were associated with U-As. For the IPA analysis, we did not include genes expressed in cell lines, but did include genes expressed in primary cells, tissues, and cells of human blood and organs. Arsenic was highlighted in the causal networks to score master regulators for their relationships to arsenic exposure.

Results

Participant characteristics

The study participants showed a large range of U-As and large variation in individual arsenic metabolites (Table 1). We performed Mann-Whitney U-tests to check for differences between the participants in the gene expression and DNA methylation analyses. The study groups for gene expression and DNA methylation did not differ significantly in characteristics, except for the use of coca: more women included in the gene expression analysis used coca than women included in the DNA methylation analysis (p-value = 0.02).

Arsenic exposure and gene expression in blood

Increasing arsenic exposure (measured as quartiles of U-As) was associated with decreased gene expression. Among the top 25 differentially expressed genes in relation to U-As (based on FDR corrected q-value), 17 (68%) had decreased expression (Supplementary Table 2), and among the top 1000 differentially expressed genes, 64% had decreased expression. U-As was significantly associated with the expression of six genes in blood (Table 2A; full gene names

in the table): *SLC7A7, PPBP, MRPL24, MRPS5, CREBBP*, and *ADAP2* (Figure 1). Pathway analysis including genes associated with U-As with a $q < 0.1$ [number of genes included (n) = 37, Table 3] showed that most of the genes were associated with cancer. The top networks associated with U-As were cell death and survival, gene expression, and organismal survival (Supplementary Figure 3). The canonical pathways and the top toxic effects associated with U-As were related to: ATM signaling, androgen signaling, and VDR/RXR activation, xenobiotic metabolism signaling, RAR activation, NRF2-mediated oxidative stress response, and gene regulation by peroxisome proliferators via PPAR. Some arsenic-related genes were associated with several of the canonical pathways, networks, or diseases (Table 3).

Arsenic exposure and gene expression in blood: the modifying effects of arsenic metabolism efficiency

We evaluated the effect of arsenic metabolism efficiency on the association between U-As and gene expression by stratifying the data for above- or below-median fraction of arsenic metabolites (%MMA or %iAs) in urine. The characteristics of these two groups (median split above and below %MMA) are presented in Supplementary Table 3. In the above-median %MMA group (mean %MMA = 10.8; range = 7.8 to 19), U-As was significantly associated with the expression of 14 genes and 11 (79%) had decreased expression, Supplementary Table 4]. In the below-median %MMA group (mean %MMA = 5.7; range = 2.4 to 7.6), U-As was significantly associated with the expression of 24 genes and 18 (75%) had decreased expression. No gene showed a statistically significant association with U-As in both %MMA groups. Pathway analyses revealed different expression patterns between %MMA groups. In the above-median %MMA group ($q < 0.1$; n = 58 genes), U-As was associated with networks and pathways related to NRF2-mediated oxidative stress, xenobiotic metabolism signaling, mitochondrial dysfunction, HIF-1α signaling, and granzyme A signaling. In the below-

median %MMA strata (q < 0.1; n = 116), U-As was associated with networks related to DNA damage signaling, DNA repair, and suppression of tumor formation (Table 4); these networks included chronic myeloid leukemia signaling, glioma signaling, role of *BRCA1* in the DNA damage response, AMPK signaling, and ATM signaling. Some genes were associated with several of the canonical pathways, networks, or diseases in relation to arsenic (Table 4).

When stratifying the data for above- or below-median %iAs, U-As was not significantly associated with the expression of any gene in either %iAs group. Also, the top 20 genes from the %iAs stratification showed no clear difference between the groups (Supplementary Table 5).

Arsenic exposure and DNA methylation in blood

Most CpG sites showed increased methylation in relation to arsenic exposure: among the top 25 CpGs (based on q-value), 22 (88%) had increased methylation (Supplementary Table 6) and among the top 1000 CpGs, 87% had increased methylation. We found that DNA methylation of six CpG sites were significantly associated with U-As. These were situated in the genes *SEC31A*, *LOC144776*, *KRTAP2-2*, *TFR2*, or near genes, with one site near *RPL9P23*, and one site near *MST1P2* (full gene names are presented in Table 2B). Pathway analysis showed that CpGs with q < 0.1 (n = 24) were present in genes that were mainly associated with cancer (n = 20) and to a lesser extent with developmental (n = 5) and hereditary disorders (n = 6). The top canonical pathways associated were D-myo-inositol (1,4,5)-trisphosphate degradation, iD-myo-inositol hexakisphosphate biosynthesis II, and D-myo-inositol (1,3,4)-trisphosphate biosynthesis. The gene associated with these pathways is *INPP5A1*, which encodes a regulator of PI3K/AKT signaling, insulin signaling, endocytosis, vesicle trafficking, cell migration, proliferation, and apoptosis (Hakim et al. 2012). Another

arsenic-related pathway was maturity-onset diabetes of young (MODY) signaling, which involves the calcium channel gene *CACNA1A*.

Arsenic exposure and DNA methylation in blood: modifying effects of arsenic metabolism efficiency

The association between U-As and DNA methylation was evaluated by stratifying the data for median %MMA in urine (Supplementary Table 7). The characteristics of the above- and below-median groups for %MMA are presented in Supplementary Table 3. The below-median %MMA group was somewhat younger and had significantly lower BMI ($p = 0.02$), weight ($p = 0.03$), %DMA ($p < 0.001$), and coca use ($p = 0.02$) compared to the above-median %MMA group (Mann-Whitney U-test). There were large differences in number of CpGs significantly associated with U-As depending on metabolism: there were 32 CpGs in the above-median %MMA group, and 498 CpGs in the below-median %MMA group. Also, the degree of increased methylation differed between the groups: 59% of the CpGs had increased methylation in above-median %MMA group; whereas 93% had increased methylation in the below-median %MMA group. Pathway analyses in the above- ($q < 0.1$; $n = 86$) and below-median %MMA ($q < 0.1$; $n = 1506$) groups identified the functions related to cancer and endocrine system disorder in both groups. In the above-median %MMA group, U-As was associated with cardiovascular disease (no. of genes associated = 6), whereas, in the below-median %MMA group, U-As was mainly associated with dermatological diseases and conditions ($n = 252$), gastrointestinal disease ($n = 451$), and metabolic disease ($n = 68$) (Supplementary Table 7). Furthermore, in the above-median %MMA group, U-As was associated with pathways related to PPAR/RXR activation, UVA-induced MAPK signaling, sphingomyelin metabolism, sphingosine-1-phosphate signaling, and type 2 diabetes mellitus signaling. By contrast, in the below-median %MMA group, U-As was associated with

pathways related to the mechanism of viral exit from host cells, ubiquinol-10 biosynthesis, glycine cleavage complex, cellular effects of sildenafil, and corticotropin-releasing hormone signaling.

Overlap between gene expression and DNA methylation

In the pathway analysis, cancer was the predominant arsenic-related disease for both gene expression and DNA methylation. However, there were no genes for which U-As was statistically significantly associated with both gene expression and DNA methylation (Supplementary Table 8).

We further used pyrosequencing to validate the methylation levels measured by 450K BeadChip. Methylation of the *CACNA1A* and *MOG* genes measured by pyrosequencing were strongly correlated with methylation measured by 450K (*CACNA1A* $r_S = 0.92$; $p < 0.001$; *MOG* $r_S = 0.84$; $p < 0.001$).

Discussion

We found, in women exposed to moderate levels of arsenic, that increasing arsenic in urine was associated with lower gene expression and higher DNA methylation in peripheral blood, suggesting that the arsenic-related down-regulation of gene expression is mediated by DNA hypermethylation. Previous work in mice supports our findings: mice exposed to arsenic via drinking water had increased DNA methylation and decreased expression of genes with low absolute methylation levels (Boellmann et al. 2010). In humans, arsenic exposure has been associated with global DNA hypermethylation in blood in adults (Niedzwiecki et al. 2013;

Pilsner et al. 2007). Still, we could not link arsenic-related changes in gene expression to changes in DNA methylation for individual genes. An explanation for this may involve the limited coverage of some gene regions in the 450K arrays. The efficiency of arsenic metabolism seemed to modify the associations between arsenic exposure and gene expression or DNA methylation to some extent: different pathways were enriched in efficient and inefficient metabolizers of arsenic. This suggests that different metabolic efficiency results in differences in the toxicity of arsenic or the biological response to arsenic (Engström et al. 2015) However, it is important to note that individuals in this population have an efficient arsenic metabolism, likely through adaptation to arsenic (Eichstaedt et al. 2015; Schlebusch et al. 2015; Schlebusch et al. 2013) and the effects of arsenic observed in this study may differ in other populations because of different efficiencies of arsenic metabolism.

Among the individual genes whose expression was significantly associated with arsenic exposure, two of these, *SLC7A7* and *CREBBP*, have previously been associated with arsenic exposure. Zebrafish exposed to low levels of arsenic (100 ppb) showed downregulation of *SLC7A7* (Mattingly et al. 2009), in line with our observation that *SLC7A7* was downregulated in relation to increasing arsenic. Human epidermal keratinocytes treated with 400 nM arsenite showed concomitant loss of G1 into the S/G2 cell cycle accompanied by stimulated binding of the transcription factors AP1 and CREBBP to their respective binding motifs, leading to increased cell proliferation (Hwang et al. 2006). In our study, *CREBBP* expression increased in relation to increased arsenic exposure; and one can speculate that increased *CREBBP* expression may also influence cell cycle progression *in vivo*. The other four genes that were associated with arsenic exposure in our study have not been reported to be associated with arsenic exposure in the literature and may potentially be newly identified targets for arsenic.

Pathways and networks upregulated with increasing arsenic exposure have previously been related to arsenic, such as DNA damage by ATM signaling, and NRF2-mediated oxidative responses. DNA damage responsive kinases ATM (ataxia-telangiectasia mutated) appears to play a crucial role in arsenite-induced G2/M phase arrest by inducing cell death or decreasing proliferation (Hu et al. 2013; Menendez et al. 2001; Tsou et al. 2006). The NRF2 pathway and early antioxidant response, key regulators of the response to increased oxidative stress, were upregulated in hepatic and kidney cells, spleen, thymus, as well as peripheral blood in arsenic-exposed (5-20 mg/kg) mice (Duan et al. 2015; Li et al. 2015).

Arsenic exposure was also associated with DNA methylation in blood and in particular with genes associated with cancer. Other pathways were enriched, but this was based on elevated methylation in only one CpG site in the gene, such as *IDH3* linked to the TCA cycle, and *CACNA1A* linked to Type I diabetes. *INPP5A*, involved in pathways related to inositol biosynthesis, showed increased methylation in our study in relation to arsenic exposure. Further evidence that arsenic targets *INPP5A* comes from a study on arsenic-related skin cancer, which indicated that genetic variation in *INPP5A* has a role in susceptibility to arsenic (Seow et al. 2015).

The association between arsenic exposure and gene expression seemed to differ depending on metabolic efficiency. The above-median %MMA group showed down-regulation of *CAT*, *ATP5A1* and *NDUFA11*. *ATP5A1* encodes a subunit of ATP synthase that catalyzes ATP synthesis in mitochondria (Hejzlarova et al. 2014). *NDUFA11* (NADH:ubiquinone oxidoreductase subunit A11) is associated with NAHD dehydrogenase, an enzyme that takes part in oxidative phosphorylation in complex 1 in mitochondria (Andrews et al. 2013; Berger et al. 2008). Down-regulation of *ATP5A1* and *NDUFA11* may thus potentially influence ATP production in complex 1 in mitochondria. The expression of *ATP5A1* has previously been associated with arsenic exposure in mice, where high

concentrations of arsenic trioxide in drinking water (1000-4000 µg/L) was associated with down-regulation of *atp5a1* expression in mice brain cells and elevated levels of reactive oxygen species (Hong et al. 2009). Arsenic was also associated with *HIF-1α* (hypoxia inducible factor) in the high %MMA group: HIF-1α is a major regulator of oxygen homeostasis, as it regulates expression of dozens of genes involved in maintaining homeostasis in hypoxia (Yoon et al. 2006). Moderate arsenic exposure (75 µg/L) induced HIF-1α accumulation, and increased reactive oxygen species production in BEAS-2B cells (He et al. 2014; Zhao et al. 2013). For individuals with below-median %MMA, *GADD45A* increased in expression with increasing arsenic concentrations. *GADD45A* was induced by arsenic through the ERK pathway in a human bronchial epithelial airway cell line treated with 20 µM As^{3+} (Chang et al. 2007; Shi et al. 2014). Arsenic induced cytotoxicity by inhibiting the AMPK pathway in neurite outgrowth in N2a cells (Wang et al. 2010); also arsenic trioxide bound to AMPK and inhibited acute myeloid leukemia precursor proliferation (Beauchamp et al. 2015). In our study population, pathway analysis showed that subjects with below-median %MMA had AMPK signaling among the top canonical pathways.

For the association between arsenic exposure and DNA methylation, genes present in both below- and above-median %MMA groups were associated with cancer. However, there were substantially more genes associated with arsenic in the below-median %MMA group, compared with the above-median %MMA group. This may be due to differences in characteristics between the groups; the below-median %MMA group was somewhat younger and had significantly lower BMI, %DMA, and coca use compared with the above-median %MMA group. Apart from the high number of arsenic-related CpGs in the below-median %MMA group, arsenic was associated with increased methylation in these individuals to a much higher extent, compared to the above-median %MMA group. In the above-median

%MMA group, type II diabetes mellitus signaling was enriched, which is of interest as arsenic exposure has been linked to increased risk of diabetes (Kuo et al. 2013).

The strengths with our study are that we studied both gene expression and DNA methylation in a group of women who have very little exposure to other potential hazards for genetic modification such as traffic, smoking, or alcohol; these subjects also have a large range in exposure to arsenic. However, our study had some limitations. For example, the fact that it is a cross-sectional study limits our ability to draw conclusions about the causality for arsenic and effects on gene regulation. We measured gene expression and DNA methylation in peripheral blood, which contains a mixture of cell types that are present at different proportions in different persons. These cell types may have different gene expression or DNA methylation patterns that may blur the results. To overcome this problem, we estimated cell type proportion, based on DNA methylation signatures and adjusted for cell type in the analysis. Although IPA analysis revealed that arsenic exposure was associated with enrichment of cancer-related genes, it should be noted that IPA can spuriously associate pathways to cancer when used in tandem with methylation microarrays (Harper et al. 2013).

Ethical standards

The Health Ministry of Salta (Argentina) and the Ethics Committee of Karolinska Institutet (Sweden) approved the study. Informed consent was obtained from all study participants. Study subjects were identified only by a number assigned in the study.

Conflict of interest statement

The authors declare they have no conflict of interest.

References

Abhyankar LN, Jones MR, Guallar E, Navas-Acien A (2012) Arsenic exposure and hypertension: a systematic review. Environ Health Perspect 120(4):494-500

Ameer SS, Xu Y, Engström K, Li H, Tallving P, Boemo A, Parada LA, Peñaloza LG, Concha G, Harari F, Vahter M, Broberg K (2016) Exposure to inorganic arsenic is associated with increased mitochondrial DNA copy number and longer telomere length in peripheral blood. Front Cell Dev Biol in press.

Andrews B, Carroll J, Ding S, Fearnley IM, Walker JE (2013) Assembly factors for the membrane arm of human complex I. Proc Natl Acad Sci U S A 110(47):18934-9

Antonelli R, Shao K, Thomas DJ, Sams R, 2nd, Cowden J (2014) *AS3MT*, *GSTO*, and *PNP* polymorphisms: impact on arsenic methylation and implications for disease susceptibility. Environ Res 132:156-67.

Argos M (2015) Arsenic Exposure and Epigenetic Alterations: Recent Findings Based on the Illumina 450K DNA Methylation Array. Curr Environ Health Rep 2(2):137-44

Bailey KA, Wu MC, Ward WO, et al. (2013) Arsenic and the epigenome: interindividual differences in arsenic metabolism related to distinct patterns of DNA methylation. J Biochem Mol Toxicol 27(2):106-15

Beauchamp EM, Kosciuczuk EM, Serrano R, et al. (2015) Direct binding of arsenic trioxide to AMPK and generation of inhibitory effects on acute myeloid leukemia precursors. Mol Cancer Ther 14(1):202-12

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Statist Soc 57(1):289-300

Berger I, Hershkovitz E, Shaag A, Edvardson S, Saada A, Elpeleg O (2008) Mitochondrial complex I deficiency caused by a deleterious *NDUFA11* mutation. Ann Neurol 63(3):405-8

Boellmann F, Zhang L, Clewell HJ, et al. (2010) Genome-wide analysis of DNA methylation and gene expression changes in the mouse lung following subchronic arsenate exposure. Toxicol Sci 117(2):404-17

Broberg K, Ahmed S, Engstrom K, et al. (2014) Arsenic exposure in early pregnancy alters genome-wide DNA methylation in cord blood, particularly in boys. J Dev Orig Health Dis 5(4):288-98

Chang Q, Bhatia D, Zhang Y, et al. (2007) Incorporation of an internal ribosome entry site-dependent mechanism in arsenic-induced *GADD45* alpha expression. Cancer Res 67(13):6146-54

Chen Y, Wu F, Liu M, et al. (2013a) A prospective study of arsenic exposure, arsenic methylation capacity, and risk of cardiovascular disease in Bangladesh. Environ Health Perspect 121(7):832-8

Chen YA, Lemire M, Choufani S, et al. (2013b) Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics 8(2):203-9

Concha G, Broberg K, Grander M, Cardozo A, Palm B, Vahter M (2010) High-level exposure to lithium, boron, cesium, and arsenic via drinking water in the Andes of northern Argentina. Environ Sci Technol 44(17):6875-80

Dangleben NL, Skibola CF, Smith MT (2013) Arsenic immunotoxicity: a review. Environ Health 12(1):73

Duan X, Li J, Zhang Y, et al. (2015) Activation of NRF2 pathway in spleen, thymus as well as peripheral blood mononuclear cells by acute arsenic exposure in mice. Int Immunopharmacol 28(2):1059-67

Eichstaedt CA, Antao T, Cardona A, Pagani L, Kivisild T, Mormina M (2015) Positive selection of *AS3MT* to arsenic water in Andean populations. Mutat Res 780:97-102

Engstrom K, Vahter M, Mlakar SJ, et al. (2011) Polymorphisms in Arsenic(+III Oxidation State) Methyltransferase (*AS3MT*) Predict Gene Expression of *AS3MT* as Well as Arsenic Metabolism. Environ Health Perspect 119(2):182-8

Engstrom KS, Hossain MB, Lauss M, et al. (2013) Efficient arsenic metabolism--the *AS3MT* haplotype is associated with DNA methylation and expression of multiple genes around *AS3MT*. PloS One 8(1):e53732

Engstrom KS, Vahter M, Fletcher T, et al. (2015) Genetic variation in arsenic (+3 oxidation state) methyltransferase (*AS3MT*), arsenic metabolism and risk of basal cell carcinoma in a European population. Environ Mol Mutagen 56(1):60-9

Fangstrom B, Moore S, Nermell B, et al. (2008) Breast-feeding protects against arsenic exposure in Bangladeshi infants. Environ Health Perspect 116(7):963-9

Farzan SF, Karagas MR, Chen Y (2013) In utero and early life arsenic exposure in relation to long-term health and disease. Toxicol Appl Pharmacol 272(2):384-90

Flora SJ (2011) Arsenic-induced oxidative stress and its reversibility. Free Radic Biol Med 51(2):257-81

Fox J, Weisberg S (2011) Robust regression in R. In: An R companion to applied Regression. 2nd ed. Thousand Oaks, CA:Sage.

Fry RC, Navasumrit P, Valiathan C, et al. (2007) Activation of inflammation/NF-kappaB signaling in infants born to arsenic-exposed mothers. PLoS Genet 3(11):e207

Gardner RM, Nermell B, Kippler M, et al. (2011) Arsenic methylation efficiency increases during the first trimester of pregnancy independent of folate status. Reprod Toxicol 31(2):210-8

Hakim S, Bertucci MC, Conduit SE, Vuong DL, Mitchell CA (2012) Inositol polyphosphate phosphatases in human disease. Curr Top Microbiol Immunol 362:247-314

Harper KN, Peters BA, Gamble MV (2013) Batch effects and pathway analysis: two potential perils in cancer studies involving DNA methylation array analysis. Cancer Epidemiol Biomarkers Prev 22(6):1052-60

He J, Wang M, Jiang Y, et al. (2014) Chronic arsenic exposure and angiogenesis in human bronchial epithelial cells via the ROS/miR-199a-5p/HIF-1alpha/COX-2 pathway. Environ Health Perspect 122(3):255-61

Hejzlarova K, Mracek T, Vrbacky M, et al. (2014) Nuclear genetic defects of mitochondrial ATP synthase. Physiol Res 63 Suppl 1:S57-71

Hong Y, Piao F, Zhao Y, Li S, Wang Y, Liu P (2009) Subchronic exposure to arsenic decreased Sdha expression in the brain of mice. Neurotoxicology 30(4):538-43

Hossain MB, Li H, Hedmer M, Tinnerberg H, Albin M, Broberg K (2015) Exposure to welding fumes is associated with hypomethylation of the *F2RL3* gene: a cardiovascular disease marker. Occup Environ Med 72(12):845-51

Hossain MB, Vahter M, Concha G, Broberg K (2012) Environmental arsenic exposure and DNA methylation of the tumor suppressor gene *p16* and the DNA repair gene *MLH1*: effect of arsenic metabolism and genotype. Metallomics 4(11):1167-75

Houseman EA, Accomando WP, Koestler DC, et al. (2012) DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 13:86

Hu YC, Hsieh BS, Cheng HL, et al. (2013) Osteoblasts survive the arsenic trioxide treatment by activation of ATM-mediated pathway. Biochem Pharmacol 85(7):1018-26

Hwang BJ, Utti C, Steinberg M (2006) Induction of cyclin D1 by submicromolar concentrations of arsenite in human epidermal keratinocytes. Toxicol Appl Pharmacol 217(2):161-7

IARC (2012) Arsenic, metals, fibres, and dusts. Monogr Eval Carcinog Risks Hum 100,11

IARC (2004) Some Drinking-water Disinfectants and Contaminants, including Arsenic. IARC Monogr Eval Carcinog Risks Hum 84:41-267

Joubert BR, Håberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, Huang Z, et al. (2012) 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect 20:1425-31.

Kuo CC, Moon K, Thayer KA, Navas-Acien A (2013) Environmental chemicals and type 2 diabetes: an updated systematic review of the epidemiologic evidence. Current Diab Rep 13(6):831-49

Lauss M, Visne I, Kriegner A, Ringnér M, Jönsson G, Höglund M (2013) Monitoring of technical variation in quantitative high-throughput datasets. Cancer Inform 12:193-201.

Li H, Engstrom K, Vahter M, Broberg K (2012) Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. Chem Res Toxicol 25(11):2333-9

Li J, Duan X, Dong D, et al. (2015) Hepatic and Nephric NRF2 Pathway Up-Regulation, an Early Antioxidant Response, in Acute Arsenic-Exposed Mice. Int J Environ Res Public Health 12(10):12628-42

Liu J, Xie Y, Ducharme DM, et al. (2006) Global gene expression associated with hepatocarcinogenesis in adult male mice induced by in utero arsenic exposure. Environ Health Perspect 114(3):404-11

Liu J, Xie Y, Ward JM, Diwan BA, Waalkes MP (2004) Toxicogenomic analysis of aberrant gene expression in liver tumors and nontumorous livers of adult mice exposed in utero to inorganic arsenic. Toxicol Sci 77(2):249-57

Mattingly CJ, Hampton TH, Brothers KM, Griffin NE, Planchart A (2009) Perturbation of defense pathways by low-dose arsenic exposure in zebrafish embryos. Environ Health Perspect 117(6):981-7

Menendez D, Mora G, Salazar AM, Ostrosky-Wegman P (2001) ATM status confers sensitivity to arsenic cytotoxic effects. Mutagenesis 16(5):443-8

Moon K, Guallar E, Navas-Acien A (2012) Arsenic exposure and cardiovascular disease: an updated systematic review. Curr Atheroscler Rep 14(6):542-55

Moon KA, Guallar E, Umans JG, et al. (2013) Association between exposure to low to moderate arsenic levels and incident cardiovascular disease. A prospective cohort study. Ann Inter Med 159(10):649-59

Nermell B, Lindberg AL, Rahman M, et al. (2008) Urinary arsenic concentration adjustment factors and malnutrition. Environ Res 106(2):212-8

Niedzwiecki MM, Hall MN, Liu X, et al. (2013) A dose-response study of arsenic exposure and global methylation of peripheral blood mononuclear cell DNA in Bangladeshi adults. Environ Health Perspect 121(11-12):1306-12

Pilsner JR, Liu X, Ahsan H, et al. (2007) Genomic methylation of peripheral blood leukocyte DNA: influences of arsenic and folate in Bangladeshi adults. Am J Clin Nutr 86(4):1179-86

Pilsner JR, Liu X, Ahsan H, et al. (2009) Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. Environ Health Perspect 117(2):254-60

Rojas D, Rager JE, Smeester L, et al. (2015) Prenatal arsenic exposure and the epigenome: identifying sites of 5-methylcytosine alterations that predict functional changes in gene expression in newborn cord blood and subsequent birth outcomes. Toxicol Sci 143(1):97-106

Schlebusch CM, Gattepaille LM, Engstrom K, Vahter M, Jakobsson M, Broberg K (2015) Human adaptation to arsenic-rich environments. Mol Biol Evol 32(6):1544-55

Schlebusch CM, Lewis CM, Jr., Vahter M, et al. (2013) Possible positive selection for an arsenic-protective haplotype in humans. Environ Health Perspect 121(1):53-8

Seow WJ, Pan WC, Kile ML, et al. (2015) A distinct and replicable variant of the squamous cell carcinoma gene inositol polyphosphate-5-phosphatase modifies the susceptibility of arsenic-associated skin lesions in Bangladesh. Cancer 121(13):2222-9

Shi Q, Sutariya V, Bishayee A, Bhatia D (2014) Sequential activation of Elk-1/Egr-1/GADD45alpha by arsenic. Oncotarget 5(11):3862-70

Smeester L, Rager JE, Bailey KA, et al. (2011) Epigenetic changes in individuals with arsenicosis. Chem Res Toxicol 24(2):165-7

Tsou TC, Tsai FY, Yeh SC, Chang LW (2006) ATM/ATR-related checkpoint signals mediate arsenite-induced G2/M arrest in primary aortic endothelial cells. Arch Toxicol 80(12):804-10

Vahter M (2002) Mechanisms of arsenic biotransformation. Toxicology 181-182:211-7

Vahter M (2009) Effects of arsenic on maternal and fetal health. Annu Rev Nutr 29:381-99

Wang X, Meng D, Chang Q, et al. (2010) Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ Health Perspect 118(5):627-34

Yoon D, Pastore YD, Divoky V, et al. (2006) Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. J Biol Chem 281(35):25703-11

Zhao F, Severson P, Pacheco S, Futscher BW, Klimecki WT (2013) Arsenic exposure induces the Warburg effect in cultured human cells. Toxicol Appl Pharmacol 271(1):72-7

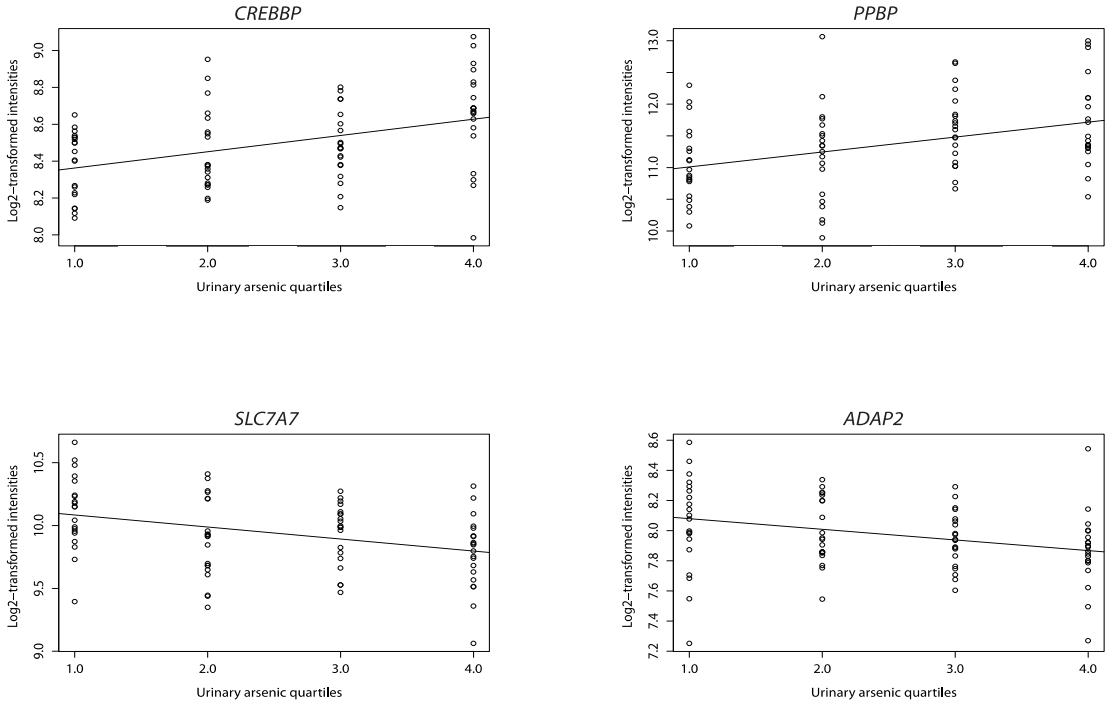


Figure 1. Associations between urinary arsenic concentrations and gene expression (fluorescence intensities) of *CREBBP* (top left), *PPBP* (top right), *SLC7A7* (bottom left), and *ADAP2* (bottom right). The fluorescence intensities from the gene expression array were log2-transformed and the arsenic concentrations were divided in quartiles [quartile 1, mean=23 µg/L range: (10.1 to 38.6); quartile 2, mean=125 µg/L range: (42.6 to 179); quartile 3, mean=239 µg/L range: (185 to 297) and quartile 4, mean=487 µg/L range: (308 to 1251)] to improve linearity.

Table 1: Characteristics of the studied women.

	Included in the gene expression analyses		**Included in the DNA methylation analyses**	
	N = 80		**N = 93**	
Variables	**Median**	**Range**	**Median**	**Range**
Age (years)	32	12 - 62	32	12 - 62
Height (cm)	153	142 - 165	153	142 - 165
Weight (kg)	56	37 - 87	55	37 - 87
BMI (kg/m^2)	24	16 – 35	24	16 - 35
Urinary arsenic (µg/L)[a]	182	10.1 - 1251	185	10.1 - 1251
iAs (%)	13	3.3 – 24.3	13.1	3.3 – 34.2
MMA (%)	7.7	2.4 – 18.5	7.6	2.4 – 18.5
DMA (%)	79	63 - 94	79	59 - 94
Coca chewing (%)	59		41	

[a]Sum of urinary arsenic metabolite adjusted for specific gravity 1.020.

Table 2: Top 10 differentially expressed genes and top 10 differently methylated CpG sites from the robust linear regression analysis between (A) urinary arsenic and gene expression, and (B) urinary arsenic and DNA methylation in peripheral blood.

A

Gene	**Full name**	**β1**[a]	**q**[b]
SLC7A7	Solute carrier family 7	-0.11	$3*10^{-6}$
PPBP	Pro-platelet basic protein	0.26	0.001
MRPL24	Mitochondrial ribosomal protein L24	-0.070	0.002
MRPS5	Mitochondrial ribosomal protein S5	-0.084	0.007
CREBBP	CREB binding protein	0.092	0.007
ADAP2	ArfGAP with dual PH domains 2	-0.070	0.047
TUFM	Tu translation elongation factor, mitochondrial	-0.097	0.058
C7orf50	Chromosome 7 open reading frame 50	-0.10	0.058
GADD45A	Growth arrest and DNA damage inducible alpha	0.053	0.058
OXA1L	Oxidase (cytochrome c) assembly 1-like	-0.082	0.058

[a]Log_2 intensity (gene expression) = α + β1 × urinary arsenic + β2 × age + β3 × coca + β4 × granulocyte + β5 × monocyte + β6 × CD4 (robust regression, estimated fractions of cell types).
[b]p-value (q) adjusted for multiple comparisons.

B

Name	**Chromosome**	**Gene**	**Full name**	**β1**	**M value**[a]	**q**[b]
cg27413543	4	*SEC31A*	SEC31 homolog A, COPII coat complex component	0.14	-3.84	$2*10^{-4}$
cg23671167	11	Near *RPL9P23*	Ribosomal protein L9 pseudogene 23	0.11	3.10	0.01
cg11516038	1	Near *MST1P2*	Macrophage stimulating 1 pseudogene 2	0.13	2.94	0.02
cg02347881	13	*LOC144776*	long intergenic non-protein coding RNA 410	0.12	2.33	0.03
cg22443249	17	*KRTAP2-2*	keratin associated protein 2-2	0.22	4.52	0.03
cg06582411	16	*TFR2*	Transferrin receptor 2	0.07	0.50	0.04
cg01983216	1	*ZNF238*	zinc finger and BTB domain containing 18	0.08	3.42	0.06
cg24221507	15	*IDH3A*	isocitrate dehydrogenase 3 (NAD+) alpha	0.10	-4.72	0.07
cg05903720	1	Near *KIF26A*	Kinase family member 26A	0.12	1.87	0.07
cg14981312	1	C1orf159	Chromosome I open reading frame 159	0.12	-4.42	0.07

[a]M-value (DNA methylation) = α + β1 × urinary arsenic + β2 × age + β3 × coca + β4 × granulocyte + β5 × natural killer cell (robust regression, estimated fractions of cell types). [b]p-value (q) adjusted for multiple comparisons.

Table 3: Pathway analysis by IPA software of genes differently expressed in peripheral blood in relation to arsenic exposure (arsenic concentrations in urine)[a]. Arrows indicate up- or down-regulation of the genes.

Networks	**Score**	**Genes**
Cell Death and Survival, Gene Expression, Organismal Survival	26	↑*CREBBP*, ↓*EIF4A1*, ↑*GADD45A*, ↓*GSTP1*, ↓*IRF5*, ↑*NCOA1*, ↑*PRKC1*, ↓*PSMB4*, ↑*PTBP3*, ↓*SLC7A7*, ↑*TSC22D1*, ↓*TUFT*, ↓*CARD9*, ↓*HCFC1R1*, ↓*RBBP6*, ↓*CENPB*, ↑*PGRMC1*, ↓*SCAMP3*, ↑*PPBP*
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	2	↓*CARD9*
Developmental Disorder, Hereditary Disorder, Metabolic Disease	2	↓*HCFC1R1*
Cell Cycle, Embryonic Development, Hair and Skin Development and Function	2	↓*RBBP6*
Cell Morphology, Organ Morphology, Renal and Urological System Development and Function	2	↓*CENBP*
Diseases and Disorders	**p-value[b] (genes)**	
Cancer	0.026 (16)	↑*GADD45A*, ↓*GSTP1*, ↑*PRKC1*, ↑*CREBBP*, ↓*EIF4A1*, ↓*RBBP6*, ↓*BRAT1*, ↑*HIST1H2BK*, ↑*IRF5*, ↓*RABGEF1*, ↑*NCOA1*, ↓*PSBM4*, ↓*CARD9*, ↓*SLC7A7*, ↓*ECHS1*, ↓*OXA1L*
Connective Tissue Disorder	0.0026 (3)	↑*CREBBP*, ↑*IRF5*, ↓*GSTP1*
Dermatological Diseases and Conditions	0.023 (3)	↓*CARD9*, ↑*PRKC1*, ↓*PSMB4*
Developmental Disorder	0.017 (3)	↑*CREBBP*, ↓*SLC7A7*, ↓*ECHS1*
Gastrointestinal Disease	0.024 (5)	↑*IRF5*, ↓*GSTP1*, ↓*BRAT1*, ↑*CREBBP*, ↓*ECHS1*
Canonical Pathways	**p-value (overlap[c])**	
ATM Signaling	1.4×10^{-4} (3/59)	↓*BRAT1*, ↑*CREBBP*, ↑*GADD45A*
VDR/RXR Activation	3.03×10^{-4} (3/77)	↑*GADD45A*, ↑*NCOA1*, ↑*PRKC1*
Androgen Signaling	8.6×10^{-4} (3/110)	↑*CREBBP*, ↑*NCOA1*, ↑*PRKC1*
Xenobiotic Metabolism Signaling	9.03×10^{-4} (4/256)	↑*CREBBP*, ↓*GSTP1*, ↑*NCOA1*, ↑*PRKC1*
NRF2-mediated Oxidative Stress Response	3.4×10^{-3} (3/177)	↑*CREBBP*, ↓*GSTP1*, ↑*PRKC1*
Top Toxic List		
VDR/RXR Activation	3.03×10^{-4} (3/77)	↑*GADD45A*, ↑*NCOA1*, ↑*PRKC1*
Xenobiotic Metabolism Signaling	1.6×10^{-3} (4/297)	↑*CREBBP*, ↓*GSTP1*, ↑*NCOA1*, ↑*PRKC1*
RAR Activation	3.9×10^{-3} (3/187)	↑*CREBBP*, ↑*NCOA1*, ↑*PRKC1*
NRF2-mediated Oxidative Stress Response	4.9×10^{-3} (3/202)	↑*CREBBP*, ↓*GSTP1*, ↑*PRKC1*
Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR	0.011 (2/94)	↑*CREBBP*, ↑*NCOA1*

[a]Log_2 intensity (gene expression) = $\alpha + \beta 1 \times$ urinary arsenic + $\beta 2 \times$ age + $\beta 3 \times$ coca + $\beta 4 \times$ granulocyte + $\beta 5 \times$ monocyte + $\beta 6 \times$ CD4 (robust regression, estimated fractions of cell types).

[b]Average p-value of the molecules associated with diseases. [c]Overlap = number of the genes matched with the genes in the canonical pathways.

Table 4: Pathway analysis by IPA of genes differently expressed in peripheral blood in relation to arsenic exposure (arsenic concentrations in urine)[a] stratified for arsenic metabolism efficiency (above and below median of %MMA). Common networks and disease in both the strata were underlined.

High MMA		**Low MMA**	
Networks	**Score**	**Networks**	**Score**
Cell Death and Survival, Cell-To-Cell Signaling and Interaction, Connective Tissue disorders	33	Cell Death and Survival, Cell Cycle, Cancer	22
Cancer, Organismal Injury and Abnormalities, Renal and Urological Disease	2	Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	16
Organismal Development, Skeletal and Muscular System Development and Function, Developmental Disorder	2	Cell-To-Cell Signaling and Interaction, Cellular Movement, Hematological System Development and Function	16
Ophthalmic Disease, Organismal Injury and Abnormalities, Gastrointestinal Disease	2	Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation, Hematological System Development and Function	16
Cell Cycle, Embryonic Development, Hair and Skin Development and Function	2	Developmental Disorder, Hereditary Disorder, Metabolic Disease	2
Diseases and Disorders	**p-value[b] (genes)**	**Diseases and Disorders**	**p-value[b] (genes)**
Cancer	0.025 (33)	Cancer	0.025 (10)
Endocrine System Disorders	0.021 (10)	Neurological Disease	0.022 (16)
Organismal Injury and Abnormalities	0.025 (34)	Organismal Injury and Abnormalities	0.025 (28)
Neurological Disease	0.025 (5)	Hematological Disease	0.025 (11)
Respiratory Disease	0.023 (4)	Infectious Disease	0.024 (9)
Canonical Pathways	**p-value (overlap[c])**	**Canonical Pathways**	**p-value (overlap[c])**
Xenobiotic Metabolism Signaling	3.4×10^{-4} (5/256)	Role of BRCA1 in DNA Damage Response	9.4×10^{-4} (4/78)
Granzyme A Signaling	7.3×10^{-4} (2/17)	Chronic Myeloid Leukemia Signaling	0.0017 (4/92)
NRF2-mediated Oxidative Stress Response	8.0×10^{-4} (4/177)	Glioma Signaling	0.0021 (4/97)
HIF 1 Signaling	0.0017 (3/100)	AMPK Signaling	0.0030 (5/176)
Mitochondrial Dysfunction	0.0056 (3/152)	ATM Signaling	0.0043 (3/59)

Top Toxic List	**p-value (overlap[c])**	**Top Toxic List**	**p-value (overlap[c])**
Xenobiotic Metabolism Signaling	6.6×10^{-4} (5/297)	Acute Renal Failure Panel (Rat)	0.0036 (3/55)
NRF2-mediated Oxidative Stress Response	0.0013 (4/203)	LXR/RXR Activation	0.031 (3/123)
Mitochodrial Dysfunction	0.0058 (3/154)	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.033 (2/51)
Oxidative Stress	0.0078 (2/56)	Oxidative Stress	0.039 (2/56)
PXR/RXR Activation	0.0098 (2/63)	Cell Cycle: G1/S Checkpoint Regulation	0.049 (2/64)

[a]Log_2 intensity (gene expression) = $\alpha + \beta1 \times$ urinary arsenic + $\beta2 \times$ age + $\beta3 \times$ coca + $\beta4 \times$ granulocyte + $\beta5 \times$ monocyte + $\beta6 \times$ CD4 (robust regression, estimated fractions of cell types).

[b]Average p-value of the molecules associated with diseases.

[c]Overlap = number of the genes matched with the genes linked to a pathway.

Table 5: Pathway analysis by IPA of genes differently methylated in peripheral blood in relation to arsenic exposure (arsenic concentrations in urine)[a]. Arrows indicate up- or down-regulation of the genes.

Networks	**Score**	**Genes**
Cell Death and Survival, Cellular Development, Tissue Development	7	↑*AMIGO2*, ↑*GATA2*, ↓*WWP2*, ↑*NAT10*,
Carbohydrate Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry	3	↑*GALNT2*
Cell-To-Cell Interaction , Cellular Movement, Hematological System Development and Function	3	↑*MOG*
Cell Cycle, Cellular Assembly and Organization, Tissue Morphology	3	↑ *SEC31A*
Cardiovascular System Development and Function, Organismal Development, Tissue Morphology	3	↑ *JAM2*
Diseases and Disorders	**p-value[b] (genes)**	
Cancer	0.024 (20)	↑*GATA2*, ↓*WWP2*, ↑*WNT3*, ↑*AMIGO2*, ↑*CACNA1A*, ↑*GALNT2*, ↑*IDH3A*, ↑*INPPA5A*, ↑*JAM2*, ↑*PDE4DIP*, ↑*SEC31A*, ↑*C1orf159*, ↑*TFR2*, ↑*TIAM2*, ↑*INPP5A*, ↑*PIGN*, ↑*KIF13A*, ↑*C1orf35*
Developmental Disorder	0.018 (5)	↑*ZBTB18*, ↑*GATA2*, ↑*TFR2*, ↑*PIGN*, ↑*WNT3*
Hematological Disease	0.019 (2)	↑*GATA2*, ↑*TFR2*
Hereditary Disorder	0.011 (6)	↑*ZBTB18*,↑*GATA2*, ↑*TFR2*, ↑*PIGN*, ↑*CACNA1A*, ↑*WNT3*
Immunological Disease	0.019 (3)	↑*GATA2*, ↑*MOG*, ↑*WNT3*
Canonical Pathways	**p-value (overlap[c])**	
D-myo-inositol (1,4,5)-trisphosphate degradation	0.022 (1/18)	↑*INPP5A*
iD-myo-inositol hexakisphosphate biosynthesis II	0.023 (1/19)	↑*INPP5A*
D-myo-inositol (1,3,4)-trisphosphate biosynthesis	0.023 (1/19)	↑*INPP5A*
Maturity Onset Diabetes of Young (MODY) Signaling	0.024 (1/20)	↑*CACNA1A*
TCA Cycle II (Eukaryotic)	0.028 (1 /23)	↑*IDH3A*

[a]DNA methylation = $\alpha + \beta1 \times$ urinary arsenic + $\beta2 \times$ age + $\beta3 \times$ coca + $\beta4 \times$ granulocyte + $\beta5 \times$ natural killer cell (robust regression, estimated fractions of cell types).
[b]Average p-value of the molecules associated with diseases. [c]Overlap = number of the genes matched with the genes available in IPA.

Supplementary Material

Arsenic exposure from drinking water is associated with lower gene expression in peripheral blood - possibly via higher DNA methylation

Syeda Shegufta Ameer[1], Karin Engström[1,2], Mohammad Bakhtiar Hossain[1], Gabriela Concha[3], Marie Vahter[2], Karin Broberg[2*]

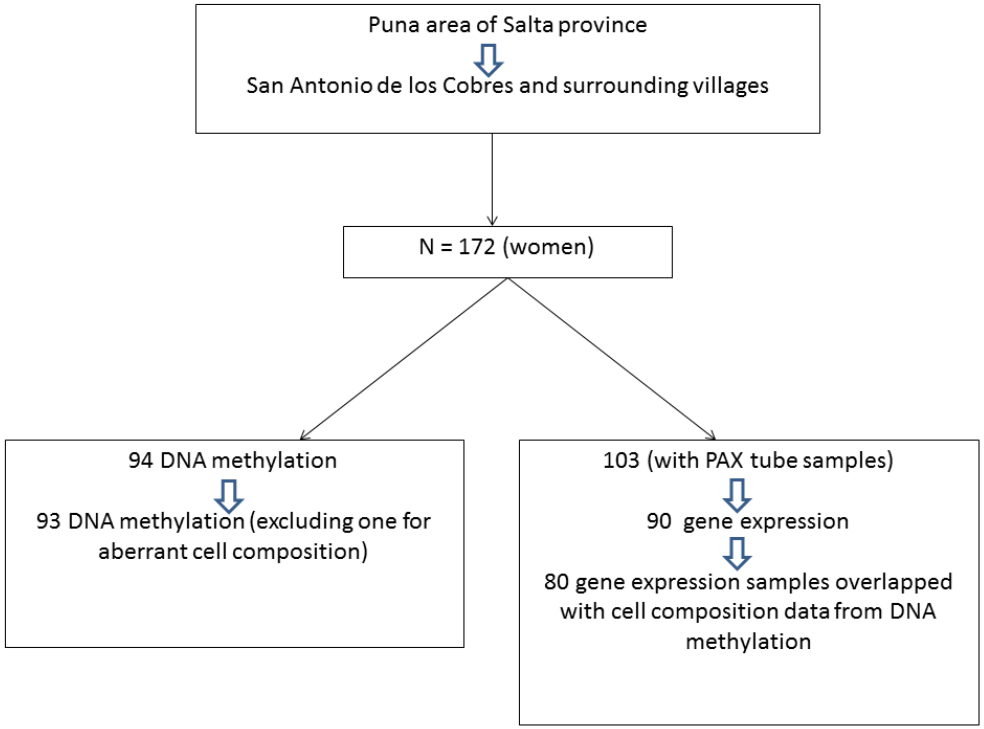
1) Department of Laboratory Medicine, Division of Occupational and Environmental Medicine, Lund University, Lund, Sweden

2) Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, Stockholm, Sweden

3) Science Department, Risk Benefit Assessment Unit, National Food Agency, Uppsala, Sweden

[*] Corresponding author

Address correspondence to K. Broberg, Institute of Environmental Medicine, Unit of Metals & Health, Karolinska Institutet, SE-17177 Stockholm, Sweden. Telephone: +46737823750. Fax: +46-8-336981; E-mail: Karin.broberg@ki.se



Supplementary Figure 1: Inclusion criteria of the women for gene expression and DNA methylation analyses.

Supplementary Table 1: Sequence of the primers (denoted 5´-3´) used in different pyrosequencing assays.

Assay	**Forward primer**	**Reverse primer**	**Sequencing primer**
CACNA1A	ATGTTTTTTTTTAGGTGAGAAGGGGATTTG*	CATCTAACCACCCCCACACC	CCCTACCCAAAAAAAAAAATAAAAA
MOG	AAAAGTTTTTTGAGGGATTTTAGATGTA	TCCCCAACACTCAACATAATATCTA*	AGATGTAAGTTTTTTAGTTGTT

*Biotinylated

Supplementary Table 2: Top 25 genes for the association between arsenic and gene expression

Gene	Description	β1[a]	q[b]
SLC7A7	Solute carrier family 7	-0.11	2.75E-06
PPBP	Pro-platelet basic protein	0.26	0.001
MRPL24	Mitochondrial ribosomal protein L24	-0.070	0.002
MRPS5	Mitochondrial ribosomal protein S5	-0.084	0.007
CREBBP	CREB binding protein	0.092	0.007
ADAP2	ArfGAP with dual PH domains 2	-0.070	0.047
TUFM	Tu translation elongation factor, mitochondrial	-0.097	0.058
C7orf50	Chromosome 7 open reading frame 50	-0.10	0.058
GADD45A	Growth arrest and DNA damage inducible alpha	0.053	0.058
OXA1L	Oxidase (cytochrome c) assembly 1-like	-0.082	0.058
RPP25L	Ribonuclease P/MRP 25kDa subunit-like	-0.071	0.062
BRAT1	BRCA1 associated ATM activator 1	-0.063	0.065
CENPB	Centromere protein B	-0.079	0.069
EIF4A1	Eukaryotic translation initiation factor 4A1	-0.066	0.069
SCAMP3	Secretory carrier membrane protein 3	-0.062	0.069
TMEM203	Transmembrane protein 203	-0.083	0.070
C16orf91	Chromosome 16 open reading frame 91	-0.054	0.073
HIST1H2BK	Histone cluster 1, H2bk	0.15	0.073
RPS2P48	Ribosomal protein S2 pseudogene 48	-0.074	0.073
GAPDHP23	Glyceraldehyde 3 phosphate dehydrogenase pseudogene 23	-0.085	0.073
IRF5	Interferon regulatory factor 5	-0.060	0.077
RABGEF1	RAB guanine nucleotide exchange factor (GEF) 1	0.063	0.077
TSC22D1	TSC22 domain family member 1	0.13	0.077
RBBP6	Retinoblastoma binding protein 6	0.013	0.077
SNORD68	Small nucleolar RNA, C/D box 68	0.078	0.077

[a]Log2 intensity (gene expression) = $\alpha + \beta1 \times$ urinary arsenic + $\beta2 \times$ age + $\beta3 \times$ coca + $\beta4 \times$ granulocyte + $\beta5 \times$ monocyte + $\beta6 \times$ CD4 (robust regression, estimated fractions of cell types).

[b]p-value adjusted for multiple comparisons

Supplementary Table 3: Characteristics of the study participants stratified for arsenic metabolism efficiency (above and below median of %MMA) for individuals included in (A) the gene expression analyses and (B) the DNA methylation analyses.

(A) Gene expression

		Above median MMA			Below median MMA	
Variables	**N**	**Median**	**Range**	**N**	**Median**	**Range**
Age (years)	40	30	12 to 62	40	33.5	14 to 60
Height (cm)	40	152	142 to 163	40	154	144 to165
Weight (kg)	40	54	37 to 74	40	59	43 to 87
BMI (kg m $^{-2}$)	40	23	16 to 30	40	25	18 to 35
Urinary arsenic (µg/L)[a]	40	172	10.1 to 1251	40	205	13.8 to 543
%iAs	40	13.4	4.9 to 24	40	10.8	3.3 to 24
%MMA	40	10.3	7.8 to 18.5	40	5.9	2.4 to 7.6
%DMA	40	75	63 to 87	40	83	69 to 94
Coca chewing	40	70% chewed coca leaves		40	48% chewed coca leaves	

(B) DNA methylation

		Above median MMA			Below median MMA	
Variables	**N**	**Median**	**Range**	**N**	**Median**	**Range**
Age (years)	46	30	12 to 62	47	34	14 to 60
Height (cm)	46	153	142 to 163	47	154	144 to165
Weight (kg)	46	54	37 to 74	47	59	43 to 87
BMI (kg m $^{-2}$)	46	23	16 to 30	47	25	18 to 35
Urinary arsenic (µg/L)[a]	46	175	10.1 to 1251	47	209	13.8 to 607
%iAs	46	13.4	4.9 to 24.3	47	12.5	3.3 to 34.2
%MMA	46	10.5	7.6 to 18.5	47	5.9	2.4 to 7.6
%DMA	46	76	63 to 87	47	82	59 to 94
Coca chewing	46	28% chewed coca leaves		47	53% chewed coca leaves	

[a]Sum of urinary arsenic metabolite adjusted for specific gravity 1.020.

Supplementary Table 4: Top 20 genes for the association between arsenic and gene expression adjusted for age, coca-use, granulocyte, monocyte and CD4 cells stratified for arsenic metabolism efficiency (above and below median of %MMA).

Above median MMA				Below median MMA			
Gene	**Description**	**β1**	**q[b]**	**Gene**	**Description**	**β1**	**q[b]**
TRIM52	Tripartite motif containing 52	-0.104	0.003	*FGFR1OP2*	FGFR1 oncogene partner 2	0.11	0.002
NAPSB	Napsin B aspartic peptidase, pseudogene	-0.22	0.017	*MRPS5*	Mitochondrial ribosomal protein S5	-0.11	0.002
SSR4	Signal sequence receptor, delta	-0.12	0.018	*HSPA8P5*	Heat shock protein family A member 8 pseudogene 5	-0.087	0.008
MRPL24	Mitochondrial ribosomal protein L24	-0.089	0.025	*CENPB*	Centromere protein B	-0.14	0.020
PPBP	Pro-platelet basic protein	0.31	0.025	*SBK1*	SH3 domain binding kinase 1	-0.10	0.020
CHCHD5	Coiled-coil-helix-coiled-coil-helix domain containing 5	-0.092	0.025	*TOP3A*	Topoisomerase (DNA) III alpha	-0.096	0.020
GIMAP7	GTPase, IMAP family member 7	-0.11	0.025	*BRAT1*	BRCA1 associated ATM activator 1	-0.088	0.022
VHL	Von Hippel-Lindau tumor suppressor	-0.087	0.025	*SLC7A7*	Solute carrier family 7 member 7	-0.11	0.029
NCOA1	Nuclear receptor coactivator 1	0.12	0.026	*GPBAR1*	G protein-coupled bile acid receptor 1	-0.13	0.030
RBBP6	Retinoblastoma binding protein 6	-0.062	0.027	*DDX24*	DEAD-box helicase 24	-0.11	0.031
CREBBP	CREB binding protein	0.11	0.036	*SMARCC2*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	-0.14	0.036
EI24	EI24, autophagy associated transmembrane protein	-0.081	0.038	*ZNF20*	Zinc finger protein 20	0.068	0.036

ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	-0.11	0.039	*RRAS*	Related RAS viral (r-ras) oncogene homolog	-0.093	0.036
ITGAM	Intergin subunit alpha M	0.089	0.053	*CHSY1*	Chondroitin sulfate synthase 1	0.11	0.036
TSC22D1	TSC22 domain family member 1	0.14	0.054	*TUBBP2*	Tubulin beta pseudogene 2	-0.11	0.037
HLA-F	Major histocompatibility complex, class I, F	-0.16	0.054	*PLXNB2*	Plexin B2	-0.087	0.037
ZNF791	Zinc finger protein 791	-0.097	0.054	*PNPLA2*	Patatin like phospholipase domain containing 2	-0.13	0.037
NAPSA	Napsin A aspartic peptidase	-0.14	0.054	*TMEM106B*	Transmembrane protein 106B	0.11	0.037
OAS2	2´- 5´-oligoadenylate synthetase 2	0.093	0.054	*COBRA1*	Cofactor of BRCA1	-0.13	0.037
ANKRD44	Ankyrin repeat domain 44	-0.101	0.054	*CORO7*	Coronin 7	-0.14	0.037

[a]Log2 intensity (gene expression) = $\alpha + \beta1 \times$ urinary arsenic + $\beta2 \times$ age + $\beta3 \times$ coca + $\beta4 \times$ granulocyte + $\beta5 \times$ monocyte + $\beta6 \times$ CD4 (robust regression, estimated fractions of cell types).
[b]p-value adjusted for multiple comparisons

Supplementary Table 5: Pathway analysis by IPA of gene expression in peripheral blood associated with arsenic exposure[a] stratified for arsenic metabolism efficiency (above and below median of %iAs).

Above median iAs		**Below median iAs**	
Networks	**Score**	**Networks**	**Score**
Lipid Metabolism, Molecular Transport, Small Molecular Biochemistry	41	Drug Metabolism, Glutathione Depletion in Liver, Cancer	50
Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry	2		
Diseases and Disorders	**p-value[b] (genes)**	**Diseases and Disorders**	**p-value[b] (genes)**
Cancer	0.051 (12)	Connective Tissue Disorders	0.0091 (2)
Dermatological Diseases and Conditions	0.034 (4)	Developmental Disorder	0.049 (4)
Developmental Disorder	0.042 (6)	Gastrointestinal Disease	0.043 (3)
Hereditary Disorder	0.0029 (3)	Hereditary Disorder	0.049 (3)
Metabolic Disease	0.015(3)	Immunological Disease	0.0017 (1)
Canonical Pathways	**p-value (Overlap[c])**	**Canonical Pathways**	**p-value (Overlap[c])**
Dendritic Cell Maturation	0.0072 (2/177)	Glutathione-Mediated Detoxification	2.6×10^{-4} (2/29)
CDP-diacylglycerol Biosynthesis 1	0.012 (1/16)	PXR/RXR Activation	0.0014 (2/67)
Phosphatidylglycerol Biosynthesis II (Non-Plastidic)	0.012 (1/18)	Aryl Hydrocarbon Receptor Signaling	0.0059 (2/140)
Lipid Antigen Presentation by CD1	0.019 (1/26)	NRF2-mediated Oxidative Stress Response	0.0096 (2/180)
Glutathione-mediated Detoxification	0.021 (1/29)	Telomere Extension Telomerase	0.012 (1/15)
Top Toxic List	**p-value (Overlap[c])**	**Top Toxic List**	**p-value (Overlap[c])**
Glutathione Depletion - Phase II Reaction	0.015 (1/20)	Glutathione Depletion - Phase II Reaction	1.2×10^{-4} (2/20)
Renal Proximal Tubule Toxicity Biomarker Panel (Rat)	0.019 (1/27)	Oxidative Stress	0.0010 (2/57)
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.037 (1/52)	PXR/RXR Activation	0.0014 (2/67)
Renal Necrosis/Cell Death	0.051 (2/501)	Aryl Hydrocarbon Receptor Signaling	0.0077 (2/161)
p53 Signaling	0.070 (1/99)	Long-term Renal Injury Anti-oxidative Response Panel (Rat)	0.015 (1/18)

[a]Log2 intensity (gene expression) = $\alpha + \beta1 \times$ urinary arsenic + $\beta2 \times$ age + $\beta3 \times$ coca + $\beta4 \times$ granulocyte + $\beta5 \times$ monocyte + $\beta6 \times$ CD4 (robust regression, estimated fractions of cell types).
[b]Average p-value of the molecules associated with diseases. [c]Overlap = number of the genes matched with the genes available in IPA

Supplementary Table 6: Top 25 CpG sites for the association between arsenic and DNA methylation adjusted for age, coca-use, granulocyte and natural killer cell

Name	CHR	Gene	Full name	β1	M value[a]	q[b]
cg27413543	4	*SEC31A*	SEC31 homolog A, COPII coat complex component	0.14	-3.84	$2*10^{-4}$
cg23671167	11	Near *RPL9P23*	Ribosomal protein L9 pseudogene 23	0.11	3.10	0.013
cg11516038	1	Near *MST1P2*	Macrophage stimulating 1 pseudogene 2	0.13	2.94	0.017
cg02347881	13	*LOC144776*	Long intergenic non-protein coding RNA 410	0.12	2.33	0.030
cg22443249	17	*KRTAP2-2*	Keratin associated protein 2-2	0.22	4.52	0.030
cg06582411	16	*TFR2*	Transferrin receptor 2	0.07	0.50	0.043
cg01983216	1	*ZNF238*	Zinc finger and BTB domain containing 18	0.08	3.42	0.057
cg24221507	15	*IDH3A*	Isocitrate dehydrogenase 3 (NAD+) alpha	0.10	-4.72	0.057
cg05903720	1	Near *KIF26A*	Kinase family member 26A	0.12	1.87	0.068
cg14981312	1	C1orf159	Chromosome I open reading frame 159	0.12	-4.42	0.068
cg24800175	19	*CACNA1A*	Calcium voltage-gated channel subunit alpha1 A	0.07	-2.65	0.068
cg17220749	1	*GALNT2*	Polypeptide N-acetylgalactosaminyltransferase 2	0.07	2.0	0.068
cg00931843	6	*TIAM2*	T-cell lymphoma invasion and metastasis 2	0.11	0.12	0.068
cg00049330	18	*PIGN*	Phosphatidylinositol glycan anchor biosynthesis class N	0.11	-4.60	0.068
cg09902061	11	*NAT10*	N-acetyltransferase 10	-0.07	-4.48	0.068
cg09723635	6	*KIF13A*	Kinase family member 13A	0.13	3.75	0.068
cg18942112	14	Quite far from *RNU3P3*	RNA, U3 small nucleolar pseudogene	0.09	3.54	0.069
cg24770927	14	*SDR39U1*	Short chain dehydrogenase/reductase family 39U member 1	0.12	-4.83	0.069
cg08419373	16	*WWP2*	WW domain containing E3 ubiquitin protein ligase 2	-0.27	5.65	0.069
cg03224396	1	*Near MARC1*	Mitochondrial amidoxime reducing component 1	0.17	3.73	0.072
cg20704602	6	*MOG*	Myelin oligodendrocyte glycoprotein	0.13	1.57	0.072
cg06093379	17	*WNT3*	Wingless-type MMTV integration site family member 3	0.18	-4.57	0.076
cg02436004	3	*GATA2*	GATA binding protein 2	0.085	-3.51	0.076
cg03382304	21	*JAM2*	Junctional adhesion molecule 2	0.083	-4.29	0.079
cg12616923	8	*Near CCAT1*	Colon cancer associated transcript 1	-0.14	-1.13	0.086

[a]M-value (DNA methylation) $= \alpha + \beta1 \times$ urinary arsenic $+ \beta2 \times$ age $+ \beta3 \times$ coca $+ \beta4 \times$ granulocyte $+ \beta5 \times$ natural killer cell (robust regression, estimated fractions of cell types).

[b]p-value adjusted for multiple comparisons.

Supplementary Table 7: Pathway analysis by IPA of DNA methylation in peripheral blood associated with arsenic exposure[a] stratified for arsenic metabolism efficiency (above and below median of %MMA).

Above median MMA		**Below median MMA**	
Networks	**Score**	**Networks**	**Score**
Cellular Development, Hematological System Development and Function, hematopoiesis	16	Cell Death and Survival, Cellular Growth and Proliferation, Carbohydrate Metabolism	36
Connective Tissue Disorders, Developmental Disorder, Gastrointestinal Disease	2	Cancer, Organismal Injury and Abnormalities, Cell Death and Survival	15
Infectious Diseases, Inflammatory Disease, Respiratory Disease	2	Cellular Development, Connective Tissue Disorders, Developmental Disorder	13
Cellular Movement, Embryonic Development, Organismal Development	2	Endocrine System Disorders, Gastrointestinal Disease, Metabolic Disease	12
Gene expression, Cellular Development, Embryonic Development	2	Cell Morphology, Cell Cycle, DNA Replication, Recombination, and Repair	12
Diseases and Disorders	**p-value[b] (genes)**	**Diseases and Disorders**	**p-value[b] (genes)**
Cardiovascular Disease	0.017 (6)	Dermatological Diseases and Conditions	0.020 (252)
Cancer	0.024 (37)	Endocrine System Disorders	0.025 (252)
Connective Tissue Disorders	0.018 (3)	Gastrointestinal Disease	0.024 (451)
Developmental Disorder	0.018 (3)	Metabolic Disease	0.024 (68)
Endocrine System Disorder	0.019 (4)	Cancer	0.025 (521)
Canonical Pathways	**p-value (Overlap[c])**	**Canonical Pathways**	**p-value (Overlap[c])**
PPAR/RXR Activation	0.0043 (3/165)	Mechanisms of Viral Exit from Host Cells	0.0014 (6/41)
UVA-Induced MAPK Signaling	0.013 (2/87)	Ubiquinol-10 Biosynthesis (Eukaryotic)	0.0080 (3/14)
Sphingomyelin Metabolism	0.016 (1/8)	Glycine Cleavage Complex	0.013 (2/6)
Sphingosine-1-phosphate Signaling	0.020 (2/108)	Cellular Effects of Sildenafil (Viagra)	0.014 (9/124)
Type II Diabetes Mellitus Signaling	0.022 (2/114)	Corticotropin Releasing Hormone Signaling	0.017 (8/107)

[a]DNA methylation = $\alpha + \beta1 \times$ urinary arsenic $+ \beta2 \times$ age $+ \beta3 \times$ coca $+ \beta4 \times$ granulocyte $+ \beta5 \times$ natural killer cell (robust regression, estimated fractions of cell types).
[b]Average p-value of the molecules associated with diseases
[c]Overlap = number of the genes matched with the genes available in IPA

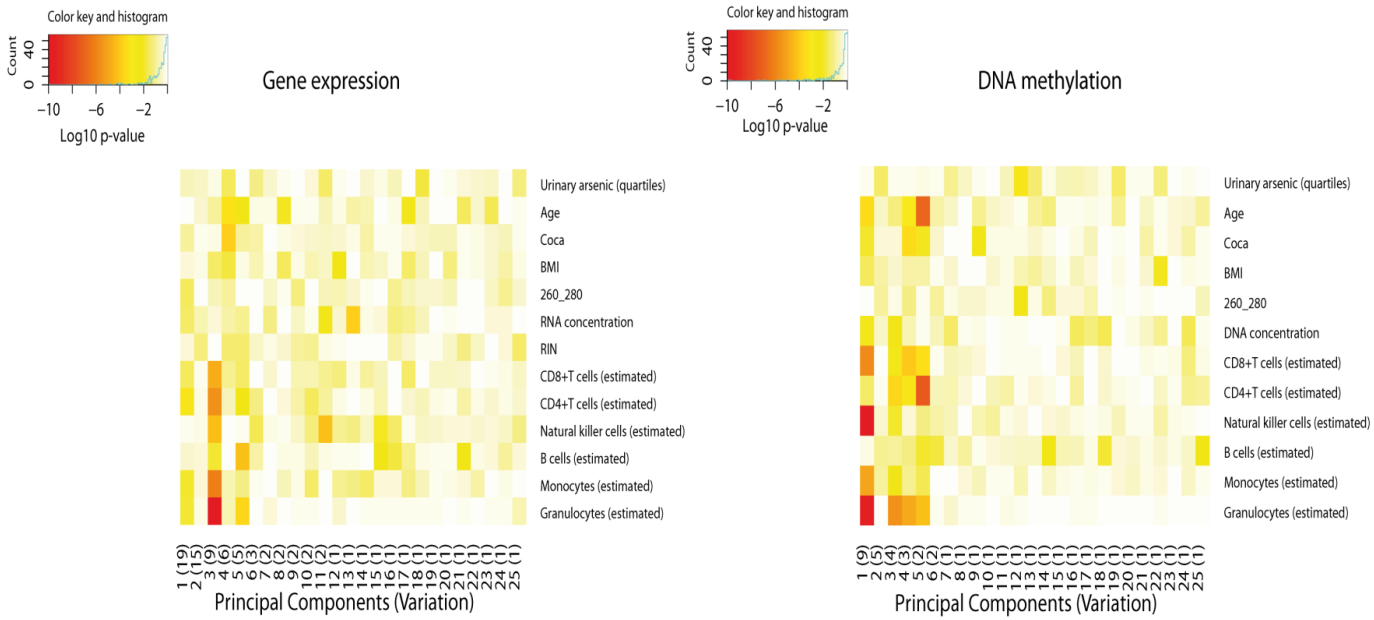
Supplementary Table 8: Overlap between gene expression and DNA methylation for the top 10 hits from the respectively association analyses with U-As. A) shows the top ten genes from the gene expression association analyses and the corresponding CpGs site from the same gene and B) shows the top ten CpGs sites from the association analyses and the corresponding gene in the gene expression analyses. When several CpGs or arrays are present from the same gene, the one with the lowest q-value is shown.

A

Gene expression analysis					**DNA methylation analysis**		
Gene expression array, llumina ID	**Gene name**	**Chromosome**	**β**	**q**	**CpG ID**	**β**	**q**
ILMN_1810275	*SLC7A7*	14	-0.11	2.75E-06	cg15070897	0.040	0.65
ILMN_1767281	*PPBP*	4	0.26	0.0013	cg21398186	0.045	0.94
ILMN_2398995	*MRPL24*	1	-0.070	0.0017	cg05375405	0.041	0.71
ILMN_1760441	*MRPS5*	2	-0.084	0.0069	cg00492362	0.047	0.78
ILMN_1809583	*CREBBP*	16	0.092	0.0069	cg07618675	0.034	0.53
ILMN_1763000	*ADAP2*	17	-0.070	0.047	cg22485810	-0.054	0.38
ILMN_1738369	*TUFM*	16	-0.097	0.058	cg02850742	0.12	0.25
ILMN_1718336	*C7orf50*	7	-0.10	0.058	cg09698220	0.039	0.37
ILMN_1694075	*GADD45A*	1	0.053	0.058	cg03705947	0.024	0.70
ILMN_1731851	*OXA1L*	14	-0.082	0.058	cg05146416	0.074	0.77

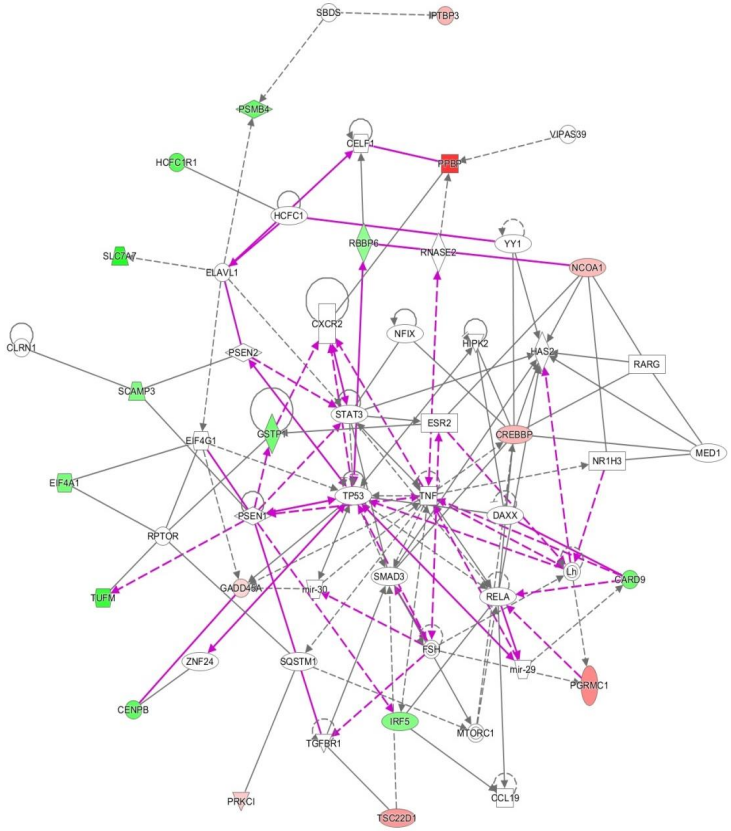
B

DNA methylation analysis				**Gene expression analysis**			
CpG ID	**β**	**Chromosome**	**q**	**Gene name**	**Gene expression array, Illumina ID**	**β**	**q**
cg27413543	0.14	4	<0.001	*SEC31A*	ILMN_1724959	0.015	0.81
cg23671167	0.11	11	0.013	Near *RPL9P23*		No match	
cg11516038	0.13	1	0.0173	Near *MST1P2*		No match	
cg02347881	0.12	13	0.030	*LOC144776*		No match	
cg22443249	0.22	17	0.030	*KRTAP2-2*		No match	
cg06582411	0.07	7	0.043	*TFR2*		No match	
cg01983216	0.08	1	0.057	*ZNF238*	ILMN_2399686	-0.0077	0.89
cg24221507	0.10	15	0.057	*IDH3A*	ILMN_1698533	0.0063	0.93
cg05903720	0.15	14	0.068	Near *KIF26A*		No match	
cg14981312	0.12	1	0.068	*C1orf159*		No match	



Supplementary Figure 2: Heatmap showing the influence of biological and technical variables on the overall variation in the gene expression data (left panel, n=80) and DNA methylation data (right panel, n=93), both measured in blood from women. The log10 of p-values is obtained by linear regression models between the principal components and the technical and biological variables.

260_280: the ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. RIN: RNA integrity number, to estimate the integrity of total RNA. Coca: some study participants chewed coca-leaves.



Supplementary Figure 3: Network representing genes expressed in blood associated with arsenic in urine.