

HUMAN GENES, GENE POLYMORPHISMS,  
AND THEIR EXPRESSION IN RELATION TO  
TRACE ELEMENTS

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**Doctoral Dissertation**  
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MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA  
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



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**Doctoral Dissertation**

HUMANI GENI IN GENSKI POLIMORFIZMI TER  
NJIHOVO IZRAŽANJE V POVEZAVI Z ELEMENTI V  
SLEDOVIH

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*“We only know a tiny proportion about the complexity of the natural world. Wherever you look, there are still things we don’t know about and don’t understand. [...] There are always new things to find out if you go looking for them.” - **David Attenborough** -*



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*“Posebna zahvala gre mojim staršem Tanji in Marjanu, ki sta se morala marsičemu odreči, da sta mi odprla vrata v mojo raziskovalno pot, ter sestri Tei. Hvala vam za vse vaše vzpodbude in potrpežljivost.”*

# Abstract

There are more questions than answers in biomarkers research at low exposure levels. The present dissertation aimed to estimate the appropriateness of exposure and effect biomarkers, the impact of single nucleotide polymorphisms (SNPs; 35 SNPs, 20 genes) – as susceptibility/adaptability biomarkers – on potentially toxic trace elements (TE) in spot urine and blood samples, and to obtain SNPs allele frequencies. Associations were estimated and tested by multivariable linear regression with the adjustment for selected confounders. Selected Slovenian, Croatian, and Kyrgyzstan populations with low to moderate environmental or diet TE exposure were chosen regarding their vulnerability related to either pregnancy, lactation, childhood, childbearing age or residential region. Some observations were tested on a smaller group of cancer patients pharmacologically exposed to metalloid-containing medicine.

Results confirmed that the normalization of spot urine biomarkers levels for inter-individual variations in hydration status, particularly by creatinine, is inappropriate at low TE exposure due to a high difference in the measurement sensitivity and specificity between creatinine and TEs, resulting in conflicting and inconsistent results. As such, TE determined in blood (plasma) have found to be a better biomarker of low level exposure. Similarly questionable is normalization of urine biomarkers of renal effect (tubular: alpha-1-microglobulin – A1M, N-Acetyl beta-D-Glucosaminidase – NAG; glomerular: Albumin – Alb, Immunoglobulin G – IgG).

Additionally, the associations were clearly influenced by physiological variability. Cd – renal effect biomarkers associations were driven by the normal physiological changes in kidney function rather than by Cd-induced renal effects. Moreover, renal protein A1M was affected also by factors unrelated to kidney function, such as smoking, pointing on its versatile physiological roles and low specificity, which at such low levels oppose its appropriateness as a biomarker of Cd effects.

Superior effect of physiological non-genetic factors versus genetic factors on TE at low exposures was observed during pregnancy. Pregnancy status silenced the most of studied SNPs/TE interactions. Among non-pregnant women, we identified the “protective” role of *AS3MT* haplotype, related to As methylation efficiency. In accordance with extremely low environmental and diet related iAs exposure we observed 7 times lower frequency of *AS3MT* haplotype for the Slovenian-Croatian population than that reported for the population of Argentina with historically high iAs exposure. Assessment of *AS3MT* haplotype and its mRNA levels in As<sub>2</sub>O<sub>3</sub>-treated cancer patients pointed out that the former rather than the latter could be used as a predictor of therapeutic efficiency.

Novel assessment of SNPs of *AQP* – coding small molecules transport channels – showed significant negative influence of *AQP4* (rs9951307) and positive influence of *AQP9* (rs2414539) on As methylation efficiency and metabolite excretion – the first one during pregnancy only and the second one during non-pregnancy. *INMT* (rs6970396) and *AQP9* both resulted, beside As, also in higher urine levels of Se pointing on their co-excretion, maybe as an As-Se complex. At moderate Pb exposures, we also confirmed influences of two *ALAD* SNPs (rs1805313 and rs1800435) on lower blood Pb. Frequencies of *ALAD*

SNPs considerably varied among three studied areas of the Kyrgyzstan population and put in question a possible role of geology and environmental pollution on observed levels. Similar differences are expected for Slovenia after genotyping industrially polluted regions including the Mežica valley (Pb and Zn mine).

The present dissertation contributed to the worldwide data on allele frequencies of 35 SNPs in 20 genes for the Slovenian-Croatian population. Significant influence of *AQPs*, *INMT* and *ALAD* SNPs points on their possible role as susceptibility or adaptability biomarkers in TE exposure. However, their role should be individually and/or in combinations tested also in populations/individuals with higher environmental, occupational and/or therapeutic exposure. Due to the observed influence, Se must be included as an association's modifier.



# Povzetek

Raziskave pri nizkih izpostavljenostih elementom v sledovih (ES) velikokrat prinašajo več vprašanj kot odgovorov. Namen disertacije je bil oceniti primernost biomarkerjev izpostavljenosti in učinka ter vpliv polimorfizmov posameznih nukleotidov (SNP; 35 SNP-jev, 20 genov) kot biomarkerjev dovzetnosti/prilagodljivosti na potencialno toksične ES v trenutnih vzorcih urina in krvi. Hkrati smo želeli pridobiti alelne frekvence izbranih SNP-jev pri proučevanih populacijah. Povezave med posameznimi parametri so bile ocenjene in testirane z multivariatno linearno regresijo z upoštevanjem vpliva so-dejavnikov. Slovenska, hrvaška in kirgizistanska populacija z nizko do zmerno izpostavljenostjo potencialno toksičnim ES preko okolja ali prehrane so bile izbrane glede na njihovo povečano občutljivost, povezano z nosečnostjo, dojenjem, otroštvom, rodno dobo ali bivanjem v industrijsko onesnaženem področju. Nekaj povezav je bilo testiranih tudi na manjši skupini bolnikov z rakom, farmakološko izpostavljenih arzenovem trioksidu ( $\text{As}_2\text{O}_3$ ).

Rezultati so potrdili, da je pri nizkih izpostavljenostih ES normiranje biomarkerjev naključnega urina (v izogib inter-individualnim razlikam v hidriranosti) večinoma neprimerno. Poseben problem predstavlja normiranje s kreatininom, ki zaradi nespecifičnosti, fiziološke variabilnosti in razlik v merilni občutljivosti med kreatininom in ES vodi v nedosledne rezultate. Zaključili smo, da so vrednosti ES v krvi ali plazmi primernejši biomarker izpostavljenosti ter da je vprašljivo tudi normiranje urinskih biomarkerjev ledvičnega učinka (npr. alfa-1-mikroglobulin – A1M).

Na asociacije je vplivala tudi fiziološka variabilnost. Povezanost koncentracij Cd in biomarkerjev ledvične funkcije je bila odvisna od razlik v normalni ledvični fiziologiji in ne od vpliva Cd na delovanje ledvic. Na protein A1M pa so vplivali tudi dejavniki, ki niso povezani z delovanjem ledvic (kajenje), kar je potrdilo njegovo večplastno fiziološko vlogo in slabo specifičnost znotraj fizioloških vrednosti. To nasprotuje njegovi ustreznosti kot biomarkerju ledvičnih učinkov pri nizki izpostavljenosti kadmiju.

Pri proučevanju povezav genetskih dejavnikov in ES smo ugotovili, da so bili pri nosečnicah učinki ne-genetskih fizioloških dejavnikov močnejši od genetskih. Nosečnost je utišala večino proučevanih SNP-SE interakcij. Pri ne-nosečnicah pa smo določili in potrdili »zaščitno« vlogo haplotipa encima *AS3MT*, ki je odgovoren za encimsko metilacijo As in njegovega izločanja. Zaradi nizke izpostavljenosti anorganskemu arzenu (iAs) so bile frekvence haplotipa pri proučevani slovensko-hrvaški populaciji nizke (10%). Ocena vpliva prisotnosti *AS3MT* haplotipa in njegovega izražanja preko *AS3MT* mRNA pri bolnikih, zdravljenih z  $\text{As}_2\text{O}_3$ , je pokazala možnost uporabe haplotipa kot napovedovalca terapevtske učinkovitosti.

Analize akvaporinov (AQP; transportni kanali majhnih molekul in As ionov) so pokazale negativen vpliv polimorfizma *AQP4* (SNP rs9951307; nosečnice) ali pozitiven vpliv *AQP9* (SNP rs2414539; ne-nosečnice) na metilacijo in izločanje metabolitov As. *AQP9* in *INMT* (SNP rs6970396) sta povzročila skupno izločanje As in selen v urinu, kar nakazuje na možno tvorbo As-Se kompleks.

Pri nepoklicni izpostavljenosti Pb smo opazili povezavo polimorfizmov encima *ALAD* za tvorbo hemoglobina (SNP rs1805313 in SNP rs1800435) z nižjimi koncentracijami Pb v krvi. Frekvence SNP-jev so se močno razlikovale med tremi onesnaženimi območji v Kirgizistanu. Zaradi obstoječih zgodovinskih industrijskih žarišč (rudnik Pb in Zn, rudnik Hg) podobne frekvenčne razlike pričakujemo tudi v Sloveniji.

Opažen vpliv SNP-jev genov *AS3MT*, *AQP*, *INMT* in *ALAD* kaže na njihovo možno vlogo kot biomarkerjev občutljivosti ali prilagodljivosti pri izpostavljenosti ES. Vloga posamičnih in kombiniranih SNP-jev mora biti preverjena pri populacijah/posameznikov z višjo okoljsko, poklicno in/ali terapevtsko izpostavljenostjo. Zaradi opaženega vpliva statusa selena na vse v nalogi testirane povezave mora biti Se v tovrstnih študijah upoštevan.

Pridobljeni podatki o frekvencah alelov 35 SNP v 20 genih za slovensko-hrvaško populacijo predstavljajo prispevek k svetovni datoteki frekvenčne porazdelitve SNP-jev.

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# Abbreviations

|                                |                                                                |
|--------------------------------|----------------------------------------------------------------|
| [(GS) <sub>2</sub> AsSe]       | ... seleno-bis(S-glutathionyl) arsinium ion                    |
| A1M                            | ... alpha-1-microglobulin                                      |
| ALAD                           | ... delta-aminolevulinic acid dehydratase                      |
| Alb                            | ... albumin                                                    |
| AML                            | ... Acute myeloid leukemia                                     |
| APL                            | ... Acute promyelocytic leukemia                               |
| AQP4                           | ... aquaporin 4                                                |
| AQP9                           | ... aquaporin 9                                                |
| As <sub>2</sub> O <sub>3</sub> | ... Arsenic trioxide                                           |
| AS3MT                          | ... Arsenic (3+) methyltransferase                             |
| AsB                            | ... Arsenobetaine                                              |
| AsIII                          | ... Arsenite                                                   |
| AsV                            | ... Arsenate                                                   |
| ATRA                           | ... all-trans retinoic acid                                    |
| B2M                            | ... 2-microglobulin                                            |
| BMI                            | ... body mass index                                            |
| CEU                            | ... Utah residents with Northern and Western European ancestry |
| CHB                            | ... China, Beijing                                             |
| DMA                            | ... dimethylarsinic acid                                       |
| DMT1                           | ... divalent metal transporter                                 |
| DNA                            | ... deoxyribonucleic acid                                      |
| DNMT1                          | ... DNA methyltransferase                                      |
| EDTA                           | ... ethylenediaminetetraacetic acid                            |
| ETAAS                          | ... electrochemical atomic absorption spectrometry             |
| GIH                            | ... Gujarati Indian from Houston, Texas                        |
| GM                             | ... geometric mean                                             |
| GSH                            | ... Glutathione                                                |
| GST                            | ... glutathione S-transferases                                 |
| GW                             | ... gestation week                                             |
| HBM                            | ... human biomonitoring                                        |
| HG-AFS                         | ... hydride generation atomic fluorescence spectrometry        |
| HMWP                           | ... high-molecular-weight protein                              |
| HPLC                           | ... high-performance liquid chromatography                     |
| HPRT1                          | ... hypoxanthine guanine phosphoribosyltransferase 1           |
| HWE                            | ... Hardy-Weinberg equilibrium                                 |
| IgG                            | ... immunoglobulin G                                           |
| INMT                           | ... indolethylamine N-methyltransferase                        |
| JPT                            | ... Japan, Tokyo                                               |
| KYR                            | ... Kyrgyzstan                                                 |
| LD                             | ... linkage disequilibrium                                     |

|                                 |                                                                           |
|---------------------------------|---------------------------------------------------------------------------|
| LOD                             | ... limit of detection                                                    |
| LWK                             | ... Luhya in Webuye, Kenya                                                |
| MAF                             | ... minor allele frequency                                                |
| MEX                             | ... Mexican Ancestry from Los Angeles                                     |
| MM                              | ... multiple myeloma                                                      |
| MMA                             | ... monomethylarsonic acid                                                |
| MPC                             | ... maximum permissible concentration                                     |
| mRNA                            | ... messenger ribonucleic acid                                            |
| MRP2                            | ... multidrug resistance associated protein 2                             |
| MT                              | ... metallothionein                                                       |
| MTHFR                           | ... methylene tetrahydrofolate reductase                                  |
| NAG                             | ... N-acetyl-beta-glucosaminidase                                         |
| ORS ICP-MS                      | ... octapole reaction system inductively coupled plasma mass spectrometer |
| p <i>AS3MT</i>                  | ... protective <i>AS3MT</i> haplotype                                     |
| p <i>AS3MT</i> hap <sup>-</sup> | ... non-carriers of protective <i>AS3MT</i> haplotype                     |
| p <i>AS3MT</i> hap <sup>+</sup> | ... carriers of protective <i>AS3MT</i> haplotype                         |
| PMI                             | ... primary methylation index                                             |
| PML-RAR- $\alpha$               | ... promyelocytic leukemia retinoic acid receptor alpha                   |
| PNP                             | ... purine nucleoside phosphorylase                                       |
| qPCR                            | ... quantitative real-time polymerase chain reaction                      |
| RBP                             | ... retinol-binding protein                                               |
| RNA                             | ... ribonucleic acid                                                      |
| RPLP0                           | ... ribosomal phosphoprotein P0                                           |
| SAC                             | ... San Antonio de los Cobros, Argentina                                  |
| SAM                             | ... S-adenosyl-methionine                                                 |
| SELENOP                         | ... selenoproteins P1                                                     |
| SG                              | ... specific gravity                                                      |
| SMI                             | ... secondary methylation index                                           |
| SNP                             | ... single nucleotide polymorphisms                                       |
| TFRC                            | ... transferrin receptor                                                  |
| TMI                             | ... total methylation index                                               |
| TSI                             | ... Italy, Tuscany                                                        |
| YRI                             | ... Yoruba in Ibadan, Nigeria                                             |
| $\Sigma$ -UAs <sub>M</sub>      | ... sum of As metabolites in urine (AsIII+ AsV + MMA + DMA)               |

# Chapter 1

## Introduction

### 1.1 Trace Elements

Trace elements (TE; microminerals or trace minerals) occur naturally and ubiquitously in the environment where humans are being constantly exposed, mostly through diet and inhalation. They are defined as chemical elements present in minor amounts in a particular sample or environment; usually less than 0.1 percent per volume/1000 part per million. Anthropogenic activities through mining, industrial settings, and agriculture can considerably increase their local or global environmental levels and consequently human exposure (Flora, 2014; Norberg et al., 2015; Prashanth et al., 2015).

In biology they are commonly classified as essential (selenium, Se; zinc, Zn; manganese, Mn; iron, Fe; copper, Cu; etc.) and non-essential (arsenic, As; cadmium, Cd; mercury, Hg; lead, Pb, nickel, Ni; aluminum, Al; chromium, Cr; etc.). However, it should be pointed out that the classification is based on current data and knowledge and it is a matter that is not settled. For some assumed non-essential trace elements it is questionable as they might be essential for humans (e.g. Cd, Ni, Al, Cr), while for others (As, Hg, Pb) their function at very low levels is possibly still not known, which might change with the future studies (Prashanth et al., 2015; Yoshida, 2012). Non-essential TE, such as As, Cd, Hg, and Pb have been found related with several negative effects on human health or at least with increased risk for developing the diseases such as: diabetes, age related neurological or neurodevelopmental disorders, cardiovascular diseases, liver and kidney dysfunction, and cancer (Flora, 2014; Norberg et al., 2015). On the other hand, the essential TE such as Se, Zn, Mn, Fe, and Cu – which are required by living organisms, usually as a part of vital enzymes – are at concentrations that are required for accomplishing their biological functions safe, while in excess or deficiency they can also be harmful (Fraga, 2005; Frazzoli, et al., 2015; Prashanth et al., 2015). Moreover, essential elements can – through various metabolic interactions – influence kinetics of non-essential elements (Chowdhury et al., 1987; Frazzoli et al., 2015; Lee & Kim, 2014).

The assessment of TE exposure of selected population and the exposure connected to health risk is generally performed by following external and internal levels of TE. With determined levels of TE in human body and adequate knowledge on their kinetics, it is possible to identify and evaluate their potential (early) biological effects as well as genetic and/or non-genetic factors of intra- and inter-individual or populational variability in exposure. Characteristics that can be objectively monitored in human tissue or fluids and evaluated as a biological indicator of chemical exposure, (patho)physiological processes, and pharmacologic responses to a therapeutic intervention, are called biomarkers (Biomarkers Definitions Working Group, 2001; Ladeira & Viegas, 2016). Their assessments are well characterized and reliable in cases of substantially increased environmental levels

of TE (hot spots), and at occupational exposure. Similar biomarkers have an important role also in clinical studies using TEs as therapeutic drugs (As, Se). However, their use at relatively low environmental exposure shows several limitations on the populational and individual level (Aylward et al., 2014).

## 1.2 Biomarkers

Biomarkers are traditionally divided into three main groups: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. Such classification of biomarkers is a general agreement between scientists but as seen from Figure 1.1, it is not always possible to assign one biomarker to a single category. They are often interchangeable between groups, depending on the aim and goals of investigation. For example, levels of plasma Se could represent a biomarker of exposure when evaluating exposure to Se, or biomarker of susceptibility since its deficiency can increase susceptibility to other TE exposure such as Cd, As etc. (Frazzoli et al., 2015).

In the literature they are most commonly defined as follows (Frazzoli et al., 2015; National Research Council, 2006; Santonen et al., 2015; WHO, 2015):

- Biomarkers of exposure are defined as levels of the exogenous chemical, its metabolites or the products of its interaction with target molecule (or cell) that are measured in the human tissues or body fluids (internal exposure). Internal biomarkers can also represent the effective dose (concentration at the target site, e.g. TE levels in kidney) and account for variations in absorption, distribution, metabolism and elimination (ADME) of the chemical between individuals. Examples of such biomarkers studied in the present dissertation are: levels of Cd, As, As metabolites (arsenite, AsIII; arsenate, AsV; monomethylarsonic acid, MMA; and dimethylarsonic acid, DMA), and Pb in blood and/or urine (see Section 3.1 – 3.4).

- Biomarkers of effect are a measurable biochemical, physiological, behavioral or other alteration within the whole organism, or at the organ, tissue, cellular or subcellular level. Based on the magnitude they can be associated with an impairment or disease. In the case of chemical exposure, they often represent a wide range of biological responses from physiological adaptation – that can ideally be reversed – to pathophysiological irreversible effects. Renal function proteins as biomarkers of effect of kidney tubule, such as  $\alpha$ -1-microglobulin (A1M) and N-acetyl-beta-D-glucosaminidase (NAG), and of kidney glomeruli, such as albumin (Alb) and immunoglobulin G (IgG) in relation to Cd exposure, are studied in the present dissertation (see Section 3.1). Additionally, in a study on cancer patients pharmaceutically exposed to As, the effect of therapy on expression of various genes in blood, (arsenic(3+) methyltransferase – *AS3MT*, aquaporin 9 – *AQP9*, glutathione peroxidase 1,3 and 4 – *GPX1,3,4*; thioredoxin reductase 1 and 2 – *TXNRD1,2*, selenoprotein P- *SELENOP*, and catalase – *CAT*) was estimated (see Section 3.4 and App A.5).

- Biomarkers of susceptibility are measurable indicators of individual inherent or acquired ability to respond to a specific chemical exposure with elevated sensitivity. Examples are genetic (e.g. (epi)genetic variations) and non-genetic factors (e.g. nutritional status, age-related physiological changes, pregnancy, children physiology) that can change the capacity to tolerate or respond effectively to exposure. Biomarkers of susceptibility studied in the present dissertation were single nucleotide polymorphisms of various genes (*AS3MT*, *SELENOP*, *AQP9*, aquaporin 4 – *AQP4*, indolethylamine N-Methyltransferase – *INMT*, methylene tetrahydrofolate reductase – *MTHFR*, metallothioneins – *MTs*, delta-

aminolevulinate dehydratase – *ALAD*; section, metal regulatory factor 1 – *MTF1*, divalent metal transporter 1 – *DMT1*, megalin – *LRP2*, Apolipoprotein receptor 2 – *LRP8*; see Section 3.2 - 3.4 and App A.2, ), gene expression (*AS3MT*, *AQP9*, *GPX1,3* and *4*, *TXNRD1* and *2*, *SELENOP* and *CAT*; Section 3.4 and App. A.5) and nutritional status of Se (see Section 3.1 - 3.3).

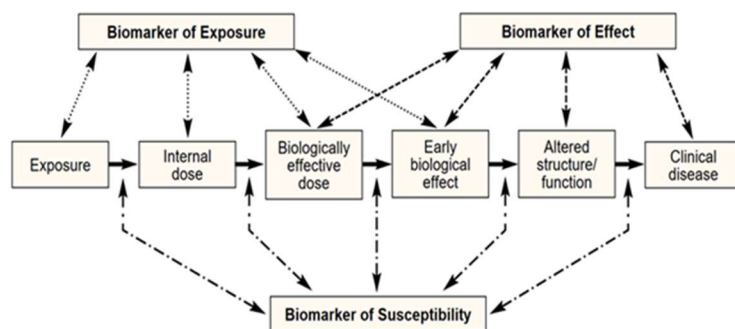


Figure 1.1: Network of biomarkers classification (source: Bearer, 2001).

In relation to health risk assessment, biomarkers of exposure have been the most extensively studied and used, while biomarkers of susceptibility are the least well understood and difficult to implement (Fan, 2014). Less often mentioned are biomarkers of adaptability, which may influence an individual's reaction to exposure by decreasing the probability of adverse health effects. They are often identified in relation to studies performed on populations with historical exposure to elevated levels of environmental contaminants. Well known is genetic adaptability to As contaminated drinking water in Andean populations of Argentina and Chile (Apata et al., 2017; Eichstaedt et al., 2015; Schlebusch et al., 2013; 2015).

In the last decade a new term “omics” biomarkers has emerged and it is informally referring to (epi)genomics (genotyping, gene expression, epigenetics), proteomics and metabolomics based biomarkers that are currently of a large interest. ‘Omics’ is a term covering technologies that measure a great amount of data representing characteristics of a large family of cellular molecules (e.g. genes, proteins, metabolites, lipids etc.) (Ward, 2014). Additionally introduced was the term of metallomics, which covers chemical elements/metals and connects them to above-mentioned biomolecules and their field of study; so we get metalloproteom, metallogenome etc. (Maret, 2018). Such biomarkers are usually developed through various advanced high-throughput technologies including bioinformatics, which results in an enormous pool of biomarker data that, consequently, exceeds the researcher's abilities to be properly validated and interpreted (Fowler, 2012).

Accordingly, the investigation and identification of various health-related biomarkers has expanded enormously. A number of them (e.g. serum cholesterol, various chromosomal translocations or deletions etc.) are regularly used in the assessment of health outcomes in clinical diseases. On the other hand, the use of biomarkers within environmental and epidemiological studies is more limited (Fowler, 2012). This limitation is mostly due to the low repeatability (between and within studies) and validity of the biomarkers because of various sources of uncertainties in biomarker determination and interpretation. Such uncertainties are frequently linked to (Aylward et al., 2014; National Research Council, 2006; Santonen et al., 2015):

- the choice of biological sample (type and time of collection),

- intra and inter-individual physiological variability resulting from changes related to (patho)physiological states (pregnancy, lactation, diseases, childhood, elderly, emotional stress, chronic subclinical diseases etc.), age, gender, physical activity, (epi)genetic variations, socio-economic or life-style factors etc.,
- difference in methodological or statistical approach.

At low levels of exposure such sources of uncertainty have important influences and their identification and evaluation is crucial for the proper and reliable interpretation of biomarkers data (Santonen et al., 2015). Selected above-mentioned sources of variability and their interactions will be discussed in more detail below and through the following chapters of the dissertation.

### 1.3 Biological Samples (Blood, Urine)

One of the critical points in human biomonitoring (HBM) studies is the collection of human samples. From logistic reasons, the majority of population studies measure biomarkers in a single so-called “spot” human sample (Aylward et al., 2014; Smolders et al., 2014). For the interpretation of such biomonitoring data it is important to be critical and keep in mind that the measured biomarker in a “spot” sample represents only a snapshot of its concentration fluctuation (Frazzoli et al., 2015).

Crucial is the *appropriate selection of the biological sample*, which depends on the time and route of exposure, physiochemical properties of the biomarker, and consequently, its kinetics in the human body (Wang et al., 2014; WHO, 2015). Accordingly, many different matrices are available for biomarker analyses (see Figure 1.2), although, most commonly chosen are *blood and urine* and much more rarely hair, nails, saliva, feces/meconium, sweat, tears, placenta and expired air. The use of blood and urine is generally defined as: blood is preferred in cases of an acute exposure and/or elements with a long biological half-time, while urine is preferred for chronic exposure and elements with fast excretion (R. Y. Wang et al., 2014). However, in practice, this guideline is not always so straightforward and is in many studies difficult to follow due to the various limiting factors, which are described in more detail in subsections and some of them through the example of cadmium inside Manuscript 1 of the thesis (see 3.1 Section).

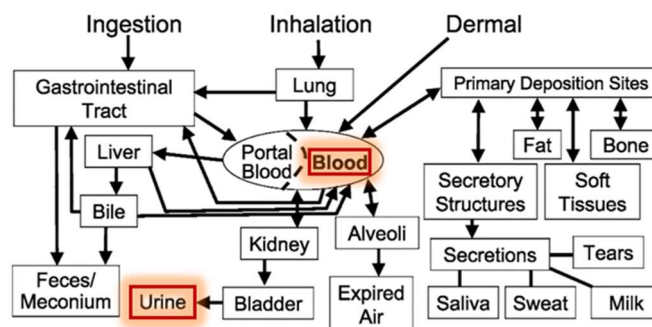


Figure 1.2: Chemicals’ routes of exposure, kinetics in human body and matrices available for their determination (source: Needham et al, 2005).

### 1.3.1 Urine

Urine is the most frequently used matrix for TE determination due to the following characteristics:

- main excretory pathway for the majority of TE,
- non-invasive,
- no need for technical skills,
- provides sufficient volume for multiple analyses,
- minimal logistic requirements.

Urine is an unregulated body fluid with considerable intra- and inter- individual variations in volume and concentration, resulting from differences in individual's hydration status and kidney physiology. Kidney function is influenced by various factors such as physical activity, circadian rhythm, age, body mass index, gender, pregnancy, diseases etc. (Aylward et al., 2014; Bernard, 2016; Cheung & Lafayette, 2013; Hoet et al., 2015; Weaver et al., 2016). For example, increased urinary excretion has been reported during pregnancy and childhood (Cheung & Lafayette, 2013; Hall et al., 2009; Poortmans, 1984). To minimize these variations the best sample would be 24h urine. But 24h urine collection which is believed to give a whole picture of the excretion is in HBM studies usually not applicable (Hoet et al., 2015; Santonen et al., 2015). So, random "spot" urine sample or first morning urine samples are used as an acceptable alternative. The latter is preferred as it is less influenced by factors such as hydration status, physical activity, and sampling time, reducing the above-mentioned variability (Witte et al., 2009) and representing the cumulative value of at least 5 hours of the night. Anyway, while in routine clinical analyses and studies the first morning samples are commonly used, human biomonitoring surveys mostly rely on random spot urine samples for practical reasons.

Biomarkers measured in such samples have to be normalized in order to reflect the situation, which would be observed in 24h urine.

#### 1.3.1.1 Normalization of urine biomarkers

The most commonly used methods are urinary concentration of *creatinine*<sup>1</sup> and *specific gravity* (SG)<sup>2</sup> of the urine sample, and less commonly osmolality of the urine (Hoet et al., 2015; Middleton et al., 2016; Santonen et al., 2015).

The method of creatinine normalization has been used as a "gold standard" for decades and relies on the assumptions of its stable excretion with little intra- and inter- individual variations and of its excretion using the same physiological pathways as the studied biomarker (Hoet et al., 2015). However, in the last years such an approach has been strongly questioned by various environmental and clinical studies, due to the clear influence of various individual's factors on creatinine excretion, namely muscle mass, diet, age, gender and a few others (Bernard, 2016; Hoet et al., 2015; Tang et al., 2015; Weaver et al., 2014). Moreover, the assumption that the studied biomarkers are using the same excretion pathway as creatinine might be misleading. Various TE are, in contrast to creatinine, bound to plasma proteins and are reabsorbed in kidney tubule (e.g. metallothionein bound Cd) while for some biomarkers excretion pathways are not yet fully understood (Hoet et al., 2015). Additionally, method sensitivity for the determination of urine creatinine is much lower than the one for the determination of TEs. Such a big difference in sensitivity

<sup>1</sup> levels of urine biomarkers are expressed per levels of urine creatinine (e.g. ngCd/ g creatinine)

<sup>2</sup> a ratio between the density of urine and density of water; the normalization is in respect to a reference SG for which mean SG of population is commonly used and carried out using the formula:  $c_n = c_m (SG_r - 1) / (SG_m - 1)$ ;  $c_n$ , normalized value;  $c_m$ , measured value;  $SG_r$ , reference SG;  $SG_m$ , individual measured SG (Suwazono et al., 2005).

can introduce important methodological uncertainties in the process of urine TE normalization. On the contrary, SG is believed to be less affected by such factors and is lately getting more attention as a better alternative for the normalization of urine biomarkers, especially for arsenic (Hoet et al., 2015; Smolders et al., 2014).

However, based on the comparisons of un-adjusted, creatinine adjusted, and specific gravity adjusted TE levels with TE levels in 24h urine, Hoet et al. (2015) concluded that for some of TE (Cd, Cr, Cu and Ni) none of the methods are recommended while for others either SG (As, Hg and Pb) or creatinine (Mo, Zn) are regarded as more suitable. As such, it seems that the choice of the normalization method might depend on the kinetics of the investigated biomarker. However, the appropriateness of either normalization method remains a point of discussion with little consensus among research groups (Hoet et al., 2015).

All these issues lead to the *uncertainty in the determination of biomarkers' concentrations in urine*, which have a profound effect particularly at low exposure levels, where they can lead to *conflicting results and misinterpretation* as is presented and discussed in the case of cadmium and urine renal proteins in Manuscript 1 of the present dissertation (see Section 3.1).

### 1.3.2 Blood

Blood or its components (erythrocytes, plasma, and serum) are the second most commonly used matrix in HBM studies. Compared to urine, disadvantages of blood sample are that its collection:

- is invasive
- is difficult to obtain for some population groups (e.g. children, elderly),
- requires technical skills,
- gives limited volume.

However, its collection is standardized and therefore more controlled than urine (WHO, 2015). Blood is commonly a preferred matrix for the determination of TE as it is in continuous contact with the organs and tissues where TE are being deposited. Additionally, contrary to urine, it is a more “regulated” matrix due to the more stable composition, resulting in lower variability in biomarkers concentrations (Santonen et al., 2015; WHO, 2015). Nevertheless, among individuals blood composition (e.g. plasma volume, cell amount) can slightly vary with age, sex, BMI, diet, etc., and to a bigger extent at specific physiological states such as pregnancy – when plasma volume can increase up to 50% – or in the case of various diseases (e.g. hypertension, anemia) (Mahlknecht & Kaiser, 2010; Richardson, 1994; Soma-Pillay et al., 2016). For proper determination and interpretation of TE levels (and other biomarkers) in blood and/or its components it is crucial to have adequate knowledge on their kinetics. For example, some trace elements (Pb, Cd, Hg, Zn) are mainly bound to erythrocytes and in order to account for above-mentioned inter-individual variability in cell/plasma proportion, such levels should be normalized by hemoglobin concentrations or hematocrit value (packed cell volume). Unfortunately, such normalization is still not a common practice in HBM studies (Santonen et al., 2015). Nevertheless, more recently some populational studies reported TE levels determined directly in erythrocytes normalized by hemoglobin (Skröder et al., 2018).

#### 1.3.2.1 Genetic biomarkers in blood

Blood is the most commonly used matrix for the determination of (epi)genetic biomarkers. In contrast to other matrices (saliva, urine etc.) it yields a higher quantity and quality of isolated nucleic acids (DNA and RNA) and consequently, better performance of molecular



techniques (Philibert et al., 2008). Additionally, previous studies have shown that “blood shares more than 80% of the transcriptome with each of the studied tissues, namely brain, colon, heart, kidney, liver, lung, prostate, spleen and stomach”, which are a target of TE accumulation and toxicity (Sunde, 2010). Hence, *gene expression (mRNA levels) in blood appears to be a useful biomarker reflecting the metabolic status of other tissues or organs* (Liu et al., 2007; Reynés et al., 2018). However, it is important to stress out that anyway the mRNA levels in blood could vary due to the composition of cell types in blood and due to the blood collection method (Reynés et al., 2018). Blood mRNA could be isolated from the whole blood (cord or peripheral blood cells, CBC or PBC) or from the fraction of peripheral mononuclear cells (PBMC); each method has advantages and disadvantages. Collection of whole blood into the RNA stabilization tubes (e.g. Paxgene and Tempus) prevents RNA degradation and ex vivo modification of expression, however due to the presence of different cell types (erythrocytes mixed with reticulocytes and PMBC) there is higher noise and variability in the results (Reynés et al., 2018). Some mRNA occurs also in red blood cells (Kabanova et al., 2009) because there are not just mature RBC in blood circulation. Erythrocytes as well as enucleated reticulocytes of different stages circulate in the blood stream and mRNA is present in the latter fraction. Additionally, gene expression can also differ based on the use of a collection tube; according to literature data the processing samples using the Tempus system seem to be more accurate (Yip et al., 2017). On the other hand, PBMC represent a more consistent and homogenous sample with reduced variability in the gene expression, though, the isolation process is more time consuming and may alter expression (Reynés et al., 2018). As such, there is no consensus regarding the ideal type of blood sample for RNA isolation, and the choice mainly depends on the aim and type of the study (Reynés et al., 2018). Expression of various genes in PBC was followed in the present dissertation (see Section 3.4 and App. A.5).

## 1.4 Association Studies

Human variability underlines characteristics, which influence an individual’s response to chemical exposure and consequently risk of developing diseases. The attempts to characterize possible interactions among exposures to potentially toxic chemicals, (epi)genetic and non-genetic factors of susceptibility/adaptability, and health or rather risk-related variables are in general referred to as population-based association studies. In the present dissertation we were primarily focused on gene-environment interactions (see Section 3.2 – 3.4, and App A.2-A.5), while non-genetic factors were used as possible effect modifiers. We tested selected genetic factors as possible susceptibility/adaptability biomarkers in relation to low-moderate levels of Cd, As and Pb, and additionally with Se levels – due to known Se role in the detoxification of TEs (Cheng & Lei, 2016). Studies were done on available data from environmentally exposed general population and in case of As also in patients with therapeutic exposure.

### 1.4.1 Gene – environment interactions

In recent years, gene-environment interaction studies expanded enormously and are being studied by researchers with a differing scientific background. As such, for good understanding, interpretation, and connection of the findings, it is important to be familiar with the basic terminology presented below, which is also used in the content of the dissertation.

In terms of genetic susceptibility/adaptability to TE exposure, the most studied biomarkers are DNA sequence variations (genetic variations), which occur naturally and – depending on the selective pressures – accumulate within populations and contribute to human variability. When a specific genetic variation equals or exceeds a frequency of 1% in the population, it is called *polymorphisms*. Among all polymorphisms (copy number variation, duplication, deletion etc.) the most common type is a *single nucleotide polymorphism (SNP)*, which is a one nucleotide change that occurs every ~500 base pairs at a specific position in the genome (see Figure 1.3) (Broberg et al, 2015; Kelada et al., 2003). Alternate versions of genes are known as *alleles*; the one present in the majority of the population is characterized as *major or common allele* and its alternate as the *minor or variant allele* (Broberg et al., 2015; Kelada et al., 2003; Reid-Lombardo & Petersen, 2010). The frequency of the variant allele is referred to as *minor allele frequency (MAF)*, which can vary among populations (Reid-Lombardo & Petersen, 2010). Based on the SNP location in the gene (coding or non-coding region) they can induce changes in the amino acid sequence, promoter activity and gene expression level, alternative splicing and stability of mRNA, etc. (Lonetti et al., 2016; Olivier, 2004; Raudenska et al., 2014). The majority of genes possess multiple SNPs and some of them tend to occur together due to their strong correlation – estimated by linkage disequilibrium (LD)<sup>3</sup> – and are commonly inherited together as a specific SNPs nucleotide sequence called *haplotype* (see Figure 1.3). Therefore, the observed association of one SNP cannot be simply interpreted as a causal relationship with the biochemical mechanism, because the genetic variation could be linked with another one within the same gene, which has the actual effect (Broberg et al., 2015). As such, the investigation of SNP haplotypes in the candidate gene could be more valuable than the investigation of a single polymorphism alone (Kelada et al., 2003; Omenn, 2013). We used such an approach when we studied the influence of *AS3MT* gene polymorphisms on methylation efficiency at low As exposure in Manuscript 2 of the dissertation (see Section 3.2).

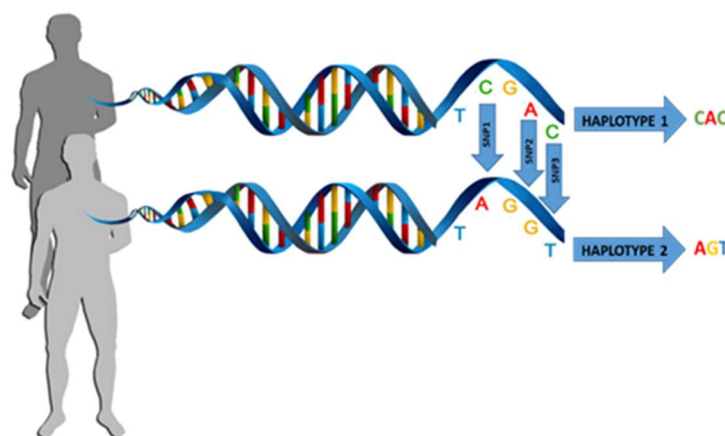


Figure 1.3: Single nucleotide polymorphisms (SNPs) and haplotype (adapted from Lonetti et al., 2016).

Various SNPs can be “associated with the diversity of the population, individuality, susceptibility to disease, and individual response to therapeutic treatment or environmental exposure” (Wang et al., 2005).

For a better interpretation and identification of possible biological pathways behind the observed SNP associations, intermediate steps such as *gene expression*, should also be

<sup>3</sup> LD; linkage disequilibrium: the non-random linkage of genetic variants that are inherited together in chunks (Moore, 2017)

studied as a possible susceptibility biomarker. SNPs' related changes in gene expression can influence the amount and activity of the encoded enzyme/protein. As such, expression level of a gene carrying studied SNP should be tested in individuals carrying different SNP alleles, and the observed difference would suggest that SNP affects gene expression and points to its functionality (Olivier, 2004). Beside SNPs, gene expression and consequently protein expression are regulated directly or indirectly by various other genetic and non-genetic factors, resulting in variability over time and reflecting individual's metabolic or physiological changes. Hence, gene expression (in blood) is getting a lot of attention in: i) medicine as a useful biomarker for the diagnosis and treatment progress of a number of diseases (immune-related diseases, cancer, liver disease etc.) (Reynés et al., 2018; Sunde, 2010) and ii) environmental studies, where altered gene expression upon exposure to a chemical could present an early biomarker of effect and provide information on the chemical's kinetics and/or dynamics (Calzolari et al., 2007). As an example, the expression of metallothionein genes – coding metal binding proteins with various physiological roles – can be induced by various factors such as metal(loid) exposure, oxidative stress, hormones, pH etc. (Raudenska et al., 2014).

Trace elements represent a good base for gene-environment studies due to their relatively well known and not so complex metabolism, which in some cases involve only a few enzymatic reactions; in such cases the identification of candidate genes involved in kinetics and/or dynamic of TE is easier (Broberg et al., 2015). Kinetics may be influenced by genes involved in TE uptake, distribution, metabolism and elimination. The TE's mode of action may be modified by the genes that affect binding proteins, genes involved in defense against oxidative stress, DNA repair, regulation of gene expression etc. (Broberg et al., 2015). Therefore, SNPs and/or changed expression of such genes could modify the degree and type of TE disposition, accumulation, retention and their biological activity and consequently susceptibility/adaptability to their adverse health effects.

There is a large number of identified candidate genes which can influence internal levels of TE like As, Pb, Cd, and Se (as main elements of present dissertation) – for which examples are presented in Table 1.1. However, most of those interactions were observed for populations with high environmental or occupational exposure while for general populations with low-level exposure interactions are poorly studied and inconsistent (Broberg et al., 2015). Frequently reported are association in relation to As or Pb. Associations between *AS3MT* SNPs and increased As methylation rates (methylation efficiency) were observed in selected populations with high inorganic As (iAs) exposure through drinking water, such as Argentina (San Antonio de los Cobres), Bangladesh and Chile (region Chamarones and Azapa) (Apata et al., 2017; Broberg et al., 2015). For Pb the association between *ALAD* SNP (*ALAD2* allele) and blood Pb levels was found in occupationally exposed populations; however the results are rather inconsistent (Broberg et al., 2015; Skerfving & Bergdahl, 2015).

Some candidate genes were identified based only on cell line or animal experiments, or were investigated in humans only on the basis of gene expression (mRNA) (see Table 1.1). An example are various genes of aquaporin's (*AQP*; e.g. *AQP4*, *AQP9* and *AQP10*) which are coding membrane proteins in cellular arsenic transport pathways in mammals including humans (Roggenbeck et al., 2016). For most of them, only the gene expression was studied while the possible influence of their SNPs in relation to As levels was not examined (see Table 1.1).

Table 1.1: Examples of gene associated with kinetics/dynamics of As, Pb, Cd and Se.

| Gene                                                                        | Gene ID | Function                                                                      | TE associated SNPs<br>or mRNA level                                                                 | H - human<br>A - animal<br>C - human cell<br>line | Ref. |
|-----------------------------------------------------------------------------|---------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|---------------------------------------------------|------|
| ARSENIC                                                                     |         |                                                                               |                                                                                                     |                                                   |      |
| Arsenic (3)<br>methyltransferase                                            | AS3MT   | As methylation                                                                | rs7085104, rs3740400,<br>rs3740393, rs3740390,<br>rs10748835, rs11191439,<br>rs12768205, rs11191453 | H                                                 | a    |
|                                                                             |         |                                                                               | mRNA level                                                                                          | H                                                 | a    |
| N-6 Adenine-Specific DNA<br>Methyltransferase 1                             | N6AMT1  | DNA and protein<br>methylation<br>(possible As<br>methylation)                | s1997605, rs2205449,<br>rs2705671, rs16983411,<br>rs1048546                                         | H                                                 | a    |
| Methylenetetrahydrofolate<br>reductase                                      | MTHFR   | one-carbon<br>metabolism<br>(metabolism of As)                                | rs1801131, rs1801133                                                                                | H                                                 | a    |
| Cystathionine-β-Synthase                                                    | CBS     |                                                                               | rs234709, rs4920037                                                                                 | H                                                 | a    |
|                                                                             |         |                                                                               | mRNA level                                                                                          | C                                                 | a    |
| 5-methyltetrahydrofolate-<br>homocysteine<br>methyltransferase<br>reductase | MTRR    |                                                                               | rs1801394                                                                                           | H                                                 | a    |
| Choline dehydrogenase                                                       | CHDH    |                                                                               | rs9001, rs7626693                                                                                   | H                                                 | a    |
| Gglutaredoxin                                                               | GLRX    | metabolism of<br>xenobiotics (As<br>reduction)                                | rs3822751                                                                                           | H                                                 | a    |
| Peroxioredoxin 2                                                            | PRDX2   |                                                                               | rs10427027, rs12151144                                                                              | H                                                 | a    |
| Glutathione S-transferase<br>omega -1 and omega-2                           | GSTO1   |                                                                               | rs4925, rs56204475,<br>rs11191979, rs2164624                                                        | H                                                 | a, b |
|                                                                             | GSTO2   |                                                                               | rs156697, rs2297235                                                                                 | H                                                 | b    |
| Purine nucleoside<br>phosphorylase                                          | PNP     |                                                                               | rs3790064, rsl713420                                                                                | H                                                 | b    |
| Aquaporins (AQP)                                                            | AQP3    | water-selective<br>membrane channels<br>(cellular uptake,<br>transport of As) | rs2228332                                                                                           | H                                                 | b    |
|                                                                             |         |                                                                               | mRNA level                                                                                          | C                                                 | b    |
|                                                                             | AQP4    |                                                                               | mRNA level                                                                                          | C                                                 | b    |
|                                                                             | AQP9    |                                                                               | mRNA level                                                                                          | H                                                 | b    |
|                                                                             | AQP10   |                                                                               | mRNA level                                                                                          | C                                                 | b    |
| Metallothioneins (MT)                                                       | MT1A    | antioxidative role,<br>homeostasis of Zn<br>and Cu, metal<br>detoxification   | mRNA level                                                                                          | H                                                 | b    |
|                                                                             | MT1E    |                                                                               | mRNA level                                                                                          | C                                                 | b, d |
|                                                                             | MT1F    |                                                                               | mRNA level                                                                                          | C                                                 | b, d |
|                                                                             | MT1X    |                                                                               | mRNA level                                                                                          | C                                                 | b, d |
|                                                                             | MT2A    |                                                                               | rs28366003, mRNA level                                                                              | H                                                 | b, c |
|                                                                             | MT3     |                                                                               | mRNA level                                                                                          | C                                                 | b, d |
| Myeloperoxidase                                                             | MPO     | antioxidative role                                                            | rs2333227                                                                                           | H                                                 | a    |
| Catalase                                                                    | CAT     |                                                                               | rs1001179                                                                                           | H                                                 | a    |
| X-ray repair cross<br>complementing 3                                       | XRCC3   | DNA repair                                                                    | rs861539                                                                                            | H                                                 | a    |
| ERCC Excision Repair 2,<br>TFIIH Core Complex<br>Helicase Subunit           | ERCC2   |                                                                               | rs13181                                                                                             | H                                                 | a    |
|                                                                             |         |                                                                               | mRNA level                                                                                          | H                                                 | b    |
| Purinic/Apyrimidinic<br>Endodeoxyribonuclease 1                             | APEX1   |                                                                               | rs1130409                                                                                           | H                                                 | a    |
| Fibrous sheath interacting<br>protein 1                                     | FSIP1   | oncoprotein                                                                   | rs10152640                                                                                          | H                                                 | a    |
| Solute carrier family 39<br>(Zinc transporter, member<br>2)                 | ZIP2    | Zn transport and<br>homeostasis                                               | rs2234636                                                                                           | H                                                 | a    |
| Tumor protein p53                                                           | TP53    | cell cycle regulation                                                         | rs1042522                                                                                           | H                                                 | a    |
| Apolipoprotein E                                                            | APOE    | lipid metabolism                                                              | rs7412, rs429358                                                                                    | H                                                 | b    |
|                                                                             |         |                                                                               | mRNA level                                                                                          | A                                                 | b    |

|                                                                    |               |                                                                         |                                                               |   |      |
|--------------------------------------------------------------------|---------------|-------------------------------------------------------------------------|---------------------------------------------------------------|---|------|
| Brain derived neurotrophic factor                                  | <i>BDNF</i>   |                                                                         | mRNA level                                                    | C | b    |
| Indolethylamine N-methyltransferase                                | <i>INMT</i>   | methylation of indoles (possible involvement in methylation of Se)      | mRNA level                                                    | A | b    |
| LEAD                                                               |               |                                                                         |                                                               |   |      |
| Aminolevulinate dehydratase                                        | <i>ALAD</i>   | heme biosynthesis (binds Pb)                                            | rs1805313, rs1800435, rs2761016, rs1139488,                   | H | a    |
|                                                                    |               |                                                                         | mRNA level                                                    | H | b    |
| Vitamin D receptor                                                 | <i>VDR</i>    | ligand-inducible transcription factor (regulation Vitamin D synthesis)  | rs10735810, rs1544410, rs731236, rs757343, rs107358, s7975232 | H | a, j |
| Homeostatic iron regulator                                         | <i>HFE</i>    | regulate iron absorption                                                | rs1799945, rs1800562,                                         | H | a    |
| Heme oxygenase-1                                                   | <i>HMOX1</i>  | heme catabolism                                                         | rs2071749, rs2071746, rs5995098                               | H | j    |
| Angiotensinogen                                                    | <i>AGT</i>    | maintaining blood pressure (role in hypertension and preeclampsia)      | rs699, rs5046                                                 | H | j    |
| Transferrin                                                        | <i>TF</i>     | Fe transport                                                            | rs1049296                                                     | H | a    |
| Cytochrome P450 family 1 (subfamily A member 1)                    | <i>CYP1A1</i> | drug metabolism and synthesis of cholesterol, steroids and other lipids | rs1048943, rs1056827, rs10568                                 | H | b    |
| Methylenetetrahydrofolate reductase                                | <i>MTHFR</i>  | one-carbon metabolism                                                   | rs1801131, rs1801133                                          | H | b    |
| Metallothioneins (MTs)                                             | <i>MT1A</i>   | antioxidative role, homeostasis of Zn and Cu, metal detoxification      | rs11076161                                                    | H | b    |
|                                                                    |               |                                                                         | mRNA level                                                    | A | b    |
|                                                                    | <i>MT1E</i>   |                                                                         | mRNA level                                                    | H | b    |
|                                                                    | <i>MT1F</i>   |                                                                         | mRNA level                                                    | H | b    |
|                                                                    | <i>MT1G</i>   |                                                                         | mRNA level                                                    | H | b    |
|                                                                    | <i>MT1H</i>   |                                                                         | mRNA level                                                    | H | b    |
|                                                                    | <i>MT1X</i>   |                                                                         | mRNA level                                                    | H | b    |
|                                                                    | <i>MT2A</i>   |                                                                         | rs28366003, rs10636                                           | H | a, b |
|                                                                    |               |                                                                         | mRNA level                                                    | H | b    |
| Catalase                                                           | <i>CAT</i>    | antioxidative role                                                      | rs769217                                                      | H | b    |
|                                                                    | mRNA level    |                                                                         | A                                                             | b |      |
| Superoxide dismutase 1                                             | <i>SOD1</i>   |                                                                         | mRNA level                                                    | A | b    |
| Transient receptor potential cation channel (subfamily V member 5) | <i>TRPV5</i>  | transcellular transport of Ca <sup>2+</sup> (bone resorption)           | rs4252424                                                     | A | e    |
| Glutamate-cysteine ligase (catalytic (C) and modifier (M) subunit) | <i>GCLM</i>   | glutathione synthesis                                                   | rs41303970                                                    | H | b    |
|                                                                    | <i>GCLC</i>   |                                                                         | rs17883901                                                    | H | b    |
| Glutathione S-transferase (pi1, theta1)                            | <i>GSTP1</i>  | metabolism of xenobiotics                                               | rs1695                                                        | H | a,j  |
|                                                                    | <i>GSTT1</i>  |                                                                         | genetic deletion                                              | H | b    |
|                                                                    |               |                                                                         | mRNA level                                                    | H | b    |
| Apolipoprotein E                                                   | <i>APOE</i>   | lipid metabolism                                                        | rs7412, rs429358, rs769446                                    | H | b, j |
| DNA methyltransferase 1                                            | <i>DNMT1</i>  | maintaining methylated cytosine pattern in genome                       | mRNA level                                                    | H | b    |
| Dopamine receptor D2                                               | <i>DRD2</i>   | regulation of dopaminergic system                                       | rs1800497                                                     | H | a    |
| Brain derived neurotrophic factor                                  | <i>BDNF</i>   | promotes neuronal survival in the adult brain                           | mRNA                                                          | A | b    |
| CADMIUM                                                            |               |                                                                         |                                                               |   |      |
| Metallothioneins (MT)                                              | <i>MT1A</i>   | antioxidative role, homeostasis of Zn                                   | rs11076161, rs8044719                                         | H | a, b |
|                                                                    |               |                                                                         | mRNA level                                                    | H | b    |

|                                                             |                |                                                                                                                    |                      |   |      |
|-------------------------------------------------------------|----------------|--------------------------------------------------------------------------------------------------------------------|----------------------|---|------|
|                                                             | <i>MT1B</i>    | and Cu, metal detoxification                                                                                       | rs1599823            | H | b    |
|                                                             | <i>MT1E</i>    |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT1F</i>    |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT1G</i>    |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT1H</i>    |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT1X</i>    |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT2A</i>    |                                                                                                                    | rs28366003, rs10636  | H | a, b |
|                                                             |                |                                                                                                                    | mRNA level           | H | b    |
|                                                             | <i>MT3</i>     |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>MT4</i>     |                                                                                                                    | mRNA level           | A | b    |
| Catalase                                                    | <i>CAT</i>     | antioxidative role                                                                                                 | mRNA level           | A | b    |
| Metal-regulatory transcriptional factor 1                   | <i>MTF1</i>    | regulation of <i>MT</i> expression                                                                                 | rs4653329            | H | b    |
| Transferrin receptors                                       | <i>TFRC</i>    | cellular Fe uptake (Fe homeostasis)                                                                                | rs3804141            | H | a    |
|                                                             |                |                                                                                                                    | mRNA level           | H | a    |
| Solute carrier family 39 (Zinc transporter member 8 and 14) | <i>ZIP8</i>    | Zn transport and homeostasis                                                                                       | rs10014145, rs233804 | H | b    |
|                                                             | <i>ZIP14</i>   |                                                                                                                    | rs4872479, rs870215  | H | b    |
| Glutathione S-transferase Pi                                | <i>GSTP1</i>   | metabolism of xenobiotics                                                                                          | rs1695               | H | b    |
| matrix metalloproteinase 2                                  | <i>MMP-2</i>   | degradation of extracellular matrix                                                                                | rs14070, rs7201      | H | b    |
| Apolipoprotein E                                            | <i>APOE</i>    | lipid metabolism                                                                                                   | mRNA level           | A | b    |
| SELENIUM                                                    |                |                                                                                                                    |                      |   |      |
| Selenoprotein (P, F, K)                                     | <i>SELENOP</i> | antioxidative role, transport of Se                                                                                | rs3877899, rs7579    | H | b, f |
|                                                             |                |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>SELENOF</i> | proposed role in protein folding                                                                                   | rs561104             | H | b    |
|                                                             | <i>SELENOK</i> | ER-associated degradation of misfolded, glycosylated proteins                                                      | rs9880056            | H | i    |
| Glutathione peroxidase (1, 3, 4)                            | <i>GPX1</i>    | antioxidative role                                                                                                 | rs1050450,           | H | b    |
|                                                             |                |                                                                                                                    | mRNA level           | H | b    |
|                                                             | <i>GPX3</i>    |                                                                                                                    | mRNA level           | H | c    |
|                                                             | <i>GPX4</i>    |                                                                                                                    | rs713041             | H | f    |
|                                                             |                |                                                                                                                    | mRNA level           | C | b    |
| Catalase                                                    | <i>CAT</i>     |                                                                                                                    | mRNA level           | H | b    |
| Superoxide dismutase                                        | <i>SOD2</i>    |                                                                                                                    | rs4880               | H | b    |
| Thioredoxin reductase (1 and 2)                             | <i>TXNRD1</i>  |                                                                                                                    | rs7310505            | H | i    |
|                                                             |                |                                                                                                                    | mRNA level           | C | b    |
|                                                             | <i>TXNRD2</i>  |                                                                                                                    | rs9605030 rs9605031  | H | i    |
| Dimethylglycine dehydrogenase                               | <i>DMGDH</i>   | one-carbon metabolism (metabolism of sulfur-containing amino acids, methionine, potentially also selenomethionine) | rs921943, rs17823744 | H | b    |
| Indolethylamine N-methyltransferase                         | <i>INMT</i>    | methylation of indoles (possible involvement TMSe formation)                                                       | rs6970396            | H | g    |
| Apolipoprotein E                                            | <i>APOE</i>    | lipid metabolism                                                                                                   | rs7412, rs429358     | H | h    |
|                                                             |                |                                                                                                                    | mRNA level           | H | c    |

a - (Broberg et al., 2015), b- (MDI Biological Laboratory & NC State University, 2019), c - (González-Martínez et al., 2018), d - (Falnog et al., 2012), e - (Liu et al., 2019), f - (Reszka et al., 2012), g - (Kuehnelt et al., 2015), h - (Trdin et al., 2019 submitted.), i - (Méplan et al., 2012); j - (Ding et al., 2016) TMSe, trimethylselenonium ion

Regardless of the large existing data on gene-TE interactions, genetic factors are still not successfully implemented as susceptibility biomarkers in health risk assessment, which is due to the inconsistencies of results between existing studies (Broberg et al., 2015; Dick et al., 2015; Kelada et al., 2003). Low repeatability of gene-TE interactions can result from:

- previously mentioned influence of biological sample type on TE levels (Section 1.3),
- different exposure levels of studied populations,
- differences in geographical distribution of SNPs frequencies,
- different methodological approaches (targeted, non-targeted) in determination of genetic factors (SNPs, mRNA levels), and
- differences in individual and/or populational physiology (non-genetic factors).

Some causes are discussed in more detail below.

#### 1.4.1.1 Genetic factors and exposure levels

As reviewed by Broberg et al., 2015 many gene-TE interactions observed at high exposure were not confirmed at lower exposure. Firstly, the range of TE levels could be too narrow and the methodological uncertainties too high to provide sufficient statistical power for identification of such associations (Ritz et al., 2017). Another point to consider is the actual magnitude of SNP's effect on TE levels. Based on the existing studies (see Table 1.1) the influence of the majority of SNPs is non-detectable, still uncertain or it is likely to be small at relatively "normal" physiological and exposure conditions (Broberg et al., 2015; Dick et al., 2015; Kelada et al., 2003). Therefore, it might be that the effect of a SNP becomes crucial only at higher exposures while at lower exposures it is minimal and overwhelmed by the effect of other modifiers of TE kinetic/dynamics, particularly non-genetic physiological parameters (Broberg et al., 2015; Gardner et al., 2012; Xu et al., 2016). On the contrary, in some cases, higher exposure may saturate enzyme activity regardless of the SNP(s) presence and the potential influence of SNP(s) could be observed only at lower or moderate exposures. However, such phenomenon is not often observed (Broberg et al., 2015). Additionally, SNPs' influence at different TE exposures levels could also depend on the specificity of the candidate gene with respect to TE. Genes coding for enzymes with various roles that are not specific for TE are expected to show effect at higher levels, while the influences of genes that are TE-specific might be observed at lower exposures (e.g. *AS3MT* in relation to As methylation (Balakrishnan et al., 2018)).

#### 1.4.1.2 Geographical distribution of SNPs frequencies

Due to the studies investigating genetic variations of the whole genome on a worldwide scale (HapMap and 1000 Genome Project) – whose data is openly accessible in the "world's largest database for nucleotide variations as a part of National Centre for Biotechnology Information (dbSNP, NCBI)" (National Centre for Biotechnology Information, 2005) – it becomes clear that the frequencies of individual SNPs vary between different ethnic groups and different parts of the world (Broberg et al., 2015; National Research Council, 2006). For example, variant allele frequencies of *AS3MT* SNPs haplotype in Argentina, Peru and Chile were found to be much higher than in other parts of the world, such as Europe (50 – 70% vs. 12%) (Engström et al., 2011), and in case of the variant allele of *ALAD* SNP (*ALAD2*) higher frequencies were observed for Caucasian and lower for Asian and African populations (19% versus 2-10%) (Broberg et al., 2015). Consequently, associations between investigated SNPs and TEs can in respect to their presence, trend and magnitude vary between populations worldwide (Broberg et al., 2015; National Research Council, 2006).

Geographical variability in the frequency of SNPs in genes related to kinetics/dynamics of TE could reflect a specific genetic adaptation of populations to a historical excess or deficiency of TE in their local environment (i.e. natural selection) (White et al., 2015). Although the discovery of such cases is rare, recently genetic adaptation of *AS3MT* related to historically increased exposure to As in drinking water was proven for the above-mentioned Andean populations of Argentina and Chile (Apata et al., 2017; Eichstaedt et al., 2015; C. M. Schlebusch et al., 2015) and genetic adaptation of selenoproteins related to deficient levels of selenium in China (White et al., 2015). With good knowledge of geographical abundance of TEs (e.g. Pb and Hg), TE kinetics/dynamics and population's history and migrations, we can expect that in the future more of such case studies will emerge. Knowledge of the genetic structure of populations is therefore important for the appropriate interpretation of gene-environmental interaction studies and could play a crucial role in emergency investigations of the major unpredicted environmental contamination (accidental exposure) (Baye et.al., 2009).

#### 1.4.1.3 Targeted versus non-targeted approach

Genetic factors and their associations are being studied by two different approaches:

- traditional hypothesis-driven targeted candidate gene association studies, which focus on a small set of genes and SNP that have been selected based on their known or predicted role (approach used in the present dissertation), and
- hypothesis-free non-targeted approach, which is via high-throughput technology analyzing hundreds of thousands of genes.

Examples of the second approach are microarrays for transcriptomes and genome wide association studies (GWAS) analyzing hundreds of thousands of SNPs across the whole genome (Reid-Lombardo & Petersen, 2010; Ritz et al., 2017).

Based on the GWAS principle, such non-targeted approach was recently also introduced for the assessment of exposure to chemicals within populational-based association studies, which is referred to as an “exposome” approach. As defined by Wild in 2005, exposome “encompasses life-course environmental exposure (including lifestyle factors) from the prenatal period onwards” complementing the genome (Wild, 2005). In other words, exposome uses a variety of high-throughput methods – “omics” – in order to unravel the nature-nurture interactions (Miller & Jones, 2014).

The traditional targeted approach, especially in gene-environment studies, gained some critics in respect to low replicability of its results with non-targeted approaches. Consequently, the need for a targeted approach has become questionable by some research groups (Border & Keller, 2017; Wilkening et al., 2009). However, as pointed out by Stingone et al. (2017), the exposome approach beside the lack of “bioinformatics and statistical methodologies to process, integrate, and analyze high-dimensional data and lack of cross-trained scientific investigators who can develop and implement these approaches”, struggles also with “the difficulty in differentiating normal physiological variations from environmentally induced changes in measures of biological response”. Therefore, the findings from such complex non-targeted analyses still need to be validated in targeted independent studies, which indicates that *exposome approach represents a complement to, rather than a replacement for, traditional targeted analyses* (Stingone et al., 2017).

#### 1.4.1.4 Non-genetic modifiers of TE levels and their associations

The kinetics and dynamics of TEs and consequently their measured internal concentrations are, beside genetic factors, primarily influenced by various non-genetic factors that shape individual (population) (patho)physiology. Changes in such factors – age, BMI, gender,



psychological status (e.g. stress, depression), physical activity, changes related to “special” physiological states (e.g. pregnancy, childhood, lactation, elderly), diet (e.g. seafood intake), habits (e.g. smoking, alcohol consumption), co-exposures etc. – lead to physiological variability, which can modify studied interactions between TE and genetic factors and/or health-related variables (Aylward et al., 2014; Santonen et al., 2015).

In the majority of association studies researchers are aware that simple comparisons between groups (e.g. Pb levels in carriers vs. non-carriers of *ALAD* SNP) could be confounded by above-mentioned non-genetic factors and that multivariable regression models using those factors as possible confounders should be applied (Broberg et al., 2015). The choice of confounders differs between researchers; some follow their knowledge and understanding of physiological mechanisms, while others follow findings from the literature in order to be more comparable. However, in both cases the availability of data presents limitation. Such data is most commonly obtained through questionnaires, which is a cost efficient approach that, however, comes with disadvantages such as (Coggon, 1995; WHO, 1995):

- lack of standardization – content regarding specific exposure assessment may differ among various studies,
- poor validity of the content due to bias introduced from: misunderstanding of the questions (when self-administered); inaccurate or incomplete recall in case of questions that rely on people’s memory or intentional false reporting in case of delicate questions (alcohol or drug use, smoking, psychological problems etc.).

Therefore, ideally questionnaires should be accompanied by or substituted with measurable characteristics, reflecting individual physiology. Some examples are listed below:

- Levels of other (non)essential TE. Status of essential trace elements is a good estimate of nutritional status and reflection of (patho)physiological changes. Elements as Zn, Se, Cu, Mn, and Fe are essential for the activity of various proteins/enzymes that are related to kinetics/dynamics of non-essential TE due to their roles as antioxidants, binding proteins, transporters etc., (Bornhorst et al., 2018; Prashanth et al., 2015). As recently reviewed by Rahman et al. (2019), Se and Zn are known to protect against toxicity of non-essential TEs (Cd, Pb, As and Hg) via different complex mechanisms. Therefore, their dishomeostasis, which could be related to some diseases, can considerably increase risk for the metal(oids) adverse effects and potential progress into a disease (Rahman et al., 2019). Iron deficiency can increase the uptake of other metal(oids), such as Cd and Pb, due to their similar absorption pathways (Lee et al., 2014). Selenium in plasma can be a rough estimation of individual antioxidant capacity (Tinggi 2008), and due to its known interaction with some non-essential TE, such as As, can influence their metabolism and excretion (Zeng et al., 2005; George et al., 2013). Additionally, levels of other non-essential elements should be evaluated in order to evaluate possible confounding due to co-exposure. For example, in the assessment of Cd effect on renal function it is necessary to evaluate also the status of other nephrotoxic elements, such as As, Hg, and Pb (Barregard & Elinder, 2015; Bernard, 2016; Hambach et al., 2013).

Such approach is not common within association studies; however, we included it in the work of the present dissertation (see Section 3.1 – 3.3).

- Levels of various hormones (estrogen, thyroid hormones etc.) are important as they can indirectly influence kinetics/dynamics of various TE (Engström et al., 2011; Lee & Kim, 2014).

- Vitamin D level is one of the indicators of adequate nutritional status. Its sufficient levels can improve the uptake of essential trace elements (e.g. Zn, Fe, and Cu), while higher levels increase uptake of some non-essential elements (e.g. Cd and Pb) (Schwalfenberg & Genuis, 2015).
- Urinary or serum level of cotinine as a good indicator of smoking (Benowitz, 1996; Leon et al., 2002). Accurate information on individual's smoking is in environmental association indispensable as a route of exposure to non-essential elements such as Cd and Pb. Moreover it is an inducer of oxidative stress and inflammation (Agarwal, 2005), which can influence expression and activity of various proteins/enzymes that are studied in relation to TE.

The structure of multivariable regression models can considerably vary between association studies dealing with the same scientific question, which could lead to different outcome and interpretation of the models, contributing to the low repeatability of the results regardless of the use of targeted or untargeted approaches.

## Chapter 2

# Aims and Hypothesis

The aim of the present dissertation was to estimate the appropriateness of exposure and effect biomarkers and the influences of selected gene single nucleotide polymorphisms on the level of potentially toxic non-essential trace elements in blood and urine of susceptible general population with very low to moderate environmental or diet-related exposure. Assessed will be populations in pregnancy, lactation, childhood, and childbearing or mixed age, with dietary (seafood), environmental and geographical variability. In the first part the study will be focused on normalization difficulties of spot urine biomarker levels, related to the insufficient sensitivity of creatinine, used as a “gold standard” to normalize for the dilution effect from drinking and for the effect of individual’s kidney physiology. The second part will deal with gene-environment interactions by studying the impact of single nucleotide polymorphisms (SNP) in genes involved in the metabolism of essential and non-essential TE (Cd, As, Pb and Se) and comparison of obtained SNP’s allele frequency distribution between studied population and those found in other populations (SNPs world database). In the last part, the relations between As exposure, As metabolites and selected gene expression will be assessed in cancer patients treated with arsenic trioxide ( $\text{As}_2\text{O}_3$ ).

### Hypothesis:

1. For Slovenian and Croatian population, with very low to moderate exposure to non-essential trace elements, we expect weak associations between biomarkers of exposure, effect and/or susceptibility and notable impact of methodological uncertainty (questionable sample normalization, shared physiological pathways), basic personal characteristics, physiological status and life style.
2. Allele frequencies of studied SNPs studied in both population will be similar to each other and to those in other European populations and different from those in Asian or African populations.
3. Arsenic treatment of cancer patients will affect blood expression of various genes and expression will differ between patients.



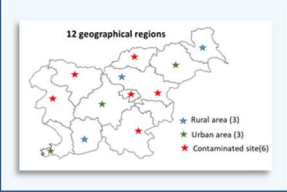
## Chapter 3

# Scientific Publications

The present dissertation consists of four publications: two published, one under review and one in preparation. They are presented correspondingly to the general hypotheses of the dissertation. The first hypothesis is covered by the first three manuscripts dealing with the appropriateness of exposure biomarkers and their associations with biomarkers of effect or with genetic variability related to cadmium (3.1), arsenic (3.2) or lead (3.3). The second hypothesis is covered by the second and third manuscript (3.2 and 3.3) as in both allele frequencies of SNPs are given in genes related to arsenic (*AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *SELENOP*, *INMT*, *MT2A*) and Pb (*ALAD*) metabolism, respectively. *AS3MT* is coding a key enzyme in As methylation; *MTHFR* is coding an enzyme involved in one-carbon metabolism; AQPs are coding selective membrane channels involved in As transitions; *SELENOP* and *INMT* are coding enzymes involved in Se transport and urine excretion in the form of trimethylselenonium ion (TMSe), respectively; *MTs* are coding for proteins involved in homeostasis of Cu and Zn and detoxification of non-essential metals; *ALAD* is coding for enzyme involved in heme biosynthesis, which can be inhibited by Pb. The third hypothesis about the individual variability in pharmacological As exposure related to gene expression is presented by the fourth manuscript (Section 3.4).

Manuscripts are accompanied by five working reports, which are added as appendixes (App. A.1-A.5). They are supplementing detailed data, which are otherwise only mentioned inside manuscripts, and data important for the given hypotheses and not published yet.

The above-mentioned studies were performed on three general populations and on a case of five cancer patients; basic information on their size, geographical distribution, physiological state, and methodology is summarized in Figure 3.1.

|                                             |                                                                                   |                                                                                    |                                                                                     |                                                                 |
|---------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------|
|                                             | <b>1<sup>st</sup> Slovenian HBM<br/>(2007 – 2015)</b>                             | <b>PHIME – CROME-LIFE<br/>(EU Projects 2007 – 2017)</b>                            | <b>Kyrgyzstan initiation HBM<br/>(2016)</b>                                         | <b>Clinical studies<br/>(2013 – 2017)</b>                       |
| <b>Population</b>                           | <b>HBM population</b>                                                             | <b>Slovenian-Croatian<br/>population</b>                                           | <b>Kyrgyzstan population</b>                                                        | <b>APL patients + control</b>                                   |
| <b>Participants (N)</b>                     | <b>1081</b>                                                                       | <b>488</b>                                                                         | <b>123</b>                                                                          | <b>5 + 14</b>                                                   |
| <b>Vulnerable* population (N)</b>           | <b>Lactating W (533)<br/>Childbearing aged M (548)</b>                            | <b>Pregnant W (136)<br/>Non-pregnant W (176)<br/>7-8y Children (176)</b>           | <b>Mixed population from<br/>industrial pollution (91W,<br/>32M)</b>                | <b>As<sub>2</sub>O<sub>3</sub> treated patients<br/>(4W,1M)</b> |
| <b>Geographical regions</b>                 |  |  |  |                                                                 |
| <b><u>Questionnaires</u></b>                | +                                                                                 | +                                                                                  |                                                                                     |                                                                 |
| <b><u>Multielemental analyses</u></b>       | +                                                                                 | +                                                                                  | +                                                                                   | +                                                               |
| <b><u>Urine renal function proteins</u></b> | +                                                                                 |                                                                                    |                                                                                     |                                                                 |
| <b>Urine As metabolites</b>                 |                                                                                   | +                                                                                  |                                                                                     | +                                                               |
| <b>Genotyping</b>                           |                                                                                   | +                                                                                  | +                                                                                   | +                                                               |
| <b>mRNA level</b>                           |                                                                                   |                                                                                    |                                                                                     | +                                                               |

\* vulnerable regarding: (patho)physiological status, age or exposure; W – women, M – men; underlined are existing databases

Figure 3.1: Basic information on studies of the present dissertation.

### 3.1 Manuscript 1: Low Cadmium Exposure in Males and Lactating Females – Estimation of Biomarkers

Published: Stajnko A., Falnoga I., Tratnik J. S., Mazej D., Jagodic M., Krsnik M., Kobal A.B., Prezelj M., Kononenko L., Horvat M. (2017). *Environmental Research*, 152, 109–119

Association studies of non-essential trace elements and biomarkers of effect are well established for occupational or high exposures and commonly used in health risk assessment. On the contrary, at low exposure levels ( $< 1 \mu\text{gCd/L}$  for blood and urine) the interpretation of such associations is challenging due to strong and undistinguished influences of methodological uncertainties and physiological variabilities (Bernard, 2016). For instance, creatinine normalization of spot urine levels of trace elements or of biomarkers of effects is introducing many biases due to low methodological sensitivity and high physiological variability of creatinine and because trace elements can use the same transporters as biomarkers of effects. Hence, such associations are not casual but physiological.

In the present manuscript, conducted on a Slovenian population from the National human biomonitoring program (2007 – 2015), we estimated the appropriateness of biomarkers at low exposure levels – regarding the choice of biological matrix – on a case of blood versus spot urine cadmium in relation to selected urine renal function biomarkers (tubular:  $\alpha$ -1 microglobulin – A1M, N-acetyl- $\beta$ -glucosaminidase – NAG; glomerular: albumin – Alb, and immunoglobulin G – IgG). Such associations are commonly used for the estimation of renal dysfunction related to Cd exposure. In our work, we assessed them on 548 men and 533 lactating women separately. Associations were tested for possible confounding effects related to physiological underlying factors (age, BMI, smoking, nutritional status of Se, co-exposure to Pb, Hg and As) and methodological (urine normalization) underlying factors. In order to test the influence of the latter, we assessed all the associations using un-normalized, creatinine normalized and specific gravity normalized urine data. As reported by Hoet et al. (2015) for some trace elements (e.g. As, Hg, Pb) specific gravity is recommended as a better alternative to creatinine, while for others (e.g. Cd, Cu) none of the methods seem suitable – as observed also in the present manuscript.

At low Cd exposure:

- i) We emphasized the awareness of the questionable assessment of association between low Cd levels and renal function biomarkers determined in spot urine, due to the introduced uncertainties from: urine normalization especially by creatinine (low sensitivity of method, excretion influenced by physiological parameters), and similar excretion pathways of biomarkers of exposure and biomarkers of effects.
- ii) Consequently, Cd in blood was pointed out as a better biomarker at low Cd levels, regardless of the hemoglobin status (the influence of hemoglobin status is presented in App. A.1. Table 1).
- iii) The usefulness of A1M, NAG, Alb and IgG as biomarkers of renal function is at their normal (low) physiological levels questionable, as they can also have a normal physiological role and thus respond to various factors unrelated to renal function. As shown on the case of A1M, which was recently reported to have an antioxidative role at physiological levels, its urine levels were significantly influenced by various factors – such as smoking, lead exposure and selenium status – pointing to its versatile biological function.

We mainly attributed the observed differences between groups of men and women to the gender issue, although they could also be a result of women's physiological and behavioral changes due to their lactation status (Moya et al., 2014). Moreover, because such changes vary during the process of lactation, we later additionally tested the studied associations for women for possible influence of sampling time, which is documented in the working report (App. A.1: Table 2). Sampling time (number of days after delivery) did not affect associations.

In the manuscript, we also pointed out the possible importance of individual genetic background on Cd levels variability and on its studied associations. Unfortunately, DNA material was not available for the population of the present manuscript. However, we checked the hypothesis in a Slovenian-Croatian pregnant and non-pregnant women; among SNPs in various *MT* genes and genes of *MTF1*, *DMT1*, *LRP2*, and *LRP8*, only one was found negatively associated with urine Cd levels (rs10636 of *MT2A*); results are documented as working report (App. A.2). In the same population we studied the influence of genetic background on the levels of As and its metabolites as presented in the next subchapter (Manuscript 2, Section 3.2).

Findings of the manuscript, related to urine biomarkers normalization, represented a good foundation for our next study conducted on the same population, in which reference values for various TE were determined in urine based on both creatinine and SG normalization (Snoj Tratnik et al., 2019).

I contributed to the present manuscript with the measurement of specific gravity in urine samples, statistical analyses, preparation of the tables and writing of the manuscript.





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## Low cadmium exposure in males and lactating females—estimation of biomarkers<sup>☆</sup>



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### ABSTRACT

**Background:** Urine cadmium (Cd) and renal function biomarkers, mostly analysed in urine spot samples, are well established biomarkers of occupational exposure. Their use and associations at low environmental level are common, but have recently been questioned, particularly in terms of physiological variability and normalisation bias in the case of urine spot samples.

**Aim:** To determine the appropriateness of spot urine and/or blood Cd exposure biomarkers and their relationships with renal function biomarkers at low levels of exposure. To this end, we used data from Slovenian human biomonitoring program involving 1081 Slovenians (548 males, mean age 31 years; 533 lactating females, mean age 29 years; 2007–2015) who have not been exposed to Cd occupationally.

**Results:** Geometric means (GMs) of Cd in blood and spot urine samples were 0.27 ng/mL (0.28 for males and 0.33 for females) and 0.19 ng/mL (0.21 for males and 0.17 for females), respectively. Differing results were obtained when contrasting normalisation by urine creatinine with specific gravity. GMs of urine albumin (Alb), alpha-1-microglobulin (A1M), N-acetyl-beta-glucosaminidase (NAG), and immunoglobulin G (IgG) were far below their upper reference limits. Statistical analysis of unnormalised or normalised urine data often yielded inconsistent and conflicting results (or trends), so association analyses with unnormalised data were taken as more valid. Relatively weak positive associations were observed between urine Cd (ng/mL) and blood Cd ( $\beta=0.11$ ,  $p=0.002$  for males and  $\beta=0.33$ ,  $p<0.001$  for females) and for females between urine NAG and blood Cd ( $\beta=0.14$ ,  $p=0.04$ ). No associations were found between other renal function biomarkers and blood Cd. Associations between Cd and renal function biomarkers in urine were stronger ( $p<0.05$ ,  $\beta=0.11–0.63$ ). Mostly, all of the associations stayed significant but weakened after normalisation for diuresis. In the case of A1M, its associations with Cd were influenced by current smoking and blood Pb in males and by pre-pregnancy smoking and blood Se in females ( $\beta$  up to 0.34,  $p<0.001$ ). Statistical analysis of unnormalised or normalised urine data often yielded inconsistent and conflicting results (or trends), so association analyses data with unnormalised were taken as more valid.

**Conclusions:** The observed uncertainties introduced by urine normalisation, particularly by creatinine, confirm blood Cd as a superior low-Cd exposure biomarker versus urine Cd in cases when 24 h urine is unattainable. Evidence that A1M can be positively related to Cd, smoking (current or pre-pregnancy), Pb, and Se status, points to the versatile biological functions of A1M.

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## 1. Introduction

Cadmium (Cd) is a non-essential toxic metal that occurs in the environment naturally and as a pollutant from industrial and agricultural activities. Lifelong human exposure in the general population occurs primarily through diet, water, and/or tobacco smoke. Cd accumulates primarily in the kidneys, with a long biological half-life (> 10 years), as well as in the liver, pancreas, bones, and lungs in case of smokers (Nordberg et al., 2012, 2015). Gastrointestinal (GI) absorption from dietary exposure is 3–5% and that from inhalation 10–50%. GI absorption is related to iron status and is generally higher in females than in males. Kidney Cd is often stated as Cd body burden, and the kidneys are the major target for Cd toxicity, especially epithelial cells of proximal tubules in the cortex (Åkerström, 2014; Nordberg et al., 2015).

Environmental Cd exposure in the general human population is primarily estimated by Cd levels in urine (U-Cd) and blood (B-Cd). Typically, B-Cd is an indicator of Cd exposure in recent months, given the short half-life of Cd in blood (2–3 months), whereas U-Cd better reflects Cd body burden (especially kidney deposits) accumulated and retained over the long term—of course, in the absence of renal damage (Agency for Toxic Substances and Disease Registry (ATSDR), 2012; Nordberg et al., 2015). However, for low levels of environmental exposure, the observed low levels of Cd in urine as well as those in blood could reflect both recent and past exposure (Åkerström et al., 2013a; Bernard, 2016), and the two types of exposure cannot be distinguished. For urine, the most reliable samples are daily cumulative samples (24-h urine), but owing to challenging collection, they are most often substituted by spot samples, especially in large human biomonitoring (HBM) studies. For inter-individual comparability, spot urine levels are regularly normalised for variations in diuresis, most often by creatinine (Crea, method regularly available in clinical labs), but also by specific gravity (SG), although neither route fully adjusts against variations in diuresis (Hoet et al., 2016; Santonen et al., 2015; Weaver et al., 2016).

'Normal' or reference Cd levels in urine are generally below 1 µg/g Crea, although they vary with age, area, and smoking habits. Blood reference levels can differ more drastically, especially due to smoking habits; general levels in non-smokers are 0.3–1.2 ng/mL, while those in smokers and heavy smokers are 0.6–3.9 ng/mL and up to 7 ng/mL, respectively (Nordberg et al., 2015).

Notably, urine Cd is a more specific measure of Cd exposure than other markers of renal function. However, Cd risk assessment in humans continues to rely on the relationship between urinary Cd and renal function biomarkers, tubular or glomerular (Bernard, 2016). A widely accepted indicator of the adverse effect of Cd on the kidneys is increased urinary excretion of low molecular weight proteins (LMWP, < 40 kDa) such as α<sub>1</sub>-microglobulin (A1M, also called protein HC, 31 kDa), β<sub>2</sub>-microglobulin (B2M, 11.8 kDa), metallothionein (MT, < 10 kDa), and retinol-binding protein (RBP, 22 kDa), and of enzymes such as N-acetyl-β-glucosaminidase (NAG, 130–140 kDa, particularly isoenzyme B). If they exceed their respective critical levels, the above-mentioned substances serve as biomarkers of impaired reabsorption of proximal tubules. Levels of high-molecular-weight protein (HMWP, > 40 kDa) such as albumin (66 kDa) and immunoglobulin G (IgG, 150 kDa) in urine primarily serve as biomarkers of glomerular damage (Delaney et al., 2012; Barregard and Elinder, 2015).

Current health risk assessments based on U-Cd and renal function biomarkers association have shown adverse effects of Cd when urinary levels of it exceed 4 µg/g Crea, which is rarely seen in the general population (Nordberg et al., 2015; Bernard, 2016). At lower levels, particularly below 1 µg/g Crea, the meanings of observed associations are much less clear, as explained below.

Various studies have reported the possibility of early kidney effects induced by Cd at lower exposure levels (U-Cd < 1 µg/g Crea) within the general population. These reports were based on the results of positive

associations between U-Cd and various urine renal function biomarkers (Bernard et al., 1995; Yamanaka et al., 1998; Jarup et al., 2000). More recently, the causality of these associations has been seriously questioned, sometimes by the same authors, but most thoroughly and convincingly by Bernard (2016). Clearly, both the level of U-Cd and renal function biomarkers in urine could be confounded by physiological sources of variability, by smoking, or silent pathological processes with no etiologic relationship with Cd (Nordberg et al., 2015; Bernard, 2016; Byber et al., 2016). Cd in blood plasma, mostly bound to low-molecular-weight MTs, but also to albumin, follows the same urinary excretion pathways (glomerular filtration and proximal tubule reabsorption) as do other plasma proteins that are used as renal function biomarkers. Therefore, variations in kidney reabsorption function owing to normal physiological processes or owing to renal damage caused by other malfunctions (mostly unidentified cardiovascular diseases and diabetes) can lead to co-excretion of protein-bound Cd (Cd-MT, Cd-Alb) and renal function biomarkers, resulting in their positive correlations, regardless of Cd exposure. Clearly, smokers can have higher U-Cd and B-Cd levels than non-smokers, but "if smoking causes proteinuria independently of Cd", then it may act as a confounder (Nordberg et al., 2015).

Additional uncertainties could be (are) introduced by normalisation of differences in urine concentration with creatinine (Crea) and/or specific gravity (SG); problems with 'incomplete corrections' and doubts pertaining to value for systematic creatinine normalisation in environmental and clinical settings are important limitations (Bernard, 2016; Hoet et al., 2016; Nordberg et al., 2015; Tang et al., 2015; Weaver et al., 2016; Zhang et al., 2015). Consequently, normalisation problems contribute to uncertainties in the association between U-Cd or B-Cd and urine renal function biomarkers, especially at levels lower than 1 or 0.5 µg/g Crea (Nordberg et al., 2015). To overcome all of the above-mentioned issues, B-Cd levels and their association to urine Cd and renal function biomarkers in blood and urine should be studied as well (Barregard and Elinder, 2015; Byber et al., 2016; Weaver et al., 2016).

The aim of present study was to assess the correlations among various biomarkers, such as B-Cd and U-Cd exposure biomarkers and four renal proteins representing renal function biomarkers (Albumin, A1M, NAG and IgG) in a Slovenian study sample comprising 533 lactating females and 548 males, aged 18–49 years and with no known occupational exposure to Cd. The focus was to identify factors that might influence these associations to estimate the appropriateness and reliability of U-Cd and/or B-Cd as biomarker(s) of choice for low levels of environmental exposure.

## 2. Materials and methods

### 2.1. Study population

For the present study, we used data on 1081 occupationally non-exposed individuals aged 18–49 years, including 533 primiparous lactating females and 548 males, who participated in a wider Slovenian Human Biomonitoring survey (HBM, 2007–2015), recruited from 12 geographically different regions covering rural, urban, and industrialized areas. All participants filled out a questionnaire covering their socio-economic status, medical history, lifestyle, and nutritional habits. Some basic personal data of the study population, stratified by gender, are summarized in Table 1. Regarding smoking, the data on current smoking and pre-pregnancy smoking are provided. Females quitting smoking due to pregnancy were classified as 'pre-pregnancy' smokers. The questionnaire did not provide any information about the life-time status of former smoking, and we could only identify current smokers and women who had stopped smoking at the beginning of pregnancy.

There was no report of diabetes or renal disease among the participants of the present study. The study population had relatively



**Table 1**  
Levels of biomarkers in studied population stratified by gender.

|                                               | All                    |                  |                                 | Male                   |                  |                                 | Female                 |                  |                                 | Male vs female p-value                     |
|-----------------------------------------------|------------------------|------------------|---------------------------------|------------------------|------------------|---------------------------------|------------------------|------------------|---------------------------------|--------------------------------------------|
| Participants N                                | 1081                   |                  |                                 | 548 (51%)              |                  |                                 | 533 (49%)              |                  |                                 |                                            |
| Smokers N                                     | 53 (5%)                |                  |                                 | 47 (9%)                |                  |                                 | 6 (1%)                 |                  |                                 |                                            |
| Former smokers N                              | 61 (6%)                |                  |                                 | ND                     |                  |                                 | 61 (11%)               |                  |                                 |                                            |
| Age, x <sup>a</sup> (min-max)                 | 30 (18–49)             |                  |                                 | 31 (18–49)             |                  |                                 | 29 (19–39)             |                  |                                 | <0.001                                     |
| BMI, kg/m <sup>2</sup> ± SD (min-max)         | 24.6 ± 4.0 (16.6–44.8) |                  |                                 | 26.0 ± 3.4 (17.5–37.6) |                  |                                 | 23.2 ± 4.0 (16.6–44.8) |                  |                                 | <0.001                                     |
| eGFR <sup>b</sup> mL/min/1.73m <sup>2</sup> N | 485 (47%)              |                  |                                 | 228 (45%)              |                  |                                 | 257 (50%)              |                  |                                 |                                            |
| eGFR > 60                                     | 948 (93%)              |                  |                                 | 507 (90%)              |                  |                                 | 491 (95%)              |                  |                                 |                                            |
| BIOMARKER                                     | N                      | GM (P25–P75)     | MEDIAN (min-max)                | N                      | GM (P25–P75)     | MEDIAN (min-max)                | N                      | GM (P25–P75)     | MEDIAN (min-max)                |                                            |
| B-Cd (ng/g)                                   | 1075                   | 0.27 (0.14–0.44) | 0.28 (0.10 <sup>a</sup> –0.45)  | 546                    | 0.22 (0.10–0.33) | 0.21 (0.10 <sup>a</sup> –0.45)  | 529                    | 0.33 (0.25–0.49) | 0.34 (0.10 <sup>a</sup> –0.49)  | <1 <sup>a</sup> / > 4 <sup>b</sup> 13 2/-  |
| B-Hg (ng/g)                                   | 1075                   | 1.12 (0.67–1.98) | 1.15 (0.10 <sup>a</sup> –29.3)  | 546                    | 1.18 (0.67–2.10) | 1.21 (0.10 <sup>a</sup> –29.3)  | 529                    | 1.06 (0.66–1.82) | 1.06 (0.10 <sup>a</sup> –9.65)  | <5 / > 15 <sup>b</sup> 0.6 2.5/-           |
| B-As (ng/g)                                   | 1076                   | 0.85 (0.49–1.22) | 0.73 (0.05 <sup>a</sup> –27.4)  | 546                    | 0.79 (0.48–1.07) | 0.69 (0.05 <sup>a</sup> –21.2)  | 530                    | 0.91 (0.50–1.45) | 0.77 (0.19–27.4)                | 0.2 0.002                                  |
| B-Pb (ng/g)                                   | 1076                   | 17.1 (12.4–22.7) | 16.6 (3.65–109)                 | 546                    | 17.7 (13.0–24.6) | 17.7 (3.65–109)                 | 530                    | 15.9 (12.3–21.1) | 15.6 (4.04–68.3)                | <5 0 <sup>b</sup> – 0.9 <0.001             |
| B-Se (ng/g)                                   | 1076                   | 99.3 (86.0–112)  | 99 (51.2–214)                   | 546                    | 109 (86.0–122)   | 107 (5.07–214)                  | 530                    | 94.0 (79.5–102)  | 90.0 (51.2–167.1)               | 58 <sup>a</sup> – 234 <sup>b</sup> <0.001  |
| U-Cd (ng/mL)                                  | 994                    | 0.19 (0.12–0.31) | 0.20 (0.015 <sup>a</sup> –3.83) | 504                    | 0.21 (0.13–0.32) | 0.22 (0.015 <sup>a</sup> –1.75) | 490                    | 0.17 (0.10–0.30) | 0.17 (0.015 <sup>a</sup> –3.83) | <0.001                                     |
| U-Cd/Crea (μg/g)                              | 981                    | 0.20 (0.13–0.30) | 0.19 (0.01–2.79)                | 498                    | 0.16 (0.11–0.22) | 0.15 (0.03–1.14)                | 483                    | 0.26 (0.17–0.39) | 0.25 (0.01–2.79)                | <1 <sup>a</sup> / > 4 <sup>b</sup> 2 2.5/- |
| U-Cd/SG (ng/mL)                               | 943                    | 0.21 (0.15–0.32) | 0.20 (0.03–3.65)                | 477                    | 0.22 (0.11–0.32) | 0.22 (0.03–1.11)                | 466                    | 0.21 (0.14–0.31) | 0.19 (0.04–3.65)                | 0.153                                      |
| U-IgG (mg/L)                                  | 857                    | 2.66 (1.70–3.90) | 1.84 (1.70 <sup>a</sup> –363)   | 421                    | 2.52 (1.70–3.80) | 1.84 (1.70 <sup>a</sup> –88.1)  | 436                    | 2.80 (1.70–4.00) | 1.84 (1.70 <sup>a</sup> –363)   | 0.293 <sup>c</sup>                         |
| U-IgG/Crea (mg/g)                             | 856                    | 2.73 (1.40–4.50) | 2.52 (0.29–5345)                | 421                    | 1.84 (1.11–2.73) | 1.72 (0.53–145)                 | 435                    | 3.99 (3.35–6.43) | 3.69 (0.28–535)                 | <0.001 <sup>c</sup>                        |
| U-IgG/SG (mg/L)                               | 821                    | 2.93 (1.66–4.43) | 2.66 (0.50–472)                 | 403                    | 2.63 (1.60–4.00) | 2.31 (0.50–152.2)               | 418                    | 3.26 (1.84–5.20) | 2.94 (0.82–472)                 | <0.001 <sup>c</sup>                        |
| U-Alb (mg/L) <sup>***</sup>                   | 981                    | 6.87 (3.50–11.9) | 6.40 (1.06 <sup>a</sup> –2190)  | 499                    | 6.80 (3.90–11.4) | 6.5 (1.06 <sup>a</sup> –1090)   | 482                    | 6.91 (3.20–12.9) | 6.15 (1.06 <sup>a</sup> –2190)  | 0.784                                      |
| U-Alb/Crea (mg/g)                             | 981                    | 7.06 (3.68–10.6) | 7.00 (0.18–3227)                | 499                    | 5.05 (3.19–6.47) | 4.30 (0.50–1791)                | 482                    | 10.0 (5.00–16.4) | 8.20 (0.18–3227)                | <0.001                                     |
| U-Alb/SG (mg/L)                               | 914                    | 7.68 (4.33–11.7) | 6.29 (0.66–2847)                | 466                    | 7.17 (4.37–10.1) | 5.96 (0.81–1883)                | 448                    | 8.24 (4.29–13.8) | 6.76 (0.66–2847)                | 0.029                                      |
| U-AIM (mg/L)                                  | 824                    | 4.13 (2.76–6.40) | 2.97 (2.62 <sup>a</sup> –116.1) | 410                    | 5.11 (2.90–8.32) | 3.89 (2.62 <sup>a</sup> –49.5)  | 414                    | 3.94 (2.76–5.97) | 2.76 (2.62 <sup>a</sup> –116.1) | <0.001 <sup>c</sup>                        |
| U-AIM/Crea (mg/g)                             | 824                    | 4.22 (2.57–6.75) | 4.13 (0.47–60.1)                | 410                    | 3.67 (2.31–5.80) | 3.73 (0.82–60.1)                | 414                    | 4.81 (2.88–7.76) | 4.64 (0.47–38.1)                | <0.001 <sup>c</sup>                        |
| U-AIM/SG (mg/L)                               | 793                    | 4.54 (2.76–7.05) | 4.43 (0.82–62.7)                | 391                    | 5.26 (3.21–8.00) | 5.38 (0.82–62.7)                | 402                    | 3.93 (2.39–5.98) | 3.79 (1.28–45.7)                | <0.001 <sup>c</sup>                        |
| U-NAG (nkat/L)                                | 926                    | 26.9 (16.0–50.0) | 31.0 (2.00 <sup>a</sup> –297)   | 482                    | 30.7 (19.0–55.0) | 39.0 (2.00 <sup>a</sup> –296)   | 444                    | 23.5 (12.0–42.0) | 24.0 (2.00 <sup>a</sup> –204)   | <0.001                                     |
| U-NAG/Crea (nkat /g)                          | 916                    | 26.9 (17.4–43.6) | 28.4 (0.17–295)                 | 478                    | 22.0 (15.5–34.0) | 23.9 (1.15–283.0)               | 438                    | 33.6 (21.4–58.0) | 36.0 (0.17–295)                 | <0.001                                     |
| U-NAG/SG (nkat/L)                             | 855                    | 29.7 (19.0–48.6) | 32.8 (0.68–293)                 | 448                    | 31.9 (21.3–49.4) | 36.9 (1.72–234.3)               | 407                    | 27.5 (17.3–46.5) | 29.7 (0.68–293)                 | 0.006                                      |
| N                                             |                        | GM (P25–P75)     | MEDIAN                          | N                      | GM (P25–P75)     | MEDIAN                          | N                      | GM (P25–P75)     | MEDIAN                          |                                            |
|                                               |                        |                  |                                 |                        |                  |                                 |                        |                  |                                 | < RV > RV %                                |
|                                               |                        |                  |                                 |                        |                  |                                 |                        |                  |                                 | < RV > RV %                                |

(continued on next page)

Table 1 (continued)

|              | All  | Male                |                     | Female |                     | Male vs female p-value |                |                     |                     |             |      |     |        |
|--------------|------|---------------------|---------------------|--------|---------------------|------------------------|----------------|---------------------|---------------------|-------------|------|-----|--------|
|              |      | P75                 | (min-max)           | P75    | (min-max)           | %                      | female p-value |                     |                     |             |      |     |        |
| U-Crea (g/L) | 1016 | 0.95 (0.90–0.99)    | 1.06 (0.09–7.09)    | 512    | 1.34 (0.92–2.10)    | 5.5                    | 504            | 0.67 (0.40–1.20)    | 0.70 (0.09–1.33)    | 0.30–3.0    | 16.3 | 1.2 | <0.001 |
| U-SG         | 943  | 1.016 (1.016–1.017) | 1.017 (1.002–1.035) | 477    | 1.019 (1.003–1.035) | 3.1                    | 466            | 1.013 (1.007–1.018) | 1.012 (1.002–1.033) | 1.010–1.030 | 39   | 0.4 | <0.001 |

BMI—body mass index; ND—not determined; y—years; N—number; GM—geometric mean; P25–25 percentile, P75–75 percentile.

\* Pre-pregnancy smoking; eGFR—estimated glomerular filtration rate.

<sup>a</sup> LOD/2; RV—reference value; URI—upper reference limit.

<sup>\*\*</sup> In the case of B-Se, 0.2% males and 0.9% females were below RV, and in the case of NAG, 19.5% males and 11.6% females were below URI.

<sup>\*\*\*</sup> Percentages of participants with Alb according to thresholds for "macroalbuminuria", males: > 200 mg/L=0.8%, > 300 mg/g Crea=0.8%; females: > 200 mg/L=1.9%, > 300 mg/g Crea =1.2% (Levey et al., 2005).

<sup>a</sup> (Barregard and Elinder, 2015; Committee on Obstetric Practice, 2013; Iwenger, 1998; Roberts et al., 2012; Schulz et al., 2011; Wilhelm et al., 2004).

<sup>b</sup> (Delaney et al., 2012; Kang et al., 2015; Levey et al., 2005).

p-values in case of A1M and IgG were determined by non-parametric test and for other biomarkers with parametric tests.

BMI—body mass index; ND—not determined; y—years; N—number; GM—geometric mean; P25–25 percentile, P75–75 percentile.

\* Pre-pregnancy smoking; eGFR—estimated glomerular filtration rate.

<sup>a</sup> LOD/2; RV—reference value; URI—upper reference limit.

<sup>b</sup> In the case of B-Se, 0.2% males and 0.9% females were below RV, and in the case of NAG, 19.5% males and 11.6% females were below URI.

<sup>c</sup> Percentages of participants with Alb according to thresholds for 'macroalbuminuria', males: > 200 mg/L=0.8%, > 300 mg/L=0.8%; females: > 200 mg/L=1.9%, > 300 mg/L=1.2% (Levey et al., 2005).

<sup>d</sup> (Barregard and Ellinder, 2015; Committee on Obstetric Practice, 2013; Iwenger, 1998; Roberts et al., 2012; Schulz et al., 2011; Wilhelm et al., 2004).

<sup>e</sup> (Delaney et al., 2012; Kang et al., 2015; Levey et al., 2005).

<sup>f</sup> p-values in case of AIM and IgG were determined by non-parametric test and for other biomarkers with parametric tests.

normal kidney function because 93% individuals had eGFR higher than 60 mL/min/1.73 m<sup>2</sup>. Twelve participants reported having cardiovascular disease. All participants signed informed consent, and the research protocol of the study was approved by the National Medical Ethics Committee, Republic of Slovenia (NMEC, number of accordance: 42/12/07 and 53/07/09). Detailed description of study population, study protocol and general results are summarized in IJS working report (IJS-DP-11985, 2015).

## 2.2. Samples and sample collection

All participants had an appointed visit in a regional health centre, where they delivered spot urine samples (~50 mL) in provided urine collection vessels; at the same time, whole blood samples (~16 mL for women, ~23 mL for men) were taken by trained personnel according to standard procedures. In total, 1045 urine samples and 1076 blood samples were obtained. The samples were partially prepared and aliquoted according to the protocol and stored at 2–8 °C or –20 °C until further analysis. Samples from females were collected in the second month after delivery.

## 2.3. Metals in urine and blood

Chemical analysis of metals was performed in 0.3 mL and 1 mL aliquots for blood and urine, respectively. Cd, As, Pb, and Se were analysed with an Octopole Reaction System (ORS) Inductively Coupled Plasma Mass Spectrometry (ICP-MS; 7500ce, Agilent Technologies) equipped with an ASX-510 autosampler (Cetac), as described in the literature (Miklavčič et al., 2013). The limits of detection (LOD)—taken as three times the standard deviation of the blank sample—of Cd, As, Pb, and Se in blood were 0.2, 0.1, 0.4, and 8 ng/g, respectively, and that for Cd in urine was 0.03 ng/mL. The concentration of total Hg in blood was determined by a direct mercury analyser DMA-80 (Milestone Srl, Italy) with a LOD of 0.2 ng/g. For analytical quality control, reagents blanks, and urine and blood reference materials were prepared and measured in the same way as the samples were. The reference materials used were ClinCheck® Urine Control (Lot 923, Recipe) and Seronorm trace elements whole blood L-1 (Lot. MR4206, Sero).

## 2.4. Urine renal proteins: IgG, Alb, A1M, and NAG

Urine renal proteins (IgG, Alb, A1M and NAG) were determined in spot urine samples stored at 2–8 °C within 24 h of collection. Albumin, IgG, and A1M proteins were measured with the standard nephelometric immunochemical method by using a BN ProSpec system (Siemens, USA). LODs varied based on the standard used, and they were 3.39–3.82 mg/L for IgG, 2.2 mg/L for Alb, and 5.2–5.7 mg/L for A1M. NAG enzyme activity was determined with the standard calorimetric method by using an Olympus AU400 chemistry analyser (Olympus, Japan) and LOD of 4.2 nkat/L.

## 2.5. Urine creatinine and specific gravity

Crea was determined with the standard Jaffe method (Larsen, 1972), rate blanked and compensated, using biochemical reagents (Catalog number 11875418) for Hitachi Roche Modular Analytices P-800. Analytical sensitivity or LOD was 0.2 mg/dL (=0.2 µg/mL) and measuring range 0.2–650 mg/dL. SG was determined by using a PAL-10S refractometer (Atago®, Japan), and the measurement range was 1.000–1.060. Both parameters, Crea and SG, were used to normalise the urine levels of biomarkers for differences in diuresis between samples. As a standard in SG normalisation calculations, SG means of 1.013 for females and 1.019 for males were used; calculation is described in the literature (Suwazono et al., 2005).



### 2.6. Statistical analysis

Values at or below the LOD were substituted with a value of LOD/2. Analyte concentrations were expressed in term of geometric mean with 25% and 75% quartile (GM, P25–P75) or median with minimum and maximum (min–max). All statistical analyses were performed on ln-transformed data to approximate normal distribution for males and females separately. We used Pearson's correlation coefficients ( $r$ ) to assess the correlations between single variables and analysis of variance (ANOVA) to examine differences between groups. In case of A1M and IgG the normal distribution was not achieved by ln-transformation therefore non-parametric tests of Spearman's rank correlation ( $r_s$ ) and Kruskal-Wallis test were used. Associations between renal proteins and Cd exposure, using B-Cd or U-Cd, were also assessed (adjusted) by multiple linear regression. Each renal protein and its association to U-Cd or B-Cd were tested using a separate model. The possible confounders tested were age, BMI, smoking status, B-Se, B-Hg, B-As, and B-Pb. Those associated significantly ( $p < 0.250$ ) with renal proteins were then included in the regression models. The level of statistical significance was set at  $p < 0.05$ , two sided. Statistical analyses were performed using STATA12/SE software and Microsoft Office Excel, 2013.

Urine-related data were used and represented in all three forms: unnormalised for diuresis, Crea-normalised, and SG-normalised. Extreme levels of Crea ( $< 0.3$  g/L and  $> 3.0$  g/L) and SG ( $< 1.010$  and  $> 1.030$ ), normally considered as very diluted or concentrated (Santonen et al., 2015), were not excluded. The results were tested additionally using a set of samples from which such cases were excluded.

### 3. Results

The main intention of Slovenian HBM was to assess the metal burden for population at their child-bearing age and possible treat for their newborns. Hence the lactating postpartum women and men (not necessary their partners) were chosen. Basic characteristics of the study population stratified by gender are summarized in Table 1.

Average age of the participants was 29 and 31 years, and average body mass index (BMI) was  $23.2 \text{ kg/m}^2$  and  $26.0 \text{ kg/m}^2$  for females ( $n=533$ ) and males ( $n=548$ ), respectively. From the responses to the questionnaire, we identified 6 current (1%) and 61 pre-pregnancy smokers among females (quit smoking ~11months ago, 11%) and 47 current smokers (9%) among males.

Table 1 lists biomarker concentration data (B-Cd, U-Cd, urine IgG, Alb, A1M, and NAG), along with basic data related to their reference values and upper reference limits. For blood, selenium levels are added, representing rough estimates of selenium status important for blood antioxidant capacity (Tinggi, 2008), in addition to a few other trace elements (Hg, As, and Pb) that could also influence kidney function. For urine, levels of Crea and SG are added as parameters used for normalisation of urine data. All urine-related parameters are consistently presented by three types of values, in raw and normalised forms by Crea and SG; in such 'triple form' are used in most of the tables and commented throughout the text. The geometric means (GMs) of both Crea and SG were found to be significantly ( $p < 0.001$ ) higher among males than in females:  $1.39 \text{ g/L}$  vs.  $0.67 \text{ g/L}$  for Crea and  $1.019$  vs.  $1.013$  for SG. Obviously, the difference between genders is lower for SG than it is for Crea. The percentage of samples within the 'normal' range for Crea (inside  $0.3 \text{ g/L}$ – $3.0 \text{ g/L}$ ) was higher than 80% (92% males, 83% female), while for SG (inside  $1.010$ – $1.030$ ), the value was similar for males and much lower for females (83% males, 61% females). Such a large difference can be attributed to the one month post-partum status of females, which is known to influence excretion of creatinine and other protein biomarkers (Capodicasa et al., 2011; Ashwood et al., 2012).

Cd geometric means (GMs) were very low:  $0.22 \text{ ng/g}$  and  $0.33 \text{ ng/g}$

in blood and  $0.16 \text{ } \mu\text{g/g}$  Crea and  $0.26 \text{ } \mu\text{g/g}$  Crea in urine for males and females, respectively. Both B-Cd and U-Cd/Crea levels were significantly higher in females than in males ( $p < 0.001$ ). Normalisation by SG diminished or even abolished gender differences in the case of U-Cd, while unnormalised data showed the opposite trend. In terms of arithmetic means, the situation is slightly different again. In any case, Cd was detectable in a greater proportion of female blood samples than in male blood samples (87% vs. 58.2%). For both genders, individual Cd levels did not exceed  $4.6 \text{ ng/g}$  in blood or  $3 \text{ } \mu\text{g/g}$  Crea in urine.

GMs of urine protein and renal proteins normalised by Crea, namely, IgG, Alb, A1M, and NAG, were  $1.84 \text{ mg/g}$  Crea,  $5.05 \text{ mg/g}$  Crea,  $3.67 \text{ mg/g}$  Crea, and  $22 \text{ nkat/g}$  Crea in males and  $3.99 \text{ mg/g}$  Crea,  $10.0 \text{ mg/g}$  Crea,  $4.81 \text{ mg/g}$  Crea, and  $33.6 \text{ nkat/g}$  Crea in females, respectively. The observed levels are higher in females, but still far below their upper reference limits (twice lower or more), as can be inferred from Table 1. In approximately half of the samples, IgG and A1M were even undetectable. Although Crea normalisation yielded significantly higher levels of all four proteins in females, normalisation by SG reversed the differences between female and male samples for A1M and NAG. SG normalised differences correspond with unnormalised relation. The proportions of participants with IgG, Alb, A1M, and NAG levels higher than their respective upper reference limits (mg/g Crea) were higher in females (9%, 12%, 16%, and 13%, respectively) than those in males (1%, 2%, 8% and 2.5%, respectively).

The levels of other protective (Se) or possibly detrimental trace elements (Hg, As, and Pb) in blood were also in the 'normal' ranges.

#### 3.1. Biomarkers in relation to smoking status

As postpartum females are mostly non-smokers the initial tendency of HBM was to recruit non-smoking males only. Recruited smokers are exception representing female's partners with special wish to be included. We decide to preserve them in the study since their data provide interesting results as is evident from Table 2.

Table 2 lists data for females and males stratified according to their smoking status. Females were divided as pre-pregnancy smokers and non-smokers. Six self-declared female current smokers were excluded because the number was too small to allow statistical analysis. Males were divided as current smokers and current non-smokers. We had no data about lifetime previous smoking for either group. As expected, blood and urine Cd levels were significantly higher in current smokers than in declared non-smokers ( $0.91 \text{ ng/g}$  versus  $0.19 \text{ ng/g}$  for blood and  $0.20 \text{ } \mu\text{g/g}$  Crea versus  $0.15 \text{ } \mu\text{g/g}$  Crea for urine). The SG-normalised U-Cd levels indicated the same trend, but with lower significance ( $p=0.054$ ), while there was no difference in unnormalised data. Undoubtedly, the influence of current smoking was stronger on blood levels than on urine levels. Among females, B-Cd levels were slightly higher in pre-pregnancy smokers ( $0.37 \text{ ng/g}$ ) than in non-smokers ( $0.32 \text{ ng/g}$ ), with only marginal significance ( $p=0.104$ ), while urine levels were unaffected.

Crea-normalised urine renal proteins showed significant increase in A1M owing to current smoking in males. The observed levels among current smokers ( $5.66 \text{ mg/g}$  Crea) were convincingly higher than those among non-smokers ( $3.53 \text{ mg/g}$  Crea). SG-normalised and unnormalised data showed significant increases in U-A1M as well ( $p < 0.05$ ). A1M levels increased slightly in females who smoked before pregnancy.

The exclusion of very diluted or concentrated urine samples (Crea levels  $< 0.3$  and  $> 3.0 \text{ g/L}$ ; SG  $< 1.010$  and  $> 1.030$ ) did not affect the overall results (data not shown).

#### 3.2. Associations between biomarkers

Table 3 presents correlations ( $r$  or  $r_s$ ) related to biomarkers and personal parameters such as age and BMI for males (Table 3A) and females (Table 3B). They were obtained by Pearson's or Spearman's correlation analysis of ln-transformed concentration data. Correlations



**Table 2**  
Cd and renal protein levels stratified by smoking<sup>a</sup> status.

| Biomarker           | Male            |                     |             |                     |                          | Female                |                     |             |                     |                    |
|---------------------|-----------------|---------------------|-------------|---------------------|--------------------------|-----------------------|---------------------|-------------|---------------------|--------------------|
|                     | Current smokers |                     | Non-smokers |                     | p-value                  | Pre-pregnancy smokers |                     | Non-smokers |                     | p-value            |
|                     | N               | GM (P25–P75)        | N           | GM (P25–P75)        |                          | N                     | GM (P25–P75)        | N           | GM (P25–P75)        |                    |
| B-Cd (ng/g)         | 46              | 0.91 (0.74–1.53)    | 491         | 0.19 (0.10–0.29)    | < 0.001                  | 60                    | 0.37 (0.26–0.52)    | 449         | 0.32 (0.24–0.47)    | 0.104              |
| B-Hg (ng/g)         | 46              | 0.89 (0.48–1.63)    | 491         | 1.21 (0.67–2.12)    | <b>0.020</b>             | 60                    | 1.08 (0.67–1.84)    | 449         | 1.06 (0.67–1.82)    | 0.899              |
| B-As (ng/g)         | 46              | 0.65 (0.41–0.77)    | 491         | 0.80 (0.50–1.11)    | <b>0.050</b>             | 60                    | 0.96 (0.49–1.57)    | 449         | 0.91 (0.50–1.49)    | 0.699              |
| B-Pb (ng/g)         | 46              | 20.6 (15.4–28.4)    | 491         | 18.0 (12.7–24.2)    | 0.076                    | 60                    | 17.5 (12.3–24.1)    | 449         | 15.7 (12.1–20.9)    | 0.063              |
| B-Se (ng/g)         | 46              | 107.9 (93.7–120)    | 491         | 109.3 (96.2–122)    | 0.683                    | 60                    | 88.1 (76.3–101)     | 449         | 90.3 (79.5–102)     | 0.330              |
| U-Cd (ng/mL)        | 40              | 0.23 (0.14–0.36)    | 457         | 0.21 (0.13–0.32)    | 0.304                    | 59                    | 0.18 (0.10–0.31)    | 413         | 0.17 (0.10–0.29)    | 0.520              |
| U-Cd/Crea (μg/g)    | 40              | 0.20 (0.14–0.31)    | 451         | 0.15 (0.11–0.21)    | <b>0.002</b>             | 59                    | 0.26 (0.16–0.42)    | 407         | 0.26 (0.18–0.40)    | 0.851              |
| U-Cd/SG (ng/mL)     | 33              | 0.27 (0.18–0.36)    | 437         | 0.22 (0.15–0.31)    | <b>0.054</b>             | 53                    | 0.21 (0.12–0.37)    | 396         | 0.21 (0.14–0.31)    | 0.846              |
| U-IgG (mg/L)        | 28              | 2.77 (1.70–3.67)    | 388         | 2.51 (1.70–3.80)    | 0.793 <sup>b</sup>       | 49                    | 3.06 (1.70–5.43)    | 368         | 2.73 (1.70–4.00)    | 0.372 <sup>b</sup> |
| U-IgG/Crea (mg/g)   | 28              | 2.42 (1.26–3.74)    | 388         | 1.81 (1.09–2.68)    | 0.207 <sup>b</sup>       | 49                    | 4.22 (2.41–6.83)    | 368         | 3.95 (2.34–6.22)    | 0.672 <sup>b</sup> |
| U-IgG/SG (mg/L)     | 25              | 3.0 (1.66–3.93)     | 373         | 2.61 (1.57–4.00)    | 0.543 <sup>b</sup>       | 47                    | 3.45 (1.92–5.53)    | 355         | 3.22 (1.84–4.94)    | 0.694 <sup>b</sup> |
| U-Alb (mg/L)        | 44              | 5.58 (3.24–7.97)    | 447         | 6.97 (4.00–11.6)    | 0.148                    | 59                    | 6.63 (2.99–12.1)    | 404         | 6.84 (3.20–12.8)    | 0.856              |
| U-Alb/Crea (mg/g)   | 44              | 4.95 (3.32–5.85)    | 447         | 5.06 (3.19–6.52)    | 0.861                    | 59                    | 9.29 (4.73–15.5)    | 404         | 10.0 (5.00–17.1)    | 0.614              |
| U-Alb/SG (mg/L)     | 33              | 7.35 (4.40–11.0)    | 425         | 7.21 (4.37–10.2)    | 0.898                    | 52                    | 7.87 (3.98–12.2)    | 379         | 8.22 (4.25–14.3)    | 0.781              |
| U-AIM (mg/L)        | 35              | 6.99 (3.70–11.4)    | 371         | 4.97 (2.83–8.20)    | <b>0.011<sup>b</sup></b> | 47                    | 3.83 (2.76–4.17)    | 351         | 3.24 (2.76–2.97)    | 0.077 <sup>b</sup> |
| U-AIM/Crea (mg/g)   | 35              | 5.66 (3.39–9.83)    | 371         | 3.53 (2.23–5.45)    | < 0.001 <sup>b</sup>     | 47                    | 5.30 (3.03–9.04)    | 351         | 4.72 (2.83–7.27)    | 0.244 <sup>b</sup> |
| U-AIM/SG (mg/L)     | 29              | 7.48 (4.77–14.1)    | 358         | 5.10 (3.12–7.51)    | <b>0.003<sup>b</sup></b> | 46                    | 4.38 (2.56–7.18)    | 341         | 3.85 (2.39–5.68)    | 0.137 <sup>b</sup> |
| U-NAG (nkat/L)      | 39              | 27.9 (15.0–57.0)    | 437         | 30.9 (19.0–55.0)    | 0.445                    | 54                    | 26.1 (16.0–55.0)    | 375         | 22.5 (12.0–42.0)    | 0.231              |
| U-NAG/Crea (nkat/g) | 39              | 22.8 (17.1–41.7)    | 433         | 21.9 (15.3–33.2)    | 0.745                    | 54                    | 34.2 (18.0–61.9)    | 369         | 33.0 (21.0–57.2)    | 0.766              |
| U-NAG/SG (nkat/L)   | 31              | 32.7 (25.8–59.9)    | 411         | 31.9 (21.2–50.2)    | 0.717                    | 47                    | 29.3 (19.1–54.4)    | 345         | 26.8 (17.2–50.5)    | 0.496              |
| U-Crea (g/L)        | 45              | 1.15 (0.87–1.88)    | 459         | 1.36 (0.95–2.12)    | 0.084                    | 61                    | 0.73 (0.36–1.22)    | 424         | 0.65 (0.39–1.18)    | 0.292              |
| U-SG                | 33              | 1.018 (1.012–1.023) | 437         | 1.019 (1.015–1.025) | 0.378                    | 53                    | 1.013 (1.007–1.019) | 396         | 1.012 (1.007–1.018) | 0.510              |

N – number of samples; GM – geometric mean; P – percentile.

<sup>a</sup> Fifteen females and nine males which did not provide answers regarding their smoking status and 6 females smoking during pregnancy were excluded from analysis.

<sup>b</sup> p-values were in case of AIM and IgG assessed by non-parametric test and for other biomarkers by parametric test; statistically significant values are bolded.

between urine biomarkers and Cd in urine or blood were further assessed for possible confounders by linear regression models, which are described supplementary Tables S1 and S2A–D. The potential confounders were age, BMI, smoking status, and blood trace elements (Pb, Se, As, Hg), but not all in each case. Table 4 summarizes beta coefficients of all tested relationships. The same assessments performed on distinct set of data (blood Cd > 0.5 ng/g, renal proteins above LOD and URL) are commented in Supplement material.

### 3.2.1. Association between B-Cd and U-Cd

B-Cd and U-Cd (unnormalised and normalised by Crea or SG) were positively and significantly correlated, regardless of the type of urine data used ( $r=0.143$ ,  $0.221$ , and  $0.214$  for males;  $r=0.224$ ,  $0.226$  and  $0.279$  for females), but the strength of correlations was low ( $R^2 < 0.1$ ) (Table 4). After inclusion of the identified confounders, the associations persisted in all cases, and p-values ranged from < 0.001 to 0.002, while  $\beta$  values were 0.11, 0.14, and 0.17 for males, and 0.33, 0.22, and 0.29 for females, for unnormalised, Crea-normalised, and SG-normalised data, respectively (Table 4 and S1A).

As in the previous cases, the exclusion of very diluted or concentrated samples did not influence the overall results.

### 3.2.2. Association between renal proteins and U-Cd

U-Cd was correlated significantly with all four renal proteins, regardless of the gender and the type of urine data used (Table 3 A and B). Again, unnormalised and normalised urine data were used, although there is no need for normalisation when comparing variables from the same spot sample, because urine dilution has no impact on this relationship. The procedure may only be used as a control for normalisation.

As expected, the strongest correlations were found in the case of unnormalised urine data ( $p < 0.001$ ,  $r=0.252$ – $0.453$ ). After urine levels were normalised by Crea or SG, the overall correlations remained significant but with considerably lower correlation coefficients, especially for Alb. The exception was AIM in females, which lost its significance.

When renal proteins were adjusted for possible confounders, overall associations with U-Cd did not change ( $p < 0.001$ ,  $\beta=0.11$ – $0.63$ ) (Table 4 and S2A–D).

### 3.2.3. Association between renal proteins and B-Cd

The only significant correlation observed was that between B-Cd and AIM in males and B-Cd and NAG in females: AIM with  $r_s=0.085$ , 0.150, and 0.158 for unnormalised, Crea-normalised, and SG-normalised data, respectively ( $p < 0.05$ ), and NAG with  $r=0.104$ , for unnormalised data only ( $p < 0.05$ ) (Table 3). After adjustment for possible confounders, the association between B-Cd and NAG remained significant (unnormalised data only), while the association between B-Cd and AIM was no longer significant (Table 4 and S2A–D). The exclusion of very diluted or concentrated samples did not change the overall results considerably, but strength of correlations decreased moderately.

### 3.2.4. AIM and smoking as confounder

Table 5 lists selected associations in which smoking was one of the confounders and for which analyses were performed with unnormalised urine data (believed to be the most relevant based on difficulties in urine normalisation). Summarized are the beta coefficient values of smoking and the accompanying significant confounders.

## 4. Discussion

The obtained data revealed very low Cd exposure in a healthy Slovenian population consisting of 548 males and 533 lactating females (Table 1). The obtained GMs of U-Cd were 0.26  $\mu\text{g/g}$  Crea and 0.16  $\mu\text{g/g}$  Crea and those of B-Cd were 0.33 ng/g and 0.22 ng/g for females and males, respectively. The GMs of other potentially toxic metals measured in blood (Hg, As, and Pb) were low as well (Table 1). Less than 5% of the participants had Cd levels over 1 ng/mL (1  $\mu\text{g/g}$  Crea) in urine or 1 ng/g (ng/mL) in blood, the which are the values set as “normal” references for healthy occupationally non-exposed population (exactly 1.4% for urine and 4.8% for blood) (Schulz et al., 2011; Nordberg et al., 2015). No participant had U-Cd levels higher than

**Table 3**  
Correlations ( $r/r_s$ )<sup>a</sup> among biomarkers and personal parameters of males (A) and females (B).

| Males A | Age                                                                              | BMI                                                                                       | B-Se                                                       | B-Pb                                                                                     | B-Hg                                                                                         | B-As                                                                                         | B-Cd                                                                                      | U-SG                                                                                        | U-Crea                                                                                      | U-NAG                                                                                     | U-A1M                                                                                     | U-Alb                                                                                     | U-IgG                                                                                     |
|---------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| U-Cd    | 0.077 <sup>*</sup><br><b>0.178</b> <sup>***</sup><br><b>0.145</b> <sup>***</sup> | 0.038<br>0.017<br>0.032                                                                   | <b>0.103</b> <sup>**</sup><br>-0.022<br>0.075 <sup>*</sup> | 0.024<br>0.076 <sup>*</sup><br>0.055 <sup>a</sup>                                        | 0.030<br>0.010<br>0.020                                                                      | 0.033<br>0.010<br>0.010                                                                      | <b>0.134</b> <sup>***</sup><br><b>0.221</b> <sup>***</sup><br><b>0.214</b> <sup>***</sup> | <b>0.567</b> <sup>***</sup><br><b>-0.195</b> <sup>***</sup><br><b>-0.137</b> <sup>***</sup> | <b>0.620</b> <sup>***</sup><br><b>-0.387</b> <sup>***</sup><br>0.079 <sup>*</sup>           | <b>0.363</b> <sup>***</sup><br><b>0.218</b> <sup>***</sup><br><b>0.233</b> <sup>***</sup> | <b>0.370</b> <sup>***</sup><br><b>0.288</b> <sup>***</sup><br><b>0.220</b> <sup>***</sup> | <b>0.433</b> <sup>***</sup><br><b>0.105</b> <sup>***</sup><br><b>0.189</b> <sup>***</sup> | <b>0.235</b> <sup>***</sup><br><b>0.209</b> <sup>***</sup><br><b>0.136</b> <sup>***</sup> |
| U-IgG   | -0.020<br>0.063<br>0.038                                                         | 0.058 <sup>a</sup><br>-0.012<br>-0.072                                                    | -0.072<br>0.005<br>-0.024                                  | 0.060<br>0.033<br>0.030                                                                  | -0.039<br>-0.002<br>-0.025                                                                   | -0.032<br>0.039<br>0.048                                                                     | 0.023<br>0.091<br>0.072                                                                   | <b>0.286</b> <sup>***</sup><br><b>-0.494</b> <sup>***</sup><br><b>-0.437</b> <sup>***</sup> | <b>0.343</b> <sup>***</sup><br><b>-0.560</b> <sup>***</sup><br><b>-0.305</b> <sup>***</sup> | <b>0.279</b> <sup>***</sup><br><b>0.298</b> <sup>***</sup><br><b>0.245</b> <sup>***</sup> | <b>0.497</b> <sup>***</sup><br><b>0.613</b> <sup>***</sup><br><b>0.539</b> <sup>***</sup> | <b>0.524</b> <sup>***</sup><br><b>0.398</b> <sup>***</sup><br><b>0.329</b> <sup>***</sup> |                                                                                           |
| U-Alb   | -0.024<br>0.000<br>0.024                                                         | 0.066 <sup>a</sup><br>0.033<br>0.022                                                      | -0.070 <sup>a</sup><br>0.028<br>0.039                      | -0.022<br>0.014<br>0.033                                                                 | 0.011<br>0.024<br>0.026                                                                      | 0.017<br>0.014<br>0.000                                                                      | -0.024<br>0.033<br>0.042                                                                  | <b>0.508</b> <sup>***</sup><br>0.000<br>0.037                                               | <b>0.599</b> <sup>***</sup><br>0.061<br><b>0.367</b> <sup>***</sup>                         | <b>0.333</b> <sup>***</sup><br><b>0.119</b> <sup>***</sup><br><b>0.189</b> <sup>***</sup> | <b>0.366</b> <sup>***</sup><br><b>0.101</b> <sup>***</sup><br><b>0.410</b> <sup>***</sup> | <b>0.539</b> <sup>***</sup><br><b>0.398</b> <sup>***</sup><br><b>0.293</b> <sup>***</sup> |                                                                                           |
| U-A1M   | 0.070 <sup>a</sup><br><b>0.144</b> <sup>***</sup><br><b>0.114</b> <sup>***</sup> | 0.045<br>-0.033<br>-0.054                                                                 | -0.003<br>-0.003<br>-0.032                                 | <b>0.112</b> <sup>**</sup><br><b>0.160</b> <sup>***</sup><br><b>0.149</b> <sup>***</sup> | <b>-0.125</b> <sup>***</sup><br><b>-0.115</b> <sup>***</sup><br><b>-0.133</b> <sup>***</sup> | <b>-0.122</b> <sup>***</sup><br><b>-0.097</b> <sup>***</sup><br><b>-0.090</b> <sup>***</sup> | <b>0.085</b> <sup>**</sup><br><b>0.150</b> <sup>***</sup><br><b>0.158</b> <sup>***</sup>  | <b>0.404</b> <sup>***</sup><br><b>-0.394</b> <sup>***</sup><br><b>-0.270</b> <sup>***</sup> | <b>0.410</b> <sup>***</sup><br><b>-0.471</b> <sup>***</sup><br><b>-0.187</b> <sup>***</sup> | <b>0.293</b> <sup>***</sup><br><b>0.248</b> <sup>***</sup><br><b>0.206</b> <sup>***</sup> |                                                                                           |                                                                                           |                                                                                           |
| U-NAG   | -0.040<br><b>0.126</b> <sup>***</sup><br><b>0.110</b> <sup>***</sup>             | <b>0.128</b> <sup>***</sup><br><b>0.105</b> <sup>***</sup><br><b>0.114</b> <sup>***</sup> | -0.082 <sup>*</sup><br>0.020<br>0.017                      | 0.035<br>0.062 <sup>a</sup><br>0.030                                                     | 0.042<br>0.085 <sup>a</sup><br>0.070 <sup>a</sup>                                            | 0.010<br>0.024<br>0.032                                                                      | -0.033<br>-0.001<br>0.022                                                                 | <b>0.397</b> <sup>***</sup><br><b>-0.199</b> <sup>***</sup><br><b>-0.162</b> <sup>***</sup> | <b>0.475</b> <sup>***</sup><br><b>-0.260</b> <sup>***</sup><br><b>0.295</b> <sup>***</sup>  |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| U-Crea  | -0.071<br>/<br>-0.057 <sup>a</sup>                                               | 0.068<br>/<br>-0.014                                                                      | <b>-0.087</b> <sup>***</sup><br>/<br>-0.070 <sup>a</sup>   | -0.034<br>/<br>-0.06 <sup>a</sup>                                                        | -0.043<br>/<br>-0.030                                                                        | -0.040<br>/<br>0.014                                                                         | -0.052<br>/<br>-0.024                                                                     | <b>0.776</b> <sup>***</sup><br>/<br><b>0.104</b> <sup>**</sup>                              | <b>0.662</b> <sup>***</sup><br>/<br><b>0.662</b> <sup>***</sup>                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| U-SG    | -0.051<br><b>0.096</b> <sup>***</sup>                                            | 0.084 <sup>*</sup><br>0.014                                                               | -0.039<br>0.024                                            | -0.017<br><b>0.219</b> <sup>***</sup>                                                    | 0.010<br><b>0.073</b> <sup>*</sup>                                                           | -0.032<br>0.036                                                                              | -0.022                                                                                    |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| B-Cd    | 0.001                                                                            | -0.066                                                                                    | <b>0.404</b> <sup>***</sup>                                | -0.017                                                                                   | <b>0.491</b> <sup>***</sup>                                                                  |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| B-As    | 0.083 <sup>*</sup>                                                               | -0.066                                                                                    | <b>0.441</b> <sup>***</sup>                                | -0.056                                                                                   |                                                                                              |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| B-Hg    | <b>0.146</b> <sup>***</sup>                                                      | 0.034                                                                                     | <b>0.115</b> <sup>**</sup>                                 |                                                                                          |                                                                                              |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| B-Pb    | 0.000                                                                            | -0.041                                                                                    |                                                            |                                                                                          |                                                                                              |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| B-Se    | <b>0.222</b> <sup>***</sup>                                                      |                                                                                           |                                                            |                                                                                          |                                                                                              |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |
| BMI     |                                                                                  |                                                                                           |                                                            |                                                                                          |                                                                                              |                                                                                              |                                                                                           |                                                                                             |                                                                                             |                                                                                           |                                                                                           |                                                                                           |                                                                                           |

| FemalesB | Age                                                                      | BMI                                                                                          | B-Se                                                                                      | B-Pb                                                                                      | B-Hg                                 | B-As                                           | B-Cd                                                                                      | U-SG                                                                                        | U-Crea                                                                                           | U-NAG                                                                                      | U-A1M                                                                                     | U-Alb                                                                                     | U-IgG                                                                                     |
|----------|--------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|--------------------------------------|------------------------------------------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| U-Cd     | -0.038<br><b>0.160</b> <sup>***</sup><br><b>0.122</b> <sup>***</sup>     | -0.033<br><b>-0.133</b> <sup>***</sup><br><b>-0.106</b> <sup>***</sup>                       | 0.056 <sup>a</sup><br>0.033<br>0.028                                                      | 0.033<br>0.078 <sup>*</sup><br>0.048                                                      | 0.044<br>0.040<br>0.020              | 0.056 <sup>a</sup><br>0.010<br>0.017           | <b>0.224</b> <sup>***</sup><br><b>0.236</b> <sup>***</sup><br><b>0.279</b> <sup>***</sup> | <b>0.646</b> <sup>***</sup><br><b>-0.136</b> <sup>***</sup><br><b>-0.053</b> <sup>***</sup> | <b>0.706</b> <sup>***</sup><br><b>-0.247</b> <sup>***</sup><br><b>0.123</b> <sup>***</sup>       | <b>0.383</b> <sup>***</sup><br><b>0.267</b> <sup>***</sup><br><b>0.209</b> <sup>***</sup>  | <b>0.170</b> <sup>***</sup><br><b>0.103</b> <sup>***</sup><br><b>0.011</b> <sup>***</sup> | <b>0.453</b> <sup>***</sup><br><b>0.141</b> <sup>***</sup><br><b>0.188</b> <sup>***</sup> | <b>0.361</b> <sup>***</sup><br><b>0.102</b> <sup>***</sup><br><b>0.112</b> <sup>***</sup> |
| U-IgG    | 0.025<br><b>0.162</b> <sup>***</sup><br><b>0.152</b> <sup>***</sup>      | <b>-0.093</b> <sup>***</sup><br><b>-0.182</b> <sup>***</sup><br><b>-0.136</b> <sup>***</sup> | 0.018<br>0.015<br>0.005                                                                   | 0.084 <sup>*</sup><br><b>0.082</b> <sup>*</sup><br>0.050 <sup>a</sup>                     | -0.038<br>0.066<br>0.080             | <b>0.125</b> <sup>**</sup><br>-0.013<br>-0.030 | -0.002<br>-0.018<br>-0.014                                                                | <b>0.403</b> <sup>***</sup><br><b>-0.719</b> <sup>***</sup><br><b>-0.452</b> <sup>***</sup> | <b>0.429</b> <sup>***</sup><br><b>-0.538</b> <sup>***</sup><br><b>-0.344</b> <sup>***</sup>      | <b>0.356</b> <sup>***</sup><br><b>0.419</b> <sup>***</sup><br><b>0.413</b> <sup>***</sup>  | <b>0.365</b> <sup>***</sup><br><b>0.609</b> <sup>***</sup><br><b>0.540</b> <sup>***</sup> | <b>0.651</b> <sup>***</sup><br><b>0.569</b> <sup>***</sup><br><b>0.543</b> <sup>***</sup> |                                                                                           |
| U-Alb    | -0.001<br>0.050<br>0.042                                                 | <b>-0.110</b> <sup>***</sup><br><b>-0.174</b> <sup>***</sup><br><b>-0.157</b> <sup>***</sup> | -0.017<br>0.030<br>0.014                                                                  | 0.000<br>0.020<br>0.014                                                                   | 0.000<br>0.009<br>0.035              | 0.069<br>0.046<br>0.001                        | 0.014<br>-0.001<br>-0.001                                                                 | <b>0.472</b> <sup>***</sup><br><b>-0.052</b><br><b>0.204</b> <sup>***</sup>                 | <b>0.516</b> <sup>***</sup><br><b>-0.098</b> <sup>***</sup><br><b>0.204</b> <sup>***</sup>       | <b>0.357</b> <sup>***</sup><br><b>0.224</b> <sup>***</sup><br><b>0.215</b> <sup>***</sup>  | <b>0.150</b> <sup>***</sup><br><b>0.138</b> <sup>***</sup><br>0.042                       |                                                                                           |                                                                                           |
| U-A1M    | 0.058<br><b>0.225</b> <sup>***</sup><br><b>0.253</b> <sup>***</sup>      | -0.023<br><b>-0.118</b> <sup>***</sup><br><b>-0.091</b> <sup>***</sup>                       | <b>0.142</b> <sup>***</sup><br><b>0.122</b> <sup>***</sup><br><b>0.124</b> <sup>***</sup> | <b>0.106</b> <sup>***</sup><br><b>0.102</b> <sup>***</sup><br><b>0.131</b> <sup>***</sup> | 0.011<br>0.095 <sup>*</sup><br>0.086 | -0.065<br>0.078<br>0.072 <sup>a</sup>          | 0.084 <sup>*</sup><br>0.036<br>0.046                                                      | <b>0.273</b> <sup>***</sup><br><b>-0.663</b> <sup>***</sup><br><b>-0.761</b> <sup>***</sup> | <b>0.240</b> <sup>***</sup><br><b>-0.836</b> <sup>***</sup><br><b>0.088</b> <sup>*</sup>         | <b>0.193</b> <sup>***</sup><br><b>0.457</b> <sup>***</sup><br><b>0.369</b> <sup>***</sup>  |                                                                                           |                                                                                           |                                                                                           |
| U-NAG    | 0.076 <sup>a</sup><br>0.061 <sup>a</sup><br><b>-0.182</b> <sup>***</sup> | -0.020<br>-0.014<br>0.072                                                                    | 0.055<br>0.017<br><b>-0.095</b> <sup>***</sup>                                            | 0.061 <sup>a</sup><br>0.063 <sup>a</sup><br><b>-0.095</b> <sup>***</sup>                  | 0.014<br>-0.017<br>-0.036            | 0.010<br>0.000<br>-0.069                       | 0.058 <sup>a</sup><br><b>0.104</b> <sup>***</sup><br><b>0.853</b> <sup>***</sup>          | <b>-0.320</b> <sup>***</sup><br><b>-0.265</b> <sup>***</sup><br><b>0.853</b> <sup>***</sup> | <b>-0.459</b> <sup>***</sup><br><b>-0.696</b> <sup>***</sup><br>/<br><b>0.155</b> <sup>***</sup> | <b>0.426</b> <sup>***</sup><br><b>-0.459</b> <sup>***</sup><br><b>0.585</b> <sup>***</sup> |                                                                                           |                                                                                           |                                                                                           |
| U-Crea   | -0.049<br><b>-0.188</b> <sup>***</sup>                                   | 0.041<br>0.046                                                                               | <b>-0.123</b> <sup>***</sup><br>-0.053                                                    | -0.075 <sup>a</sup><br>0.001                                                              | -0.017<br>-0.068                     | -0.000<br>-0.058                               | 0.024<br>0.042                                                                            | <b>0.155</b> <sup>***</sup>                                                                 | <b>0.585</b> <sup>***</sup>                                                                      |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| B-Cd     | <b>0.084</b> <sup>***</sup>                                              | <b>0.091</b> <sup>***</sup>                                                                  | 0.060 <sup>a</sup>                                                                        | <b>0.135</b> <sup>***</sup>                                                               | 0.026                                | 0.036                                          |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| B-As     | <b>0.098</b> <sup>***</sup>                                              | -0.080 <sup>*</sup>                                                                          | <b>0.281</b> <sup>***</sup>                                                               | <b>0.095</b> <sup>***</sup>                                                               | <b>0.444</b> <sup>***</sup>          |                                                |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| B-Hg     | 0.017                                                                    | -0.054                                                                                       | <b>0.248</b> <sup>***</sup>                                                               | -0.010                                                                                    |                                      |                                                |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| B-Pb     | <b>0.100</b> <sup>***</sup>                                              | <b>-0.087</b> <sup>***</sup>                                                                 | <b>0.141</b> <sup>***</sup>                                                               |                                                                                           |                                      |                                                |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| B-Se     | -0.041                                                                   |                                                                                              |                                                                                           |                                                                                           |                                      |                                                |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |
| BMI      | -0.014                                                                   |                                                                                              |                                                                                           |                                                                                           |                                      |                                                |                                                                                           |                                                                                             |                                                                                                  |                                                                                            |                                                                                           |                                                                                           |                                                                                           |

\*\*\* p < 0.001.

\*\* p < 0.05.

\* p < 0.1.

<sup>a</sup> p < 0.250 as the lowest level for inclusion in the linear regression model.

<sup>\*</sup> for IgG and A1M are given Spearman's correlation coefficients ( $r_s$ ) and for other biomarkers Pearson's correlation coefficients ( $r$ ). Statistically significant data are bolded. All parameters with the exception of BMI and age were ln-transformed. Urine data are given in triplicate: unnormalised (first row), Crea-normalised (second row), and SG-normalised (third row).

4 µg/g Crea, and only one participant had B-Cd level higher than 4 ng/mL (4.5 ng/g). Both levels are considered as action levels (Schulz et al., 2011; Nordberg et al., 2015). The GMs of all four urine renal function biomarkers, were far lower than their critical levels, although some individuals exceeded the critical levels (Table 1).

U-Cd is as a well-established biomarker of Cd body burden and a biomarker of exposure at elevated exposure levels (> 4 µg/g Crea). U-Cd extrapolation for use as an exposure biomarker in the general

population is less accurate. Particularly, the use of urine samples when studying the effect of low Cd levels on kidney function (< 1 µg/g Crea) has recently been questioned (Bernard, 2016). The results of the present study (GM < 0.35 µg/g Crea, range 0.01–2.79 µg/g Crea), as of many other studies, imply that low levels of Cd in urine are also influenced by recent exposure or factors unrelated to body burden, for example, normal physiological variability in kidney function influences excretion of renal function biomarkers along with Cd excretion



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**Table 4**Beta coefficients ( $\beta$ ) of associations between IgG, Alb, AIM, NAG and Cd estimated by linear regression models (Tables S1 and S2A–D).

| Biomarkers | Urine Cd                         |                                  | Blood Cd                         |                                  |
|------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|            | Male $\beta$ (N)                 | Female $\beta$ (N)               | Male $\beta$ (N)                 | Female $\beta$ (N)               |
| U-IgG      | <b>0.22</b> <sup>***</sup> (405) | <b>0.33</b> <sup>***</sup> (410) | 0.02 (408)                       | −0.02 (418)                      |
|            | <b>0.23</b> <sup>***</sup> (411) | <b>0.16</b> <sup>***</sup> (411) | 0.03 (414)                       | −0.07 (418)                      |
|            | <b>0.15</b> <sup>***</sup> (403) | <b>0.16</b> <sup>***</sup> (403) | 0.06 (401)                       | −0.05 (403)                      |
| U-Alb      | <b>0.61</b> <sup>***</sup> (473) | <b>0.63</b> <sup>***</sup> (481) | 0.04 (483)                       | −0.01 (465)                      |
|            | <b>0.14</b> <sup>***</sup> (488) | <b>0.22</b> <sup>***</sup> (451) | 0.03 (496)                       | −0.05 (465)                      |
|            | <b>0.21</b> <sup>***</sup> (465) | <b>0.31</b> <sup>***</sup> (435) | 0.04 (464)                       | −0.04 (433)                      |
| U-AIM      | <b>0.34</b> <sup>***</sup> (396) | <b>0.11</b> <sup>***</sup> (390) | 0.01 (404)                       | 0.02 (395)                       |
|            | <b>0.30</b> <sup>***</sup> (396) | 0.08 (392)                       | 0.0s (404)                       | −0.06 (398)                      |
|            | <b>0.22</b> <sup>***</sup> (384) | −0.04 (383)                      | 0.01 (387)                       | −0.04 (383)                      |
| U-NAG      | <b>0.42</b> <sup>***</sup> (459) | <b>0.39</b> <sup>***</sup> (415) | −0.02 (469)                      | <b>0.14</b> <sup>***</sup> (427) |
|            | <b>0.26</b> <sup>***</sup> (455) | <b>0.36</b> <sup>***</sup> (420) | −0.01 (465)                      | 0.06 (432)                       |
|            | <b>0.29</b> <sup>***</sup> (433) | <b>0.28</b> <sup>***</sup> (402) | 0.01 (435)                       | 0.13 (403)                       |
| U-Cd       | –                                | –                                | <b>0.11</b> <sup>***</sup> (469) | <b>0.33</b> <sup>***</sup> (486) |
|            | –                                | –                                | <b>0.14</b> <sup>***</sup> (488) | <b>0.22</b> <sup>***</sup> (467) |
|            | –                                | –                                | <b>0.17</b> <sup>***</sup> (467) | <b>0.29</b> <sup>***</sup> (450) |

N – Number of samples;  $\beta$  – beta coefficient.

\*\*\* p &lt; 0.001.

\*\* p &lt; 0.05; statistically significant values are bolded; Urine data are given in triplicate: unnormalised (1st row), Crea-normalised (2nd row), and SG-normalised (3rd third row). All data were ln-transformed.

**Table 5**

Influence of smoking, Pb and Se on associations between AIM and U-Cd or B-Cd (according to unnormalised data).

| Dependent variable | Independent variables | MALE $\beta$ (p-value)          | FEMALE $\beta$ (p-value)        |
|--------------------|-----------------------|---------------------------------|---------------------------------|
| U-AIM              | U-Cd                  | <b>0.34 (&lt; 0.001)</b>        | <b>0.11 (&lt; 0.001)</b>        |
|                    | B-Pb                  | <b>0.14 (0.022)</b>             | –                               |
|                    | B-Se                  | –                               | <b>0.33 (0.001)</b>             |
| U-AIM              | Smoking               | <b>0.26 (0.019)<sup>a</sup></b> | <b>0.18 (0.001)<sup>b</sup></b> |
|                    | B-Cd                  | 0.01 (0.981)                    | 0.02 (0.562)                    |
|                    | B-Pb                  | <b>0.15 (0.023)</b>             | –                               |
|                    | B-Se                  | –                               | <b>0.26 (0.013)</b>             |
|                    | Smoking               | <b>0.28 (0.042)<sup>a</sup></b> | <b>0.17 (0.004)<sup>b</sup></b> |

<sup>a</sup> Current smoking.<sup>b</sup> Pre-pregnancy smoking;  $\beta$  – beta coefficient; All data were ln-transformed; statistically significant results are bolded.

(Åkesson et al., 2014; Barregard and Elinder, 2015) and normalisation bias in the case of urine spot samples (Hoet et al., 2016; Tang et al., 2015; Bernard, 2016; Weaver et al., 2016). According to Byber et al. (2016), ‘the recent cross-sectional studies showing a Cd-related effect at low environmental exposure may have been affected by several sources of errors causing inconsistent non-causal associations’.

#### 4.1. Cd levels and smoking

As expected, both blood and urine slightly increased owing to current smoking, with a greater effect on Cd in blood; a marginal increase in blood Cd was also observed in pre-pregnancy smokers (Table 2). Consistent with a few reports (Chaumont et al., 2013; Ikeda et al., 2005), no influence of pre-pregnancy smoking on U-Cd levels was observed in our study, which is an unexpected observation for U-Cd as a biomarker of cumulative exposure. These results could be explained either by recent suggestions that U-Cd in most smokers returns to a normal level within 1–2 years after smoking cessation (Bernard, 2016) (in our case, approximately 11 months) or owing to the presence of un-identified former smokers among females who reported as non-smokers. Because such imprecise data on participants’ smoking history and smoking frequency (cigarettes/day) from self-reported questionnaires could lead to a crucial uncertainty in the statistical results, it is necessary to measure urine or plasma cotinine (major nicotine metabolite) as a more reliable indicator of tobacco exposure (Benowitz, 1996; Leon et al., 2002). In any case, our findings indicate that both U-Cd and B-Cd can be influenced by current

exposure to Cd, and B-Cd also by previously accumulated Cd (according to our smoking males and pre-pregnancy smoking females, respectively). It is impossible to evaluate the contribution of recent or past Cd exposure when such low levels of Cd are observed.

#### 4.2. Inter-individual variability and normalisation of biomarkers

In the present study, biomarkers measured in spot urine samples were normalised for inter-individual variation in diuresis. The most common normalisation methods that employ urine creatinine concentration (Crea) and specific gravity (SG) were used. Although the values of Crea and SG were strongly correlated in the present study (irrespective of gender, Table 3A and B), important inaccuracies in the statistical analysis were observed, depending on the type of normalisation. For example, a significant gender difference in U-Cd was observed only in Crea-normalised data but not in SG-normalised data (Table 1), as reported in the literature (Åkerström et al., 2012). As expected, correlations between unnormalised U-Cd levels and four renal proteins were substantially stronger than those when biomarkers were normalised for either Crea or SG (Table 3 A and B). Similar observations have been reported in the literature (Moriguchi et al., 2003; Ikeda et al., 2011; Zhang et al., 2015). Hence, when measuring associations between biomarkers obtained from the same individual’s spot urine sample, unnormalised data yields more accurate results, as suggested by some other authors (Ikeda et al., 2003; Zhang et al., 2015). By contrast, when studying biomarker associations in different matrixes or the differences between groups, normalisation for diuresis



**Table 6**  
Comparison of correlations between B-Cd and U-Cd in present study with those from reports in the literature.

| Study                                 | Population characteristics                                 | U-Cd/Crea (μg/g)                      | B-Cd (ng/g or ng/mL)                  | r                  |
|---------------------------------------|------------------------------------------------------------|---------------------------------------|---------------------------------------|--------------------|
| Present study                         | 548 M, 533 F, mean age 30 y, ever smokers 11% <sup>a</sup> | GM: 0.20, $\tau$ : 0.25, Median: 0.19 | GM: 0.28, $\tau$ : 0.38, Median: 0.29 | ~0.20 <sup>*</sup> |
| Akerstrom et al. (2013a) <sup>a</sup> | 49 M, 60 F, mean age 51 y, ever smokers 62%                | $\tau$ : 0.29                         | $\tau$ : 0.51                         | 0.55 <sup>*</sup>  |
| Chaumont et al. (2012) <sup>b</sup>   | 332 M, 404 F, median age 15 y                              | Median: 0.09                          | Median: 0.18                          | 0.16 <sup>*</sup>  |
| Haddam et al. (2011) <sup>c</sup>     | 134 M, mean age ~40 y                                      | $\tau$ : 0.48–1.00                    | $\tau$ : 0.40–1.20                    | 0.43 <sup>*</sup>  |
| Ikeda et al. (2011) <sup>d</sup>      | 1403 F, mean age 44 y, current smokers 14.7%               | GM: 1.00                              | GM: 1.23                              | 0.56 <sup>*</sup>  |

y - years; r - Pearson correlation coefficient; <sup>\*</sup>not all identified; M - male, F - female.  
p < 0.001.

<sup>a</sup> Akerstrom et al. (2013a).

<sup>b</sup> Chaumont et al. (2012).

<sup>c</sup> Haddam et al. (2011).

<sup>d</sup> Ikeda et al. (2011).

remains a common practice. However, in a recent study on 39 healthy occupationally non-exposed participants (15 male, 24 female; 20–59 years old), Hoet et al. (2016) reported that either Crea or SG normalisation of Cd levels in spot urine samples did not reliably reflect a 24-h Cd excretion rate. Moreover, they pointed out that Cd and Crea are not excreted by the same pathway, which creates an additional difficulty in normalisation. Therefore, they suggested that normalisation of low levels U-Cd for diuresis is not recommended (Hoet et al., 2016). Similar suggestion was given by Zhang et al. (2015) in their recent article. Additionally, Crea is determined by a far less accurate method than U-Cd (μg/mL vs. ng/mL; LODs in our case were 2 μg/mL vs. 0.03 ng/mL), what can significantly contribute to uncertainty and lead to inconsistent and conflicting results and misinterpretation. From that viewpoint, blood, as a more stable matrix, is better suited for the assessment of exposure to low levels of environmental Cd. However, because blood Cd is mainly bound to red blood cells, Cd levels should be normalised for haemoglobin concentration or haematocrit value to account for inter-individual variability (Santonen et al., 2015).

#### 4.3. Associations between blood and urine Cd

Correlations between blood and urine Cd levels were significant and relatively weak (weaker for males than females), regardless of the type of urine data used for calculation—unnormalised, and normalised by Crea and SG (p < 0.001, r = 0.20; Table 3 A and B). A few authors have reported stronger correlations (r = 0.43–0.56; Akerstrom et al., 2013a, 2013b, Haddam et al., 2011; Ikeda et al., 2011), but other have reported weaker correlations (r = 0.16; Chaumont et al., 2012). A detailed comparison is given in Table 6. The differences can be attributed to study population characteristics such as age, gender, and proportion of smokers. Comparison was possible for Crea-normalised data only, although normalisation can introduce serious bias at very low Cd levels (Hoet et al., 2016), as discussed above.

Additionally, associations stayed significant after U-Cd adjustment for possible confounders (p < 0.002, β = 0.11–0.33), (Table 4 and S1).

#### 4.4. Associations between urine renal proteins and Cd in urine and blood

Similar to a few studies on populations exposed to low levels of Cd (Akerstrom et al., 2013b; Chaumont et al., 2013, 2012; Haddam et al.,

2011; Wallin et al., 2014), we observed significant positive associations between urine Cd and all four urine renal proteins (IgG, Alb, A1M, and NAG; Table 4). Interestingly, the association of A1M (mg/L) with Cd (ng/mL) was positively influenced by current smoking and Pb levels in males, while in females, it was influenced by pre-pregnancy smoking and Se levels (Table 5 and S2A–D).

Regarding associations between urine renal proteins and blood Cd, the only significant association was for NAG in females (Table 4), while A1M was again influenced by smoking, Pb, and Se (Table 5). Pb was most probably related to current smoking, while the positive influence of Se in females could point to compensation for elevated oxidative stress (Tinggi, 2008). NAG is known to be physiologically elevated during pregnancy in lactating females, and it normalizes gradually thereafter (Capodicasa et al., 2011); hence, the observed increased NAG levels may not indicate renal damage.

Based on recent relevant reports (Åkesson et al., 2014; Barregard and Elinder, 2015; Bernard, 2016), the observed significant positive association between the examined renal proteins and U-Cd at such low levels of Cd exposure (GM < 0.35 ng/mL or ng/g) are a consequence of (patho)physiological inter-individual variability rather than a consequence of Cd-induced renal effects. In the literature, such associations are explained by Cd co-excretion because Cd bound to MT (also albumin) and renal proteins share similar excretion pathways and are affected in the same way by normal variations in renal function. Moreover, there are possibilities of the presence of unidentified pathological states such as diabetes, cardiovascular disease, urinary stones, and urinary tract infection, as well as possible co-exposure of Cd with other potentially nephrotoxic metals (Pb, As and Hg), which could lead to altered renal function, irrespective of Cd exposure, and influence the excretion of urine biomarkers including Cd (Delaney et al., 2012; Barregard and Elinder, 2015; Bernard, 2016). As pointed out above, the associations between A1M and B-Cd in males (Table 3A) were confounded by current smoking (Table 5). Smoking increased the levels of both B-Cd and A1M (Table 2), independently of B-Cd. Similar findings were reported in a study of 143 industrial male workers (mean age 40 years; B-Cd 0.30–1.70 ng/mL) (Haddam et al., 2011). Because A1M is considered to have anti-inflammatory and anti-oxidative roles (Andersson et al., 2008; Åkerström and Gram, 2014) and smoking is a well-known inducer of oxidative stress and inflammation (Agarwal, 2005), the observed increased levels of A1M among smokers could be explained by its (patho)physiological role unrelated to low-Cd exposure. In a study involving 109 healthy kidney donors (49 males, 60 females; mean age 49 years; arithmetic mean B-Cd: 0.51 ng/mL), Wallin et al. (2014) found a significant positive association between urine A1M and B-Cd; positive confounders were smoking, age, gender, and weight (β = 0.2, p = 0.02). Because A1M in males was also significantly associated with kidney Cd (β = 0.009, p = 0.04), they suggested that this protein is a sensitive renal function biomarker and that the observed associations may be consequences of Cd's renal effects. However, they also pointed out that the function and metabolism of A1M are not completely understood, and its increased excretion may be caused by factors other than impaired renal function.

#### 4.5. Limitations

Undoubtedly, the influence of smoking (together with Pb and Se status) is an important topic related closely to the association of Cd with renal function marker A1M (Table 5). Self-reported smoking data and incomplete information about participants' smoking history (former smokers among males not identified) and an unbalanced group of smokers versus non-smokers (Table 2) are major limitations of the present study. They may have introduced bias in the assessment of the effect of smoking on biomarker levels and their associations.

Another limitation was the high proportion of renal proteins with levels below LOD, because the LODs were rather high and not optimal, especially for A1M and IgG, which makes it impossible to determine



their exact values, leading to possible misinterpretation of the results.

Regarding confounding factors, it would be important to include genetic polymorphisms in the context of Cd metabolism and renal effects of biomarkers. They can have considerable influence on Cd levels and physiological responses to Cd exposure (Nordberg et al., 2015). Various studies have reported changes in blood and urine Cd levels due to specific genetic variations such as polymorphism in metallothioneins (MTs) (Adams et al., 2015; Kayaalti et al., 2011), transferrin receptor (TFRC) (Rentschler et al., 2013), and divalent metal transporter (DMT1) (Kayaalti et al., 2015).

## 5. Conclusion

Normalisations of low-Cd urine (like GM 0.19 ng/mL in our study) by creatinine is giving conflicting and unacceptable results, because the difference in measurement sensitivity for Cd and Crea is too high (0.03 ngCd/mL vs 2 µgCrea/mL, LODs in our case). Our results are additionally supporting the observation of Hoet et al. (2016) that 'in the context of environmental exposure systematic creatinine adjustment is not recommended for urinary concentrations of trace elements'. Due to collection and storage complications of 24 h urine, B-Cd represents a superior biomarker over U-Cd, in such cases.

No association between IgG, Alb, A1M and Cd were found in blood, although all of them were positively associated with Cd in urine (regardless of the type of urine data).

Smoking and B-Pb positively influenced the associations between A1M and B-Cd or U-Cd in males, while the same associations were positively influenced by pre-pregnancy smoking and B-Se in females. Connections between smoking, Cd, Pb, Se, and A1M could be related to their physiological roles (Åkerström and Gram, 2014) and are worth testing more rigorously.

The highest individual A1M levels did not coincide with the highest metal levels. This is additionally supporting that positive associations were a consequence of (patho)physiology rather than consequences of Cd (or Pb) - induced renal effects.

## Conflict of interest

The authors have no conflict of interest.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2016.09.025.

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## SUPPLEMENTAL MATERIALS

### **Low cadmium exposure in males and lactating females—estimation of biomarkers**

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**Table S1.** Associations between U-Cd and B-Cd estimated by linear regression models (all data except for BMI and age was ln-transformed; bolded are levels with  $p < 0.1$ ).

| Gender | Dependent variable | Independent variable | Unnormalised urine data |         | Crea-normalised urine data |         | SG-normalised urine data |         |
|--------|--------------------|----------------------|-------------------------|---------|----------------------------|---------|--------------------------|---------|
|        |                    |                      | $\beta$ (95% CI)        | p-value | $\beta$ (95% CI)           | p-value | $\beta$ (95% CI)         | p-value |
| MALE   | U-Cd               | B-Cd                 | 0.11 (0.04, 0.19)       | 0.002   | 0.14 (0.06, 0.21)          | <0.001  | 0.17 (0.09, 0.25)        | <0.001  |
|        |                    | B-Pb                 | -                       | -       | -0.003 (-0.11, 0.10)       | 0.948   | -0.003 (-0.10, 0.10)     | 0.995   |
|        |                    | B-Se                 | -0.42 (-0.73, -0.11)    | 0.009   | -                          | -       | -0.24 (-0.51, 0.03)      | 0.076   |
|        |                    | Age                  | 0.02 (-0.00, 0.03)      | 0.094   | 0.02 (0.007, 0.03)         | 0.001   | 0.01 (0.003, 0.02)       | 0.008   |
|        |                    | Current smoking      | -                       | -       | 0.05 (-0.17, 0.26)         | 0.682   | -0.13 (-0.36, 0.11)      | 0.289   |
| FEMALE | U-Cd               | B-Cd                 | 0.33 (0.20, 0.46)       | <0.001  | 0.22 (0.12, 0.31)          | <0.001  | 0.29 (0.19, 0.39)        | <0.001  |
|        |                    | B-As                 | -0.03 (-0.13, 0.07)     | 0.535   | -                          | -       | -                        | -       |
|        |                    | B-Pb                 | -                       | -       | 0.05 (-0.09, 0.19)         | 0.458   | -                        | -       |
|        |                    | B-Se                 | -0.28 (-0.71, 0.14)     | 0.184   | -                          | -       | -                        | -       |
|        |                    | BMI                  | -                       | -       | -0.02 (-0.03, 0.002)       | 0.023   | -0.01 (-0.03, 0.003)     | 0.123   |
|        |                    | Age                  | -                       | -       | 0.02 (-0.01, 0.03)         | 0.006   | 0.01 (-0.001, 0.03)      | 0.069   |

**Table S2.** Association between renal proteins and Cd estimated by linear regression model: A) IgG vs. U-Cd or B-Cd, B) Alb vs. U-Cd or B-Cd, C) AIM vs. U-Cd or B-Cd and D) NAG vs. U-Cd or B-Cd (bolded are levels with  $p < 0.1$ ; all data except for BMI and age were ln-transformed).

A)

| Gender                    | Dependent variable | Independent variable | Unnormalised urine data<br>$\beta$ (95% CI) | p-value          | Crea-normalised urine data<br>$\beta$ (95% CI) | p-value          | SG-normalised urine data<br>$\beta$ (95% CI) | p-value      |
|---------------------------|--------------------|----------------------|---------------------------------------------|------------------|------------------------------------------------|------------------|----------------------------------------------|--------------|
| MALE                      | IgG                | U-Cd                 | <b>0.22 (0.14, 0.31)</b>                    | <b>&lt;0.001</b> | <b>0.23 (0.10, 0.35)</b>                       | <b>&lt;0.001</b> | <b>0.15 (0.04, 0.27)</b>                     | <b>0.010</b> |
|                           |                    | BMI                  | 0.01 (-0.01, 0.028)                         | 0.177            | -                                              | -                | -                                            | -            |
|                           |                    | Current smoking      | -                                           | -                | 0.23 (-0.05, 0.50)                             | 0.103            | -                                            | -            |
|                           |                    | B-Cd                 | 0.02 (-0.06, 0.09)                          | 0.625            | 0.03 (-0.07, 0.13)                             | 0.519            | 0.06 (-0.02, 0.14)                           | 0.148        |
| FEMALE                    | IgG                | BMI                  | <b>0.02 (-0.00, 0.03)</b>                   | <b>0.094</b>     | -                                              | -                | -                                            | -            |
|                           |                    | Current smoking      | -                                           | -                | 0.25 (-0.08, 0.59)                             | 0.136            | -                                            | -            |
|                           |                    | U-Cd                 | <b>0.33 (0.25, 0.40)</b>                    | <b>&lt;0.001</b> | <b>0.16 (0.02, 0.30)</b>                       | <b>0.021</b>     | <b>0.16 (0.04, 0.29)</b>                     | <b>0.009</b> |
|                           |                    | B-As                 | -0.04 (-0.13, 0.04)                         | 0.333            | -                                              | -                | -                                            | -            |
|                           |                    | B-Pb                 | 0.01 (-0.17, 0.18)                          | 0.944            | 0.02 (-0.18, 0.22)                             | 0.830            | 0.008 (-0.19, 0.20)                          | 0.938        |
|                           |                    | BMI                  | <b>-0.02 (-0.04, -0.02)</b>                 | <b>0.030</b>     | <b>-0.03 (-0.05, -0.01)</b>                    | <b>0.001</b>     | <b>-0.03 (-0.05, -0.007)</b>                 | <b>0.011</b> |
|                           |                    | Age                  | -                                           | -                | <b>0.03 (0.01, 0.05)</b>                       | <b>0.002</b>     | <b>0.03 (0.01, 0.05)</b>                     | <b>0.005</b> |
|                           |                    | B-Cd                 | -0.02 (-0.15, 0.11)                         | 0.745            | -0.07 (-0.21, 0.06)                            | 0.281            | -0.05 (-0.19, 0.08)                          | 0.435        |
| $\beta$ —beta coefficient |                    | B-As                 | -0.07 (-0.17, 0.02)                         | 0.141            | -                                              | -                | -                                            | -            |
|                           |                    | B-Pb                 | -0.05 (-0.14, 0.24)                         | 0.602            | 0.05 (-0.14, 0.24)                             | 0.602            | 0.03 (-0.17, 0.23)                           | 0.775        |
|                           |                    | BMI                  | <b>-0.03 (-0.05, -0.01)</b>                 | <b>0.011</b>     | <b>-0.04 (-0.06, -0.02)</b>                    | <b>&lt;0.001</b> | <b>-0.03 (-0.05, -0.01)</b>                  | <b>0.003</b> |
|                           |                    | Age                  | -                                           | -                | <b>0.04 (0.02, 0.06)</b>                       | <b>&lt;0.001</b> | <b>0.03 (0.01, 0.05)</b>                     | <b>0.001</b> |



C)

| Gender                    | Dependent variable    | Independent variable  | Unnormalised urine data<br>$\beta$ (95% CI) | p-value          | U-Crea-normalised urine data<br>$\beta$ (95% CI) | p-value          | SG-normalised urine data<br>$\beta$ (95% CI) | p-value          |
|---------------------------|-----------------------|-----------------------|---------------------------------------------|------------------|--------------------------------------------------|------------------|----------------------------------------------|------------------|
| MALE                      | AIM                   | U-Cd                  | <b>0.34 (0.26, 0.42)</b>                    | <b>&lt;0.001</b> | <b>0.30 (0.18, 0.42)</b>                         | <b>&lt;0.001</b> | <b>0.22 (0.11, 0.33)</b>                     | <b>&lt;0.001</b> |
|                           |                       | B-As                  | -0.03 (-0.12, 0.06)                         | 0.464            | -0.02 (-0.12, 0.08)                              | 0.766            | -0.01 (-0.11, 0.09)                          | 0.887            |
|                           |                       | B-Pb                  | <b>0.14 (0.02, 0.25)</b>                    | <b>0.022</b>     | <b>0.13 (0.003, 0.26)</b>                        | <b>0.045</b>     | <b>0.14 (0.02, 0.26)</b>                     | <b>0.022</b>     |
|                           |                       | B-Hg                  | <b>-0.06 (-0.13, 0.01)</b>                  | <b>0.083</b>     | -0.06 (-0.16, 0.02)                              | 0.153            | <b>-0.07 (-0.14, 0.005)</b>                  | <b>0.067</b>     |
|                           |                       | Age                   | 0.00 (-0.01, 0.01)                          | 0.694            | 0.01 (-0.003, 0.02)                              | 0.122            | 0.01 (-0.01, 0.02)                           | 0.277            |
|                           |                       | Current smoking       | <b>0.26 (0.04, 0.48)</b>                    | <b>0.019</b>     | <b>0.35 (0.11, 0.59)</b>                         | <b>0.005</b>     | <b>0.32 (0.08, 0.55)</b>                     | <b>0.008</b>     |
|                           |                       | B-Cd                  | 0.01 (-0.09, 0.09)                          | 0.981            | 0.03 (-0.07, 0.12)                               | 0.576            | 0.06 (-0.03, 0.15)                           | 0.218            |
|                           |                       | B-As                  | -0.04 (-0.14, 0.06)                         | 0.408            | -0.01 (-0.12, 0.09)                              | 0.791            | -0.01 (-0.10, 0.09)                          | 0.892            |
|                           |                       | B-Pb                  | <b>0.15 (0.02, 0.28)</b>                    | <b>0.023</b>     | <b>0.12 (-0.01, 0.26)</b>                        | <b>0.068</b>     | <b>0.12 (0.000, 0.25)</b>                    | <b>0.049</b>     |
|                           |                       | B-Hg                  | -0.06 (-0.14, 0.02)                         | 0.119            | -0.05 (-0.13, 0.03)                              | 0.180            | <b>-0.06 (-0.14, 0.01)</b>                   | <b>0.084</b>     |
| FEMALE                    | AIM                   | Age                   | 0.01 (-0.01, 0.02)                          | 0.442            | <b>0.01 (-0.002, 0.03)</b>                       | <b>0.019</b>     | 0.01 (-0.003, 0.02)                          | 0.130            |
|                           |                       | Current smoking       | <b>0.28 (0.01, 0.54)</b>                    | <b>0.042</b>     | <b>0.38 (0.11, 0.66)</b>                         | <b>0.007</b>     | <b>0.23 (-0.04, 0.50)</b>                    | <b>0.058</b>     |
|                           |                       | U-Cd                  | <b>0.11 (0.08, 0.15)</b>                    | <b>&lt;0.001</b> | 0.08 (-0.04, 0.19)                               | 0.194            | -0.04 (-0.13, 0.05)                          | 0.408            |
|                           |                       | B-As                  | -                                           | -                | -                                                | -                | 0.01 (-0.07, 0.09)                           | 0.793            |
|                           |                       | B-Pb                  | 0.06 (-0.03, 0.14)                          | 0.201            | 0.06 (-0.11, 0.19)                               | 0.482            | 0.08 (-0.07, 0.22)                           | 0.284            |
|                           |                       | B-Hg                  | -                                           | -                | 0.03 (-0.06, 0.11)                               | 0.509            | 0.05 (-0.03, 0.12)                           | 0.257            |
|                           |                       | B-Se                  | <b>0.33 (0.14, 0.52)</b>                    | <b>0.001</b>     | 0.30 (-0.04, 0.75)                               | 0.078            | 0.19 (0.15, 0.52)                            | 0.272            |
|                           |                       | BMI                   | -                                           | -                | <b>-0.02 (-0.04, 0.000)</b>                      | <b>0.051</b>     | -0.01 (-0.03, 0.003)                         | 0.115            |
|                           |                       | Age                   | -                                           | -                | <b>0.04 (0.02, 0.06)</b>                         | <b>&lt;0.001</b> | <b>0.04 (0.21, 0.06)</b>                     | <b>&lt;0.001</b> |
|                           |                       | Pre-pregnancy smoking | <b>0.18 (0.07, 0.28)</b>                    | <b>0.001</b>     | 0.13 (-0.08, 0.35)                               | 0.219            | 0.13 (0.05, 0.31)                            | 0.148            |
| $\beta$ -beta coefficient | AIM                   | B-Cd                  | 0.02 (-0.05, 0.08)                          | 0.562            | -0.06 (-0.18, 0.06)                              | 0.323            | -0.04 (-0.15, 0.06)                          | 0.441            |
|                           |                       | B-As                  | -                                           | -                | -                                                | -                | 0.01 (-0.07, 0.09)                           | 0.809            |
|                           |                       | B-Pb                  | 0.05 (-0.03, 0.14)                          | 0.236            | 0.08 (-0.08, 0.25)                               | 0.337            | 0.07 (-0.07, 0.21)                           | 0.322            |
|                           |                       | B-Hg                  | -                                           | -                | 0.03 (-0.05, 0.11)                               | 0.450            | 0.04 (-0.04, 0.12)                           | 0.288            |
|                           |                       | B-Se                  | <b>0.26 (0.05, 0.46)</b>                    | <b>0.013</b>     | <b>0.34 (0.04, 0.74)</b>                         | <b>0.083</b>     | 0.21 (-0.13, 0.55)                           | 0.223            |
|                           |                       | BMI                   | -                                           | -                | <b>-0.02 (-0.04, 0.003)</b>                      | <b>0.027</b>     | -0.01 (-0.01, 0.03)                          | 0.110            |
|                           |                       | Age                   | -                                           | -                | <b>0.04 (0.004, 0.02)</b>                        | <b>&lt;0.001</b> | <b>0.04 (0.03, 0.06)</b>                     | <b>&lt;0.001</b> |
| $\beta$ -beta coefficient | Pre-pregnancy smoking | Pre-pregnancy smoking | <b>0.17 (0.06, 0.28)</b>                    | <b>0.004</b>     | 0.12 (-0.09, 0.34)                               | 0.247            | 0.14 (-0.04, 0.32)                           | 0.138            |



D)

| Gender | Dependent variable | Independent variable  | Unnormalised urine data    |                   | Crea-normalised urine data  |                  | SG-normalised urine data   |                   |
|--------|--------------------|-----------------------|----------------------------|-------------------|-----------------------------|------------------|----------------------------|-------------------|
|        |                    |                       | $\beta$ (95% CI)           | p-value           | $\beta$ (95% CI)            | p-value          | $\beta$ (95% CI)           | p-value           |
| MALE   | NAG                | U-Cd                  | <b>0.42(0.32, 0.53)</b>    | <b>&lt; 0.001</b> | <b>0.26 (0.14, 0.38)</b>    | <b>&lt;0.001</b> | <b>0.29 (0.17, 0.41)</b>   | <b>&lt; 0.001</b> |
|        |                    | B-Pb                  | -                          | -                 | 0.07 (-0.06, 0.20)          | 0.304            | -                          | -                 |
|        |                    | B-Hg                  | -                          | -                 | <b>0.07 (-0.002, 0.14)</b>  | <b>0.043</b>     | <b>0.07 (-0.005, 0.14)</b> | <b>0.067</b>      |
|        |                    | B-Se                  | -0.13(-0.49, 0.23)         | 0.472             | -                           | -                | -                          | -                 |
|        |                    | Age                   | -                          | -                 | <b>0.020 (-0.002, 0.04)</b> | <b>0.032</b>     | 0.01 (-0.005, 0.02)        | 0.234             |
|        |                    | BMI                   | <b>0.03(0.01, 0.05)</b>    | <b>0.006</b>      | 0.01 (-0.004, 0.02)         | 0.175            | <b>0.02 (0.002, 0.04)</b>  | <b>0.028</b>      |
|        |                    | B-Cd                  | -0.02(-0.11, 0.07)         | 0.633             | -0.01 (-0.10, 0.07)         | 0.793            | 0.01 (-0.07, 0.10)         | 0.755             |
|        |                    | B-Pb                  | -                          | -                 | 0.07 (-0.07, 0.21)          | 0.304            | -                          | -                 |
|        |                    | B-Hg                  | -                          | -                 | <b>0.08 (0.006, 0.15)</b>   | <b>0.033</b>     | 0.06 (-0.02, 0.13)         | 0.127             |
|        |                    | B-Se                  | -0.29(-0.67, 0.09)         | 0.133             | -                           | -                | -                          | -                 |
|        |                    | Age                   | -                          | -                 | <b>0.02 (0.003, 0.03)</b>   | <b>0.017</b>     | 0.01 (-0.00, 0.02)         | 0.115             |
|        |                    | BMI                   | <b>0.03(0.01, 0.05)</b>    | <b>0.007</b>      | 0.02 (-0.002, 0.04)         | 0.076            | <b>0.03 (-0.01, 0.05)</b>  | <b>0.011</b>      |
|        |                    | U-Cd                  | <b>0.39(0.30, 0.48)</b>    | <b>&lt; 0.001</b> | <b>0.36 (0.24, 0.49)</b>    | <b>&lt;0.001</b> | <b>0.28 (0.15, 0.40)</b>   | <b>&lt;0.001</b>  |
|        |                    | B-Pb                  | -                          | -                 | 0.09 (-0.10, 0.29)          | 0.347            | 0.10 (-0.10, 0.29)         | 0.335             |
| FEMALE | NAG                | Age                   | -0.01(-0.03, 0.01)         | 0.287             | 0.01 (-0.10, 0.03)          | 0.513            | 0.004 (-0.02, 0.03)        | 0.636             |
|        |                    | Pre-pregnancy smoking | 0.09(-0.13, 0.31)          | 0.408             | -                           | -                | -                          | -                 |
|        |                    | B-Cd                  | <b>0.14(0.01, 0.28)</b>    | <b>0.040</b>      | 0.06 (-0.08, 0.20)          | 0.365            | 0.13 (-0.09, 0.27)         | 0.168             |
|        |                    | B-Pb                  | -                          | -                 | 0.09 (-0.10, 0.28)          | 0.354            | 0.09 (-0.11, 0.29)         | 0.355             |
|        |                    | Age                   | <b>-0.02(-0.04, -0.00)</b> | <b>0.040</b>      | -0.1 (-0.007, 0.04)         | 0.199            | 0.010 (-0.12, 0.03)        | 0.437             |
|        |                    | Pre-pregnancy smoking | 0.11(-0.13, 0.35)          | 0.370             | -                           | -                | -                          | -                 |
|        |                    | — beta coefficient    |                            |                   |                             |                  |                            |                   |

#### Comments related to associations:

Associations in models were additionally assessed using

- participants with B-Cd > 0.5 ng/g (124 females and 77 males)
  - participants with renal proteins above their LODs
  - participants with renal proteins above their URLs
  - participants with B-Cd > 0.5 ng/g (124 females and 77 males) and with renal proteins above their LODs or above URLs;
- no significant associations were observed (data not shown).

### 3.2 Manuscript 2: Arsenic Metabolites; Selenium; and *AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *SELENOP*, *INMT*, and *MT2A* Polymorphisms in Croatian-Slovenian Population from PHIME-CROME Study

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Relationships between inorganic arsenic metabolism, genetic polymorphisms and status of selenium have been frequently studied in populations with high environmental As exposure levels (Broberg et al., 2015), while at low exposures such gene-environment associations are scarce and for some genes non-existing.

In the manuscript, we tested possible influences of 15 single nucleotide polymorphisms (SNPs) of 7 genes on As methylation efficiency (estimated by urine As metabolites) in a Croatian-Slovenian population with low inorganic As exposure (recruited between 2007-2017 within PHIME and CROME study). Beside previously studied SNPs of *AS3MT*, *MTHFR*, *INMT* and *MT2A*, we also tested SNPs of *AQP4*, *AQP9* and *SELENOP*, which can all be related to As methylation. Relations of SNPs of the latter three genes are to the best of our knowledge assessed for the first time. We also constructed *AS3MT* SNPs' haplotype and tested it for its previously reported beneficial role in As methylation (Broberg et al., 2015). Additionally, we followed Se levels and SNPs influences on its urine excretion, due to known interrelated metabolism of Se and As (George et al., 2013).

Study population was stratified into a subgroup of pregnant women (n =136), non-pregnant women (n =176) and their children (n =176), which enabled the estimation of As methylation efficiency, Se status and gene-environment interactions at three different physiological states. Interactions within each group were also tested for possible confounding factors such as BMI, age, gender of fetus, gender, parity, blood As, nutritional status of Se, ever-smoking, which were specific within each studied group.

Furthermore, we presented allele frequency distribution of studied SNPs obtained in our study population. For *AS3MT*, frequencies of SNPs minor alleles and of their constructed haplotype were also compared with those reported for other worldwide populations. For other genes, studied in the present manuscript (and in dissertation as a whole), similar comparisons were elaborated and collected in a working report (App. A.3).

At low iAs exposure we:

- i) confirmed the influence of physiological status (pregnancy, non-pregnancy, and childhood) on As methylation efficiency, on As and Se levels, and on gene-environmental interactions;
- ii) confirmed the “protective” role of *AS3MT* SNPs/haplotype on As methylation;
- iii) identified possible role(s) of some genes and their SNPs in the As metabolism (SNPs of *INMT*, *AQP9*, *AQP4*, and *MT2A*) and Se metabolism (SNPs of *INMT* and *AQP9*);
- iv) found obtained *AS3MT* SNPs/haplotype allele frequencies to be similar with those reported for European countries, higher from those reported for Africa and lower from populations with historically elevated As exposures (Argentina, Bangladesh, and Chile).

In the present manuscript the confounding by the seafood-borne DMA was expected to be insignificant, as DMA levels in fish were expected to be negligible due to observations from our previous study on As speciation in fish (rays) from the Northern Adriatic Sea (Šlejkovec et al., 2014).

My contribution to the present manuscript consisted of participation in study subjects' samplings in the CROME part of the project, participation in DNA extractions, genotyping of the majority of included SNPs, statistical analyses, preparation of tables and figures, and writing of the manuscript.



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## Arsenic metabolites; selenium; and *AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *SELENOP*, *INMT*, and *MT2A* polymorphisms in Croatian-Slovenian population from PHIME-CROME study

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### ABSTRACT

The relationships between inorganic arsenic (iAs) metabolism, selenium (Se) status, and genetic polymorphisms of various genes, commonly studied in populations exposed to high levels of iAs from drinking water, were studied in a Croatian-Slovenian population from the wider PHIME-CROME project. Population consisted of 136 pregnant women in the 3<sup>rd</sup> trimester and 176 non-pregnant women with their children (n = 176, 8–9 years old). Their exposure to iAs, defined by As (speciation) analyses of biological samples, was low. The sums of biologically active metabolites (arsenite + arsenate + methylated As forms) for pregnant women, non-pregnant women, and children, respectively were: 3.23 (2.84–3.68), 1.83 (1.54–2.16) and 2.18 (1.86–2.54) ng/mL<sub>SG</sub>; GM (95 CI). Corresponding plasma Se levels were: 54.8 (52.8–56.9), 82.3 (80.4–84.0) and 65.8 (64.3–67.3) ng/mL; GM (95 CI). As methylation efficiency indexes confirmed the relationship between pregnancy/childhood and better methylation efficiency. Archived blood and/or saliva samples were used for single nucleotide polymorphism (SNP) genotyping of arsenic(3+) methyltransferase - *AS3MT* (rs7085104, rs3740400, rs3740393, rs3740390, rs11191439, rs10748835, rs1046778 and the corresponding *AS3MT* haplotype); methylene tetrahydrofolate reductase - *MTHFR* (rs1801131, rs1801133); aquaporin - *AQP* 4 and 9 (rs9951307 and rs2414539); selenoprotein P1 - *SELENOP* (rs7579, rs3877899); indolethylamine N-methyltransferase - *INMT* (rs6970396); and metallothionein 2A - *MT2A* (rs28366003). Associations of SNPs with As parameters and urine Se were determined through multiple regression analyses adjusted using appropriate confounders (blood As, plasma Se, ever smoking, etc.). SNPs' influence on As methylation, defined particularly by the secondary methylation index (SMI), confirmed the 'protective' role of minor alleles of six *AS3MT* SNPs and their haplotype only among non-pregnant women. Among the other investigated genes, the carriers of *AQP9* (rs2414539) were associated with more efficient As methylation and higher urine concentration of As and Se among non-pregnant women; poorer methylation was observed for carriers of *AQP4* (rs9951307) among pregnant women and *SELENOP* (rs7579) among non-pregnant women; *MT2A* (rs28366003) was associated with higher urine concentration of AsIII regardless of the pregnancy status; and *INMT* (rs6970396) was associated with higher As and Se concentration in non-pregnant women. Among confounders, the strongest influence was observed for plasma Se; it reduced urine AsIII concentration during pregnancy and increased secondary methylation index among non-pregnant women. In the present study of populations with low As exposure, we observed a few new As-gene associations (particularly with *AQPs*). More reliable interpretations will be possible after their confirmation in larger populations with higher As exposure levels.

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## 1. Introduction

Human exposure to arsenic (As) compounds can be (1) environmental, primarily due to ingestion of contaminated drinking and cooking water and food (especially seafood and rice); (2) occupational, mainly due to smelting of nonferrous metals, wood combustion, manufacture of glass, etc.; or (3) therapeutic, for example, owing to the recent FDA- and EMA-approved use of arsenic trioxide ( $As_2O_3$ ) for treating patients with acute promyelocytic leukaemia (APL) (Fowler et al., 2015; Khairul et al., 2017). Inorganic arsenic (iAs), including arsenite (AsIII) and arsenate (AsV) after its bioconversion to AsIII, is primarily biotransformed in the liver via reduction/oxidation and methylation. Methylation conducted in the presence of cofactor glutathione (GSH) with arsenic (3+) methyltransferase (AS3MT) using S-adenosyl-methionine (SAM) as a substrate generates methylated (monomethylarsonic acid, MMA) and dimethylated (dimethylarsinic acid, DMA) arsenicals as major products that are then eliminated in urine (Cullen, 2014; Fowler et al., 2015). In comparison, other methyltransferases and non-enzymatic methylation routes have been less investigated (Aposhian et al., 2004; Gardner et al., 2012). Methylation is a crucial factor that influences As tissue distribution and elimination and, consequently, its adverse health effects (Fowler et al., 2015). Arsenic is mainly eliminated through urine (iAs, MMA, and DMA) and less so through bile (faeces) (iAs and MMA) (Roggenbeck et al., 2016). The average reported values of eliminated As metabolites in the urine of populations environmentally exposed to low levels of iAs from water are 20–25% for iAs, 15–25% for MMA, and 40–75% for DMA (Fowler et al., 2015). Several studies also noted considerable individual and populational variations in metabolite profiles (Vahter, 1999). The As metabolite pattern in urine is influenced by age, body mass index (BMI), gender, dietary habits (As-rich seafood and rice), behavioural factors (smoking and alcohol consumption), As exposure dosage and duration, and various (patho)physiological conditions such as pregnancy or diseases, especially those of the liver, kidney, and cardiovascular system (Taylor et al., 2017; Tseng, 2009). Some studies, although with some inconsistencies, have also noted the influence of nutritional status, especially that of selenium (Se), folate (vitamin B9), and other B vitamins (B2, B6, and B12) on As metabolism (Gamble et al., 2006; Gardner et al., 2011; Li et al., 2008; Spratlen et al., 2017; Thomas and Bradham, 2016; Tseng, 2009).

Selenium is an essential element (Schwarz and Foltz, 1957) with multiple physiological roles, mainly as a component of various selenoproteins in the form of the amino acid selenocysteine (Alexander, 2015). Selenium has been shown to play a complex role in As metabolism, elimination, and toxicity (Fahnoga et al., 2014; George et al., 2013; Sun et al., 2014; Zeng et al., 2005). Elements are mutually antagonistic, and the addition of one increases the elimination of the other. In 2002, seleno-bis(S-glutathionyl) arsinium ion  $[(GS)_2AsSe]^+$  was isolated and identified as a major metabolite eliminated into the bile of rabbits (Gailer et al., 2002); in 2011, it was also detected in human blood (La Porte, 2011). Because, the transporter of the  $[(GS)_2AsSe]^+$  complex, multidrug resistance associated protein 2 (MRP2), was also identified in kidney cells (Carew and Leslie, 2010), the elimination of this complex through urine was suggested (Skröder Löveborn et al., 2016).

Considerable variations in urine As metabolite profiles observed between different ethnical groups exposed to similar levels of As in drinking water suggest the influence of genetic variations, as has been frequently noted in the last decade (Agusa et al., 2015; Broberg et al., 2015; Hernández and Marcos, 2008). The AS3MT gene has been widely studied as the key enzyme in As methylation. Studies conducted in populations historically exposed to high levels of As in drinking water identified several noncoding single nucleotide polymorphisms (SNPs) of AS3MT, such as rs7085104, rs3740400, rs3740393, rs3740390, rs11191453, rs10748835, and rs1046778, with minor alleles mostly associated with lower iAs% and MMA% and higher DMA% (Agusa

et al., 2015; Broberg et al., 2015; Engström et al., 2011, 2013; Hernández and Marcos, 2008). This consistent association of various SNPs with more efficient iAs methylation could be explained by the strong correlation between these alleles (as estimated by linkage disequilibrium (LD)) in populations. As such, these SNP alleles tend to occur together and are usually inherited as a cluster representing specific nucleotide sequences called haplotypes. By contrast, one coding SNP was associated with less-efficient As methylation (rs11191439). Studies have introduced various clusters of selected SNP alleles associated with more efficient As methylation as 'protective' haplotypes (Agusa et al., 2015, 2009; Broberg et al., 2015; De la Rosa et al., 2017; Engström et al., 2015, 2011, 2007). AS3MT genetic variations may impact enzyme function by affecting their gene expression and/or translation (Engström et al., 2011, 2013). The population frequency of the 'protective' AS3MT haplotype varies in terms of dosage and long-term duration of iAs exposure. In populations with high historic iAs exposure in drinking water (200–1000  $\mu gAs/L$ ; for example, Argentina, Peru, and Chile), observed frequencies of 50–70% indicate genetic adaptations to arsenic-enriched environments (Apata et al., 2017; Eichstaedt et al., 2015; Schlebusch et al., 2015a, 2015b, 2013). In European populations exposed to low to moderate iAs levels in drinking water (0.01–167  $\mu gAs/L$ ; for example, Slovakia, Hungary, and Romania), the reported frequencies are lower, being 10–14% (Engström et al., 2015).

In addition to AS3MT, variations in various other genes may also influence the urine As metabolite pattern. Among them are SNPs in the gene coding for methylene tetrahydrofolate reductase (MTHFR), one of the genes involved in one-carbon metabolism (Gambao-Loira et al., 2018), like AS3MT. The one-carbon metabolism is a network of various physiological processes including biosynthesis of methionine, which is a substrate of SAM- a methyl donor for As methylation (Broberg et al., 2015). MTHFR SNPs were found to be associated with less-efficient arsenic methylation, as reflected by the higher MMA% and/or lower DMA% among populations with moderate to high iAs exposure (Engström et al., 2007; Lindberg et al., 2007).

Many studies have investigated genes involved in the GSH metabolism; variations in genes of glutathione S-transferases (GSTs) are related to GSH capacity. Variations in the gene of purine nucleoside phosphorylase (PNP) are possibly involved in AsV to AsIII reduction (Broberg et al., 2015; Hernández and Marcos, 2008) and variations in the gene of DNA methyltransferases (DNMT1a and DNMT1b) (Broberg et al., 2015; Chen et al., 2017; De la Rosa et al., 2017; Gardner et al., 2012) and the genes of metallothioneins (MTs) (González-Martínez et al., 2018), were also found to be associated with the methylation capacity or elimination (or better urine concentrations) of As. Additionally, possible candidate variations in relation to As kinetics could be those in genes coding for aquaporins (AQPs), as AQP3, AQP4, AQP7, AQP9, and AQP10 were shown to be responsible for AsIII uptake (Calatayud and Laparra Llopis, 2015; Calatayud et al., 2012; Mukhopadhyay et al., 2014; Roggenbeck et al., 2016) and in genes involved in elimination of Se through urine, such as indolethylamine N-methyltransferase (INMT) (Amorós et al., 2018; Kuehnelt et al., 2015; Skróder et al., 2018), or Se transport and storage, such as selenoprotein P1 (SELENOP) (Burk and Hill, 2005; Mao et al., 2016).

The evaluation of the population genotype/haplotype frequencies of SNPs associated with more- or less- efficient As metabolism in combination with Se status is essential for the health risks assessment of arsenic exposure, especially in the case of major unpredicted environmental As contamination, and for the individual estimation of  $As_2O_3$  effectiveness during acute promyelocytic leukaemia therapy. However, it should be noted that the magnitude of the genetic influences on arsenic metabolism is basically unknown. It may depend on various factors like the degree of As exposure (Xu et al., 2016) pregnancy status (Gardner et al., 2012), nutritional status (Gardner et al., 2012; Spratlen et al., 2017) and type of SNP (Xu et al., 2016).

The present study aimed to examine the influence of genetic

predisposition (SNPs in *AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *SELENOP*, *INMT*, and *MT2A*) and selenium status on arsenic methylation efficiency and arsenic urine concentrations in Croatian-Slovenian woman (pregnant and non-pregnant) and children exposed to low levels of inorganic arsenic. The influence of SNPs on Se urine concentrations was simultaneously tested as well.

## 2. Material and methods

### 2.1. Study population

Study includes a Croatian-Slovenian population ( $n = 506$ ) with low As exposure mainly through seafood consumption. Participants were recruited between 2006 and 2011 within the wider EU-funded PHIME-FP6 project ('Public Health Impact of long-term, low-level, mixed element exposure in susceptible population strata') and/or between 2013 and 2017 within the follow-up EU-funded CROME-LIFE+ project ('Cross-Mediterranean Environment and Health Network'). All participants provided signed informed consent, and the research protocol of the study was approved by the National Ethics Committee of Croatia and Slovenia.

As shown in Fig. 1, the study population consisted of three main groups: pregnant women (A;  $n = 136$ , aged 20–44 years; residents of the coastal city of Rijeka, Croatia, and its surroundings) with a small subgroup of the same women resampled 8–9 years after pregnancy (A<sub>1</sub>;  $n = 21$ , aged 29–48 years); non-pregnant mothers (B;  $n = 176$ , aged 30–51 years, residents of Ljubljana and its surroundings); and children of non-pregnant women (C;  $n = 176$ , aged 7–8 years; C<sub>1</sub>: 86 girls and C<sub>2</sub>: 90 boys). Information regarding their basic characteristics (age, weight, height...), socioeconomic status, lifestyle, and nutritional habits were acquired through interviewer-administered questionnaires. The residential areas—Rijeka and Ljubljana—were not far apart (< 150 km) and before 1991, they both belonged to the former Yugoslavia and had very balanced socioeconomic status.

The reported seafood intake frequency data (intake frequency of fish, crustaceans, molluscs, etc.) with serving of 150 g were transformed into a daily seafood intake index, as previously explained by Valent et al. (2013) and Miklavčič et al. (2013).

The As levels in drinking water were < 0.1–0.55 µg/L in Ljubljana (unpublished data, Zuliani and Šlejkočec 2014, 2018) and 0.05–1 µg/L in Rijeka (measured in 2007, 2014 and 2016) (Fiket et al., 2007; Piškur, 2016, 2014). WHO's permissible exposure limit is 10 µg/L (World Health Organization, 1993).

### 2.2. Samples and sampling data

Peripheral venous blood/plasma and urine spot samples were obtained for all study groups (A(A<sub>1</sub>), A<sub>1</sub>, B, and C). Saliva was obtained for non-pregnant women and children (B and C). Fig. 1 shows the details of the collected samples. Pregnant women (A(A<sub>1</sub>)), sampling was performed during a regular medical exam at the University Hospital of Rijeka in the 3<sup>rd</sup> trimester of pregnancy; as previously described in detail by (Miklavčič et al., 2013; Valent et al., 2013).

For non-pregnant women and children, samples were collected at appointed visits in paediatric clinics in Ljubljana, Slovenia (B and C), or the University Hospital of Rijeka (A<sub>1</sub>). Urine samples (~50 mL) and peripheral blood (16 mL for mothers and 6 mL for children) were obtained by trained personnel according to the standard protocol. Before analysis, samples were divided into aliquots and stored at -80 °C. Saliva samples (~1 mL) were collected using an Oragene DNA self-collection kit (OG-500) (DNA Genotek Inc), and stored at room temperature (~25 °C).

### 2.3. As and Se determination

#### 2.3.1. Total As and Se determination in blood, urine and plasma (Se only)

**Pregnant women (A):** Total As and Se levels in blood and urine were measured at the Jožef Stefan Institute, Department of Environmental Sciences, Slovenia, using an octapole reaction system inductively coupled plasma mass spectrometer (ORS ICP-MS, 7500ce, Agilent Technologies, Santa Clara, CA, USA) equipped with an ASX-510 Autosampler (Cetac), as described in detail in a previous study (Miklavčič et al., 2013). The limit of detection (LOD) calculated as three times the standard deviation of blanks for As and Se was respectively 0.1 and 5.0 ng/g in blood and 0.3 and 4.0 ng/mL in urine.

Se in plasma was measured at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre, Ljubljana, Slovenia, by electrochemical atomic absorption spectrometry (ETAAS) with LOD of 0.2 ng/mL.

**Non pregnant women and children (B, A<sub>1</sub> and C):** Total As and Se levels in blood, plasma (Se only), and urine were measured at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre Ljubljana, Slovenia. An aliquot (0.2 mL) of the sample was diluted with 2 mL of 1.02% (v/v) ammonium hydroxide (Fluka Analytical TraceSELECT Ultra) solution containing 0.06% (v/v) Triton X-100 (Aldrich Chemistry, Trace Metal Basis), 1.8% (v/v) 1-butanol (Sigma Aldrich, ACS reagent), 0.06% (w/v) ethylenediamine-tetraacetic acid disodium salt dehydrate (Aldrich Chemistry, Trace

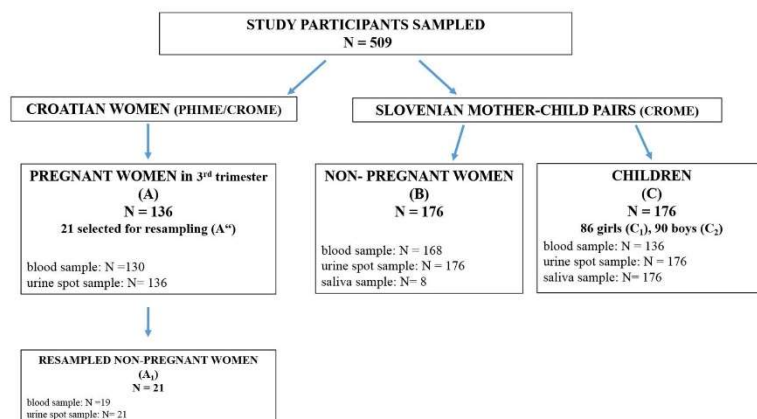


Fig. 1. Study selection scheme: participant groups with collected samples.



Metal Basis), and internal standard solution (ICP-MS Internal Std Mix, Agilent Technologies). External calibration with a multi calibrator IV-STOCK-27 (Inorganic Ventures) was used for quantification. Prepared solutions were measured using an ORS ICP-MS (7700 ×, Agilent) equipped with an Integrated Autosampler (Agilent). The instrument conditions were as follows: micromist nebuliser, Scott-type spray chamber with lower temperature of 2 °C and higher temperature of 16 °C, plasma gas flow rate of 15 L/min, carrier gas flow rate of 1.07 L/min, RF power of 1550 W, and reaction cell helium gas flow rate of 10 mL/min for Se and 5 mL/min for As. The instrument was tuned daily. The reference materials used to check the accuracy of the measured trace elements were whole Blood L-1 (lot no. 1406263, Sero) and L-2 (lot no. 1406264, Sero), Seronorm Trace Elements Serum L-1 (lot no. 1309438, Sero), and Seronorm Trace Elements Urine L-1 (lot no. 1011644, Sero) and L-2 (lot no. 1403081, Sero). The values found were in good agreement with the reference values. LODs were calculated based on 3-day measurements (in 20 repetitions) of Seronorm Trace Elements Urine L-1 following the recommendation of the Environmental Protection Agency. LODs in blood, plasma (Se only), and urine were 0.1 ng/mL for As and 1.0 ng/mL for Se.

The use of different LODs in the measurement of As (urine) and Se levels should not have had a significant impact on the statistical analysis because the number of participants with levels below LODs was negligible (11 pregnant women and 3 non-pregnant women for Se in urine and one non-pregnant woman for As in urine).

### 2.3.2. Determination of As metabolites in urine

Biologically active arsenic metabolites, namely AsIII, AsV, MMA, and DMA, were measured in all urine samples, and their sum ( $\Sigma\text{As}_M$ ) was used as an estimation of the exposure to iAs. Nontoxic, readily eliminated arsenobetaine (AsB), generally the major component of seafood As, was measured in a subset of urine samples ( $n = 31$ ) or was otherwise calculated (U-cAsB) by subtracting the sum of AsIII, AsV, MMA and DMA from total urine As (U-As). U-cAsB was used as an estimation of foodborne AsB, although this estimation also included potential traces of undefined metabolites from food consumption (arsenocholine, arsenolipids, arsenosugars, etc.). The proportion (%) of calculated AsB was calculated based on U-As.

AsIII, AsV, MMA, and DMA were determined in thawed urine samples using high-performance liquid chromatography (HPLC) with an anion exchange column (Hamilton PRP-X100; 20 mM  $\text{KH}_2\text{PO}_4$ , pH 6.1 as mobile phase) coupled with hydride generation atomic fluorescence spectrometry (HG AFS), as described previously (Šlejko et al., 2008). Arsenobetaine (AsB) was determined using a cation exchange column (Zorbax 300-SCX; 10 mM pyridine, pH 2.75 as mobile phase) and UV-decomposition (3% NaOH/3%  $\text{K}_2\text{S}_2\text{O}_8$ , 3 mL/min) before HG. The LODs were 0.1 ng/mL for AsIII, 0.2 ng/mL for MMA and DMA, and 1 ng/mL for AsB.

The presence of undefined As species was tested by mass balance analysis (total As versus the sum of measured metabolites—AsIII, AsV, MMA, DMA and AsB—in samples with exact measurements of all As variables in urine ( $n = 31$ ). The average (arithmetic) share of analysed metabolites was 89% in the range of 66–100%. Mass balance was particularly good in samples with higher As levels. Difference in samples with low As levels ( $n = 9$ , 3.44–22.0 ng/mL) can be attributed to methodological reasons and analytical measurement uncertainty related to very low concentration levels. Differences for samples with higher levels ( $n = 5$ , 273–429 ng/mL) might be explained by the matrix effect binding of some As species (As-metallithionein, As-Cys, etc.) on the HPLC column that could not be detected; this phenomenon was described previously by Šlejko et al. (2008), Cullen (2014), and Stice et al. (2016).

### 2.3.3. Adjustment of urine biomarkers for differences in diuresis between samples

Specific gravity (SG) was used for adjusting the total As and Se in

urine, as described previously (Suwazono et al., 2005). The SG of each sample was determined using a PAL-10S refractometer (Atago®, Japan) with measurement range of 1.000–1.060.

Adjustment by creatinine has previously been reported as being inappropriate because (1) physiological interactions between creatinine and arsenic in urine (through common one-carbon metabolism) lead to possible underestimation of As exposure (Tseng, 2009) and (2) creatinine at low levels of exposure normalisation can even introduce mathematical uncertainties (Bernard, 2016; Hoet et al., 2015; Stajanko et al., 2017; Tang et al., 2015).

### 2.3.4. As methylation efficiency

Methylation efficiency or methylation capacity is generally estimated by proportions of biologically active urine metabolites (AsIII%, MMA%, and DMA%) considering the total urine As or their sum in case of known seafood consumption to exclude nontoxic arsenobetaine. In addition, primary, secondary and total methylation index ( $\text{PMI} = \text{MMA}/\text{iAs}$ ,  $\text{SMI} = \text{DMA}/\text{MMA}$  and  $\text{TMI} = (\text{MMA} + \text{DMA})/\text{iAs}$ , respectively) are usually used as indicators for evaluating the methylation capacity of individuals. We used both types to achieve better comparison with literature. Shares of biologically active metabolites were calculated based on their sum.

### 2.4. DNA extraction and SNP genotyping

DNA was mostly extracted from blood samples (1–3 mL) using a FlexiGene® DNA Kit (Qiagen, Hilden, Germany), and in cases where blood samples for extraction were unavailable (group C and eight women from group B), it was extracted from saliva samples (0.5 mL) using PrepIT-L2P (DNA Genotek Inc.). The concentration and purity of isolated DNA was determined by spectrophotometry using a NanoDrop 2000c UV–VIS spectrophotometer (ThermoFisher Scientific, USA).

SNPs in seven genes—AS3MT, MTHFR, AQP4, AQP9, SELENOP, INMT, and MT2A—were selected based on a (1) literature search, (2) position in gene (preferably: exon > regulatory regions > intron), and (3) availability of pre-designed TaqMan® SNP Genotyping Assays. Accordingly, we have chosen 17 SNPs (Table S1): eight for AS3MT (rs7085104, rs3740400, rs3740393, rs3740390, rs11191439, rs10748835, rs1046778, and rs7897654); two each for MTHFR (rs1801131 and rs1801133), SELENOP (rs7579 and rs3877899), and AQP9 (rs2414539 and rs17240643); and one each for AQP4 (rs9951307), INMT (rs6970396), and MT2A (rs28366003). Two SNPs, rs7897654 in AS3MT and rs17240643 in AQP9, were genotyped only on a subset of participants; owing to their complete LD with two other SNPs (rs1046778 and rs2414539, respectively) in the corresponding genes, they were excluded from further analysis. The remaining seven AS3MT SNPs were marked as SNP1–7 based on their subsequent position in the gene.

All SNPs were genotyped using pre-designed TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Detailed information on the studied SNPs, including chromosome position, functional region, nucleotide or amino acid position, minor allele frequency (MAF), and TaqMan ID, is shown in the supplement (Table S1). The reaction volume was 5  $\mu\text{L}$ , and it contained 2.5  $\mu\text{L}$  of TaqMan® Universal Master Mix II with UNG

(Applied Biosystems, Foster City, CA, USA), 1.875  $\mu\text{L}$  of ultrapure nuclease-free water (Life Technologies, CA, USA), 0.125  $\mu\text{L}$  of 44X TaqMan probe/primer mix, and 0.5  $\mu\text{L}$  of genomic DNA. Amplifications and fluorescence detection were performed using LightCycler® 480 Instrument II and LightCycler® 480 Software version 1.5.1 (Roche). PCR cycling included (1) pre-PCR (1 cycle: 50 °C for 2 min), (2) activation (1 cycle: 95 °C for 10 min), (3) annealing and amplification (45 cycles for rs7579 and 50 cycles for all others: 95 °C for 15 s and 61 °C for 1 min), and (4) post-PCR (1 cycle: 40 °C for 30 s). In each genotyping run, a subset of randomly selected samples was repeated as a control (~10%).



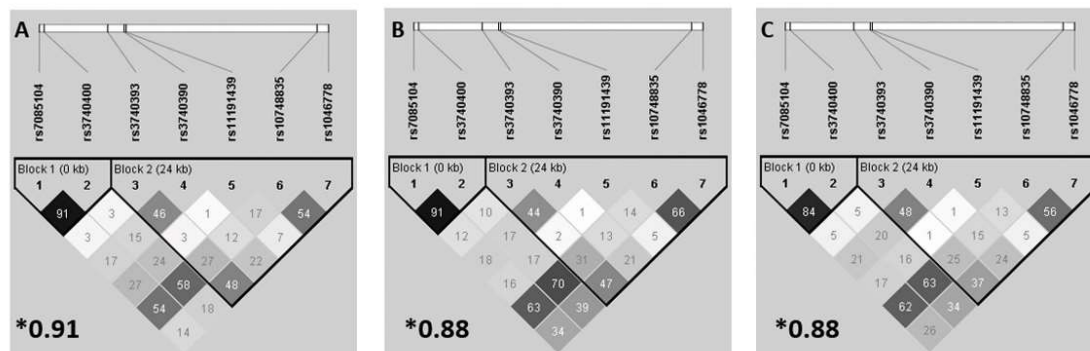


Fig. 2. LD plots and haplotype blocks for seven *AS3MT* SNPs in populations of pregnant women (A) and non-pregnant women (B) with children (C). The strength of the correlation between SNPs is presented in greyscale (darker colour indicates stronger correlation) and correspondingly by its pairwise  $R^2$  value. (SNPs are shown in 5'–3' order based on their position in the gene; \*multilocus D prime: measure of LD between block 1 and block 2 haplotypes; (Broad Institute, 2018).

### 2.5. Construction of *AS3MT* haplotypes

In comparison with haplotypes reported for populations from Argentina and Bangladesh constructed from eight SNPs (Engström et al., 2011), we selected seven SNPs and constructed our 'protective'/'neutral' haplotypes using six of them (as described below).

We performed LD analysis and construction of *AS3MT* haplotypes by using Haploview (version 4.2, Day Lab at the Broad Institute Cambridge, USA). Fig. 2 shows LD ( $R^2$ ) patterns for seven *AS3MT* SNPs in the main studied groups. They were similar for all study groups, with the strongest LD observed for SNP1 and SNP2 ( $R^2 = 0.84$ – $0.91$ ) and no or very low LD for SNP5 compared to the other SNPs ( $R^2 \leq 0.27$ ). Such results were observed in other studies as well (De la Rosa et al., 2017; Drobná et al., 2016; Engström et al., 2015). Owing to the strong LD between both haplotype blocks (multilocus D prime, 0.88–0.91; Fig. 2), we combined them into one haplotype but excluded SNP5 (rs11191439) owing to its very low LD compared with other SNPs. As such, we identified eight haplotype versions (with a sequence of six SNPs in 5'–3' order based on their position in the gene, as seen in Fig. 2) that had frequency above 1%. In the present study, statistics are presented for the carriers versus non-carriers of at least one copy of the 'protective' *AS3MT* haplotype with a sequence of minor alleles GGCTAC and carriers versus non-carriers of two copies of common alleles, namely, 'neutral' *AS3MT* haplotype with sequence ATGCGT.

### 2.6. Statistical analysis

Descriptive statistics, performed separately for each (sub)group, was used to assess the analyte concentrations that are expressed in terms of geometric means with 95 confidence interval and median with minimum and maximum (GM, 95 CI and P50, min-max), and age, weight, height, and BMI were expressed in terms of arithmetic means with minimum and maximum ( $\bar{x}$ , min-max). The frequencies of genotypes and alleles were expressed in terms of number and/or percentage N (%). Analyte values below the LOD were substituted with a value of LOD/2.

#### 2.6.1. Comparisons of As and Se parameters and SNP frequencies between groups

The differences in the distribution of As and Se parameters between (sub)groups were assessed using a non-parametric Mann-Whitney *U* test. Pearson's chi-squared test was used for comparing the SNP genotypes and allelic frequencies between groups and to evaluate the consistency of genotype frequencies with the Hardy-Weinberg equilibrium (HWE,  $p > 0.05$ ).

#### 2.6.2. Comparison of As and Se parameters between carriers and non-carriers of minor alleles inside each group

Owing to the relatively low number of participants, low number of minor homozygotes in the case of some SNPs (Table SP2), and low As exposure biomarkers, all SNPs and *AS3MT* haplotypes in statistical analysis were used as binary variables. Therefore, the participants of each study group were stratified based on the presence/absence of minor alleles in the case of individual SNPs or the presence/absence of *AS3MT* haplotypes. The differences in urinary As and Se concentrations and As methylation parameters (all representing dependent continuous variables) between carriers and non-carriers of the studied SNPs alleles or haplotypes (representing independent categorical variables) were tested by a non-parametric Mann-Whitney *U* test.

#### 2.6.3. Linear regression models

The possible influences of SNPs and *AS3MT* haplotypes for each group separately were further evaluated in multiple linear regression models by adjusting for several variables. Possible collinearity between variables was assessed by the variance inflation factor test. The age, weight, height, BMI, B-As, and P-Se were included as continuous variables and parity (0 = zero delivery versus 1 = 1–3 deliveries), child sex, gender, ever-smoking status (in last two years: yes vs. no), and smoking parents (at least one vs. none), as categorical variables. Regression models with AsIII%, MMA%, DMA%, PMI, SMI, TMI, AsIII, MMA, DMA, E- $UAs_M$ , or U-Se as outcome variables (Y) were specific for each of the main study groups as follows:

- pregnant women (A),  

$$Y = \beta_1 \times (\text{SNP of interest/haplotype}) + \beta_2 \times (\text{age}) + \beta_3 \times (\text{pre-pregnancy BMI}) + \beta_4 \times (\text{child sex}) + \beta_5 \times (\text{ever smoking}) + \beta_6 \times (\text{parity}) + \beta_7 \times (\text{P-Se}) + \beta_8 \times (\text{B-As})$$
- non-pregnant women (B),  

$$Y = \beta_1 \times (\text{SNP of interest/haplotype}) + \beta_2 \times (\text{age}) + \beta_3 \times (\text{BMI}) + \beta_4 \times (\text{ever smoking}) + \beta_5 \times (\text{P-Se}) + \beta_6 \times (\text{B-As})$$
- children (C),  

$$Y = \beta_1 \times (\text{SNP of interest/haplotype}) + \beta_2 \times (\text{gender}) + \beta_3 \times (\text{height}) + \beta_4 \times (\text{weight}) + \beta_5 \times (\text{smoking parents}) + \beta_6 \times (\text{P-Se}) + \beta_7 \times (\text{B-As})$$

Statistical analysis was performed using STATA12/SE statistical software on ln-transformed data to approximate the normal distribution with the level of statistical significance set at  $p < 0.05$  (two-sided). The results were visualized using OriginPro 2017 software (OriginLab, Northampton, USA) and Microsoft Excel 2016.

## 2.7. Confounders

Confounders (blood As, plasma Se, age, BMI, ever smoking status, parity, child sex, gender, weight, height, smoking of parents) were selected based on their potential biological relevance in As metabolism, as reported previously (Drobná et al., 2016; Khairul et al., 2017; Taylor et al., 2017; Tseng, 2009) and based on the availability of their data in the present study. The known influence of gestation week (GW) at sampling was not included owing to the missing information for 55 individuals (Gardner et al., 2011; Hopenhayn et al., 2003). When tested as a confounder in regression models for the remaining 81 women, its influence was insignificant. Despite the importance of folate supplementation (increase in DMA%; Gamble et al., 2007, 2006), its influence during pregnancy was not included either. The questionnaire data were too scarce. However, gynaecologists regularly prescribe its supplementation in both countries.

The possible release of residual tissue-bound As as DMA or/and as iAs and MMA during pregnancy, as mentioned in literature (Hopenhayn et al., 2003), is believed to be negligible in the present study owing to the very low levels of total As.

As in blood was chosen as a confounder, unlike in most similar studies, where the sum of urine As metabolites was used to adjust for iAs exposure levels. The range of iAs exposure levels for our population is relatively small; therefore, we did not expect its significant modification on the tested associations. Additionally, at low levels of As exposure, recent food consumption (especially seafood) could considerably influence the assessment of urine As metabolites; therefore, food intake for at least 2–3 days before urine sampling should be recorded (Molin et al., 2015; Taylor et al., 2017). We were missing such information, and the reliability of self-reported seafood intake could be questionable. As in blood well reflects recent exposure through food (Fowler et al., 2015) and internal As levels influenced by physiological variations (Balakrishnan et al., 2018).

Plasma selenium was used as the optimal (available) indicator of selenium nutritional status, as it is known to have an impact on As excretion (Alexander, 2015; Sun et al., 2014).

The gender of the foetus was included due to its recently reported effect on associations between genetic polymorphisms (AS3MT, AQP9) and urine arsenic metabolites in pregnant women (Drobná et al., 2016; Winterbottom et al., 2017).

## 3. Results and discussion

The three main study groups were age-matched pregnant women in the 3rd trimester and non-pregnant women and 7–8-year-old children of non-pregnant women (girls and boys). A subset of 21 pregnant women from group A was resampled 8–9 years after pregnancy to perform a direct individual comparison of their As and Se status during and after pregnancy.

Fig. 1 shows the sampling scheme for each group and subgroup and Table 1, their demographic and lifestyle characteristics. Table 2 shows arsenic and selenium parameters in the collected samples.

### 3.1. Arsenic levels

Observed total As levels were very low to moderate. GMs for main groups were 0.38–2.59 ng/g (ng/mL) in blood and 4.33–24.9 ng/mL SG in urine (Table 2). They were below or within the ranges usually reported for a general population with no known high As exposure: 2–23 ng/mL for blood (as a reference interval) (International Programme on Chemical Safety, 2004) and 5–50 ng/mL for urine (Fowler et al., 2015). In agreement with the self-reported daily seafood intake, the highest levels were observed in the group of pregnant women.

The sum of biologically active metabolites in urine was expected to be similar and very low for all groups; that for individuals with no

**Table 1**  
Basic characteristics of population groups as shown in Fig. 1.

|                                    | Pregnant women (A)<br>N = 136 |  | Non-pregnant (A <sub>1</sub> ) N = 21 |  | Non-pregnant women (B)<br>N = 176 |  | Children (C)<br>N = 176 |  | Girls (G <sub>1</sub> ) N = 86 |  | Boys (G <sub>2</sub> ) N = 90 |  |
|------------------------------------|-------------------------------|--|---------------------------------------|--|-----------------------------------|--|-------------------------|--|--------------------------------|--|-------------------------------|--|
| Age <sup>a</sup> years             | 30; 20–44 (127)               |  | 30; 20–38 (21)                        |  | 39; 30–51 (176) <sup>b</sup>      |  | 7; 7–8 (176)            |  | 7; 6; 7–8 (86)                 |  | 7; 7; 7–8 (90)                |  |
| Weight <sup>b</sup> kg             | 65; 45–122 (134) <sup>c</sup> |  | 62; 48–83 (21)                        |  | 69; 45–123 (176)                  |  | 29; 21–50 (176)         |  | 28; 21–43 (86)                 |  | 30; 21–50 (90)                |  |
| Height <sup>b</sup> cm             | 168; 153–186 (134)            |  | 169; 156–180 (21)                     |  | 167; 150–181 (176)                |  | 132; 99–155 (175)       |  | 131; 99–147 (85)               |  | 133; 99–155 (90)              |  |
| BMI <sup>b</sup> kg/m <sup>2</sup> | 23; 17–41 (134) <sup>c</sup>  |  | 22; 18–29 (21)                        |  | 24.8; 17.6–43.8 (176)             |  | —                       |  | —                              |  | —                             |  |
| GW <sup>c</sup> week               | 38; 33–41 (81)                |  | 39; 37–41 (18)                        |  | —                                 |  | —                       |  | —                              |  | —                             |  |
| Daily seafood intake               | 0.38; 0–2.21 (136)            |  | 0.46; 0.08–1.21 (20)                  |  | 0.18; 0–1.39 (176) <sup>b</sup>   |  | 0.14; 0–1.14 (176)      |  | 0.14; 0–0.69 (89)              |  | 0.14; 0–1.14 (90)             |  |
| Parity (0/1–3 deliveries) N        | 79/56                         |  | 13/8                                  |  | —                                 |  | —                       |  | —                              |  | —                             |  |
| Current smokers <sup>d</sup> N (%) | 11 (8.1)                      |  | 2 (10)                                |  | 28 (15.9)                         |  | —                       |  | —                              |  | —                             |  |
| Ever smokers <sup>d</sup> N (%)    | 54 (39.7)                     |  | 10 (48)                               |  | 33 (18.8)                         |  | —                       |  | —                              |  | —                             |  |
| Smoking parents <sup>d</sup> N (%) | —                             |  | —                                     |  | —                                 |  | 30 (17)                 |  | 16 (18)                        |  | 14 (16)                       |  |

Age, weight, height, BMI, and GW presented as  $\bar{x}$ ; min-max (N); daily seafood intake presented as GM; min-max (N).

<sup>a</sup> Significant difference ( $p < 0.05$ ) between non-pregnant (B) and pregnant women (A).

<sup>b</sup> Pre-pregnancy weight and pre-pregnancy BMI.

<sup>c</sup> Current smokers and those that quit smoking in the last two years.

<sup>d</sup> Children with at least one smoking parent; GW, gestational week at the time of sampling; Daily seafood intake factor was calculated as presented in Miklavčič et al. (2013).



**Table 2**  
Levels of As and Se parameters in population groups as shown in Fig. 1.

| Pregnant women (A)                                                 |                       | Non-pregnant (A <sub>N</sub> ) N = 21 |                                   | Non-pregnant women (B)       |                              | Children (C)                 |                              | Girls (C <sub>G</sub> ) N = 86 |  | Boys (C <sub>B</sub> ) N = 90 |  |
|--------------------------------------------------------------------|-----------------------|---------------------------------------|-----------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|--------------------------------|--|-------------------------------|--|
| N = 136                                                            |                       | N = 21                                |                                   | N = 176                      |                              | N = 176                      |                              | N = 86                         |  | N = 90                        |  |
| ARSENIC LEVELS GM, 95 CI; P50, min-max (N)                         |                       |                                       |                                   |                              |                              |                              |                              |                                |  |                               |  |
| B-As <sub>g</sub> <sup>a</sup><br>ng/gor ng/mL                     | 2.59, 2.12–3.15       | 3.91, 2.30–6.62                       | 2.00, 0.85–4.70                   | 0.65, 0.56–0.76 <sup>a</sup> | 0.38, 0.31–0.88 <sup>b</sup> | 0.48, 0.37–0.62 <sup>c</sup> | 0.31, 0.23–0.38 <sup>d</sup> |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 2.78, 0.27–29.2 (130) | 4.20, 0.41–22.1 (21)                  | 1.64, 0.19–135.2 (19)             | 0.53, 0.06–19.0 (168; 1)     | 0.33, 0.06–7.02 (136)        | 0.39, 0.08–7.02 (62)         | 0.25, 0.06–5.35 (73)         |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 22.9, 17.6–29.8       | 45.5, 20.7–99.7                       | 14.7, 5.47–39.5 <sup>e</sup>      | 3.42, 2.81–4.16 <sup>a</sup> | 3.58, 3.03–4.23              | 3.85, 3.04–4.86              | 3.35, 2.64–4.26              |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 29.4, 1.18–1199 (136) | 52.6, 1.18–664.6 (21)                 | 11.8, 0.66–1663 (21)              | 3.48, 0.06–291 (176)         | 3.69, 0.25–255 (176)         | 3.58, 0.45–255 (86)          | 3.84, 0.26–57.7 (90)         |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 24.9, 19.3–32.1       | 51.7, 26.7–100.4                      | 16.3, 6.42–41.3 <sup>e</sup>      | 4.27, 3.44–5.20 <sup>a</sup> | 4.33, 3.60–5.20              | 4.63, 3.58–5.99              | 4.06, 3.11–5.30              |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 27.6, 2.15–1279 (136) | 64.7, 4.95–680 (21)                   | 9.42, 1.80–3089 (21)              | 3.91, 0.05–145.6 (174)       | 4.26, 0.15–185 (175)         | 4.46, 0.37–185 (85)          | 4.06, 0.15–160 (90)          |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 0.11, 0.10–0.13       | 0.14, 0.09–0.20                       | 0.14, 0.09–0.20                   | 0.08, 0.07–0.09              | 0.08, 0.07–0.09              | 0.09, 0.07–0.10              | 0.08, 0.07–0.09              |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 0.10, 0.05–13.3 (136) | 0.10, 0.05–5.30 (21)                  | 0.10, 0.05–0.60 (21)              | 0.05, 0.05–0.63 (176)        | 0.05, 0.05–0.63 (176)        | 0.05, 0.05–0.63 (86)         | 0.05, 0.05–0.62 (90)         |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 0.12, 0.10–0.15       | 0.16, 0.09–0.28                       | 0.15, 0.10–0.22                   | 0.11, 0.09–0.12              | 0.10, 0.9–0.12               | 0.12, 0.09–0.15              | 0.09, 0.07–0.12              |                                |  |                               |  |
| U-As <sub>g</sub> <sup>a</sup><br>ng/mL                            | 0.11, 0.03–17.7 (136) | 0.13, 0.03–8.48 (21)                  | 0.16, 0.04–1.11 (21)              | 0.10, 0.02–2.33 (174)        | 0.09, 0.03–3.87 (175)        | 0.10, 0.03–1.90 (85)         | 0.07, 0.03–3.87 (90)         |                                |  |                               |  |
| U-MMA <sup>a</sup><br>ng/mL                                        | < LOD                 | < LOD                                 | < LOD                             | < LOD                        | < LOD                        | < LOD                        | < LOD                        |                                |  |                               |  |
| U-MMA <sup>a</sup><br>ng/mL                                        | 0.15, 0.13–0.35       | 0.18, 0.13–0.25                       | 0.28, 0.20–0.40 <sup>e</sup>      | 0.16, 0.14–0.18              | 0.17, 0.15–0.19              | 0.16, 0.14–0.18              | 0.18, 0.15–0.21              |                                |  |                               |  |
| U-MMA <sup>a</sup><br>ng/mL                                        | 0.10, 0.01–7.3 (136)  | 0.10, 0.10–0.6 (21)                   | 0.20, 0.10–0.90 (21)              | 0.10, 0.10–1.50 (176)        | 0.10, 0.10–1.31 (176)        | 0.10, 0.10–0.98 (90)         | 0.10, 0.10–0.98 (90)         |                                |  |                               |  |
| U-MMA <sup>a</sup><br>ng/mL                                        | 0.17, 0.14–0.19       | 0.22, 0.15–0.31                       | 0.31, 0.25–0.38                   | 0.20, 0.17–0.23              | 0.22, 0.19–0.25              | 0.21, 0.17–0.27              | 0.22, 0.18–0.27              |                                |  |                               |  |
| U-DMA <sup>a</sup><br>ng/mL                                        | 0.15, 0.05–16.7 (136) | 0.20, 0.06–0.96 (21)                  | 0.32, 0.13–0.83 (21)              | 0.20, 0.04–4.50 (174)        | 0.18, 0.05–3.36 (175)        | 0.18, 0.06–2.84 (85)         | 0.19, 0.05–3.36 (90)         |                                |  |                               |  |
| U-DMA <sup>a</sup><br>ng/mL                                        | 2.43, 2.06–2.85       | 3.02, 1.97–4.63                       | 2.27, 1.25–4.12                   | 1.12, 0.96–1.30 <sup>a</sup> | 1.45, 1.25–1.68 <sup>b</sup> | 1.45, 1.20–1.77 <sup>c</sup> | 1.44, 1.16–1.81              |                                |  |                               |  |
| U-DMA <sup>a</sup><br>ng/mL                                        | 2.35, 0.10–38.4 (136) | 2.70, 0.40–19.8 (21)                  | 2.00, 0.20–23.9 (21)              | 1.22, 0.10–15.8 (176)        | 1.79, 0.10–9.87 (176)        | 1.62, 0.10–9.87 (86)         | 1.90, 0.10–8.85 (90)         |                                |  |                               |  |
| U-DMA <sup>a</sup><br>ng/mL                                        | 2.67, 2.32–3.07       | 3.56, 2.57–4.92                       | 2.51, 1.56–4.04                   | 1.41, 1.17–1.69 <sup>a</sup> | 1.85, 1.56–2.19 <sup>b</sup> | 1.96, 1.55–2.46 <sup>c</sup> | 1.76, 1.40–2.27              |                                |  |                               |  |
| U-DMA <sup>a</sup><br>ng/mL                                        | 2.29, 0.22–38.4 (136) | 3.04, 1.40–14.4 (21)                  | 1.95, 0.58–29.3 (21)              | 1.39, 0.04–37.9 (174)        | 2.02, 0.06–37.1 (175)        | 2.03, 0.12–19.7 (85)         | 2.02, 0.06–37.1 (90)         |                                |  |                               |  |
| <sup>a</sup> U-AsB <sup>a</sup><br>ng/mL                           | 299, 124–942 (8)      | –                                     | –                                 | 6.50, 0.5–287 (18)           | 58.2, 15.4–389 (5)           | –                            | –                            |                                |  |                               |  |
| <sup>b</sup> U-AsB <sup>a</sup><br>ng/mL                           | 278, 104–1045 (8)     | –                                     | –                                 | 11.0, 0.86–133.2 (18)        | 70.6, 57.6–283 (5)           | –                            | –                            |                                |  |                               |  |
| U-CAsB <sup>a</sup><br>ng/mL                                       | 18.3, 13.5–24.7       | 37.8, 15.8–90.5                       | 9.62, 2.84–32.5 <sup>e</sup>      | 1.76, 1.31–2.34 <sup>a</sup> | 1.78, 1.39–2.28              | 2.11, 1.53–2.90              | 1.51, 1.04–2.20              |                                |  |                               |  |
| U-CAsB <sup>a</sup><br>ng/mL                                       | 25.8, 0.00–1191 (136) | 50.7, 0.00–645 (21)                   | 9.24, 0.00–1661 (21)              | 1.61, 0.00–287 (176)         | 1.24, 0.00–246 (176)         | 1.39, 0.00–246 (86)          | 1.02, 0.00–50.3 (90)         |                                |  |                               |  |
| U-CAsB <sup>a</sup><br>ng/mL                                       | 19.8, 14.8–26.5       | 43.0, 20.3–90.9                       | 10.5, 3.25–33.6                   | 2.16, 1.59–2.94 <sup>a</sup> | 2.15, 1.66–2.78              | 2.54, 1.80–3.61              | 1.82, 1.24–2.68              |                                |  |                               |  |
| U-ZAs <sub>g</sub> <sup>a</sup><br>ng/mL                           | 24.5, 0.00–1269 (136) | 62.4, 2.55–677 (21)                   | 7.90, 0.33–3094 (21) <sup>e</sup> | 2.21, 0.00–144 (174)         | 1.77, 0.00–179 (175)         | 2.06, 0.17–9 (85)            | 1.44, 0.00–119 (90)          |                                |  |                               |  |
| U-ZAs <sub>g</sub> <sup>a</sup><br>ng/mL                           | 2.93, 2.54–3.38       | 3.56, 2.36–5.36                       | 2.84, 1.64–4.91                   | 1.45, 1.27–1.66 <sup>a</sup> | 1.81, 1.59–2.05 <sup>b</sup> | 1.79, 1.50–2.13              | 1.82, 1.51–2.13              |                                |  |                               |  |
| U-ZAs <sub>g</sub> <sup>a</sup><br>ng/mL                           | 2.75, 0.55–38.6 (136) | 3.40, 0.55–20.0 (21)                  | 2.60, 0.35–24.8 (21)              | 1.46, 0.25–16.3 (176)        | 2.12, 0.25–10.0 (176)        | 1.89, 0.25–10.0 (86)         | 2.15, 0.25–9.68 (90)         |                                |  |                               |  |
| U-ZAs <sub>g</sub> <sup>a</sup><br>ng/mL                           | 3.23, 2.84–3.68       | 4.19, 3.03–5.80                       | 3.15, 2.04–4.84                   | 1.83, 1.54–2.16 <sup>a</sup> | 2.18, 1.86–2.54 <sup>b</sup> | 2.14, 1.72–2.66              | 2.20, 1.76–2.77              |                                |  |                               |  |
| <sup>a</sup> U-ZAs <sub>g</sub> <sub>g</sub> <sup>a</sup><br>ng/mL | 3.03, 0.57–38.5 (136) | 3.28, 1.80–20.0 (21)                  | 2.44, 0.79–29.9 (21)              | 1.76, 0.10–39.1 (174)        | 2.24, 0.15–41.2 (175)        | 2.16, 0.27–20.0 (85)         | 2.28, 0.15–41.2 (90)         |                                |  |                               |  |
| U-As <sub>g</sub> + AsV + MMA <sup>a</sup><br>ng/mL                | 21.5, 0.5–1007 (136)  | 37.2, 0.15–100 (21)                   | 15.1, 0.47–51.2 (21)              | 53.4, 1.44–100 (176)         | 58.2, 3.33–100 (176)         | 54.6, 3.33–100 (86)          | 61.7, 4.22–100 (90)          |                                |  |                               |  |
| U-As <sub>g</sub> + AsV + MMA <sup>a</sup><br>ng/mL                | 0.30, 0.26–0.34       | 0.35, 0.23–0.52                       | 0.44, 0.32–0.61                   | 0.26, 0.23–0.28              | 0.27, 0.24–0.30              | 0.26, 0.24–0.31              | 0.28, 0.24–0.32              |                                |  |                               |  |
| U-As <sub>g</sub> + AsV + MMA <sup>a</sup><br>ng/mL                | 0.25, 0.15–13.4 (136) | 0.30, 0.15–5.90 (21)                  | 0.40, 0.15–1.30 (21)              | 0.15, 0.15–2.27 (176)        | 0.15, 0.15–1.94 (176)        | 0.15, 0.15–1.94 (86)         | 0.19, 0.15–0.94 (90)         |                                |  |                               |  |
| U-As <sub>g</sub> + AsV + MMA <sup>a</sup><br>ng/mL                | 0.33, 0.28–0.38       | 0.41, 0.26–0.65                       | 0.48, 0.38–0.62                   | 0.32, 0.28–0.37              | 0.33, 0.28–0.38              | 0.32, 0.26–0.39              | 0.34, 0.27–0.42              |                                |  |                               |  |
| U-As <sub>g</sub> + AsV + MMA <sup>a</sup><br>ng/mL                | 0.29, 0.08–17.9 (136) | 0.35, 0.09–9.44 (21)                  | 0.49, 0.20–1.49 (21)              | 0.32, 0.06–6.30 (174)        | 0.27, 0.08–6.3 (175)         | 0.27, 0.08–6.3 (85)          | 0.29, 0.08–6.3 (90)          |                                |  |                               |  |
| METHYLATION EFFICIENCY GM, 95 CI; P50, min-max (N)                 |                       |                                       |                                   |                              |                              |                              |                              |                                |  |                               |  |
| AsIII <sub>g</sub>                                                 | 3.83, 3.16–4.62       | 3.81, 2.20–10.9                       | 4.81, 3.27–7.01                   | 5.78, 5.10–6.53 <sup>a</sup> | 4.50, 3.96–5.12 <sup>b</sup> | 4.85, 4.05–5.80 <sup>c</sup> | 4.19, 3.47–5.06              |                                |  |                               |  |
| AsIII <sub>g</sub>                                                 | 3.77, 0.13–71.4 (136) | 4.17, 0.24–42.4 (21)                  | 5.88, 1.21–24.0 (21)              | 6.02, 0.31–29.6 (176)        | 4.03, 0.50–32.2 (176)        | 4.33, 0.50–25.8 (86)         | 3.63, 0.82–32.2 (90)         |                                |  |                               |  |
| MMA <sub>g</sub>                                                   | 5.13, 4.40–5.98       | 5.13, 3.20–8.21                       | 9.81, 6.45–14.9 <sup>e</sup>      | 10.9, 9.83–12.2 <sup>a</sup> | 9.37, 8.44–10.4 <sup>b</sup> | 8.82, 7.80–10.2 <sup>c</sup> | 9.92, 8.56–11.5              |                                |  |                               |  |
| DMA <sub>g</sub>                                                   | 82.7, 77.2–88.5       | 84.9, 79.5–90.6                       | 12.1, 0.62–28.6 (21)              | 11.2, 0.74–40 (176)          | 9.37, 0.99–40.0 (176)        | 8.85, 0.99–40.0 (86)         | 10.1, 1.64–40.0 (90)         |                                |  |                               |  |
| DMA <sub>g</sub>                                                   | 88.1, 1.34–99.6 (136) | 88.2, 52.8–99.2 (21)                  | 80.0, 57.1–98.1 (21)              | 77.1, 74.8–79.4 <sup>a</sup> | 80.4, 78.2–82.6 <sup>b</sup> | 81.3, 78.6–84.1 <sup>c</sup> | 79.5, 76.1–83.0              |                                |  |                               |  |
| PMI                                                                | 1.34, 1.10–1.63       | 1.34, 0.99–2.00                       | 2.03, 1.49–2.77 <sup>e</sup>      | 1.89, 1.71–2.10 <sup>a</sup> | 2.08, 1.83–2.36 <sup>b</sup> | 1.82, 1.53–2.16              | 2.36, 1.97–2.84 <sup>c</sup> |                                |  |                               |  |
| PMI                                                                | 2.00, 0.01–146 (136)  | 2.00, 0.11–4.00 (21)                  | 2.00, 0.33–6.00 (21)              | 2.00, 0.14–12.0 (176)        | 2.78, 0.16–19.6 (176)        | 2.00, 0.17–10.0 (86)         | 2.00, 0.16–19.6 (90)         |                                |  |                               |  |
| SMI                                                                | 16.1, 13.3–19.5       | 16.5, 9.93–27.6                       | 8.13, 5.08–13.0 <sup>e</sup>      | 7.05, 6.18–8.04 <sup>a</sup> | 8.58, 7.56–9.73 <sup>b</sup> | 9.21, 7.73–11.0 <sup>c</sup> | 8.01, 6.67–9.62              |                                |  |                               |  |
| SMI                                                                | 15.5, 0.01–384 (136)  | 15.3, 3.25–198 (21)                   | 6.75, 2.00–158 (21)               | 7.25, 0.10–133 (176)         | 8.94, 1.098.7 (176)          | 9.10, 1.00–98.7 (86)         | 8.20, 1.00–59.2 (90)         |                                |  |                               |  |
| TMI                                                                | 24.2, 19.7–29.7       | 24.3, 13.5–43.6                       | 19.4, 12.8–29.2                   | 15.9, 13.9–18.2 <sup>a</sup> | 20.7, 18.0–23.8 <sup>b</sup> | 19.2, 15.8–23.3 <sup>c</sup> | 22.2, 18.2–27.2              |                                |  |                               |  |
| TMI                                                                | 25.5, 0.40–770 (136)  | 25.0, 1.36–398 (21)                   | 16.0, 3.17–81.0 (21)              | 15.6, 2.38–325 (176)         | 23.8, 2.10–199 (176)         | 22.1, 2.87–199 (86)          | 26.6, 2.10–120 (90)          |                                |  |                               |  |

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(continued on next page)

Table 2 (continued)

| SELENIUM LEVELS GM, 95 CI; F50, min-max (N) |                          |                         |                              |                                       |                                       |                                      |                              |  |  |
|---------------------------------------------|--------------------------|-------------------------|------------------------------|---------------------------------------|---------------------------------------|--------------------------------------|------------------------------|--|--|
| B-Se <sub>ng/g#</sub>                       | 94.8, 91.4–98.3          | 90.2; 79.5–102          | 120, 113–129 <sup>e</sup>    | 105; 103–107 <sup>a</sup>             | 82.3, 79.9–84.7 <sup>b</sup>          | 85.0, 81.6–88.7 <sup>c</sup>         | 79.8, 76.6–83.2 <sup>d</sup> |  |  |
| P-Se <sub>ng/ml</sub>                       | 94.8, 42.4–182.4 (130)   | 93.0 42.0–142 (21)      | 115, 104–166 (19)            | 106, 68.7–142 (168)                   | 84, 42.2–133 (136)                    | 86.0, 55.4–133 (62)                  | 82.3, 42.2–109 (73)          |  |  |
| U-Se <sub>ng/ml</sub>                       | 54.8, 52.8–56.9          | 55.9, 51.4–60.7         | 98.8, 92.8–105 <sup>e</sup>  | 82.3, 80.4–84.0 <sup>a</sup>          | 65.8, 64.3–67.3 <sup>b</sup>          | 64.6, 62.6–67.0 <sup>c</sup>         | 66.8, 64.8–68.9              |  |  |
| U-Se <sub>ng/ml</sub>                       | 56, 33.0–96.0 (135)      | 57.0, 38.0–81.0 (21)    | 96.0, 78.0–126 (19)          | 81.6, 54.9–118 (168)                  | 66, 48.8–108 (136)                    | 65.9, 52.2–95.1 (62)                 | 67.1, 48.8–108 (73)          |  |  |
| U-Se <sub>ng/ml</sub>                       | 19.2, 16.6–21.9          | 17.3, 11.1–26.9         | 12.9, 9.50–17.4 <sup>e</sup> | 7.42, 6.14–8.50 <sup>a</sup>          | 11.5, 10.2–13.1 <sup>b</sup>          | 10.3, 8.49–12.2                      | 13.0, 10.9–15.5              |  |  |
| U-Se <sub>ng/ml</sub>                       | 22.0, 2.00–64.6 (136)    | 17.7, 2.00–64.6 (21)    | 13.6, 2.57–40.8 (21)         | 6.89, 0.06–95.3 (176)                 | 14.1, 1.33–58.9 (176)                 | 13.1, 1.33–54.1 (86)                 | 15.4, 1.69–58.9 (90)         |  |  |
| U-Se <sub>ng/ml</sub>                       | 20.8, 19.3–22.5          | 19.6, 15.4–25.1         | 14.3, 12.9–15.8 <sup>e</sup> | 9.33, 7.89–11.1 <sup>a</sup>          | 13.8, 11.9–16.1 <sup>b</sup>          | 12.1, 9.76–14.9                      | 15.8, 12.7–19.6              |  |  |
| U-Se <sub>ng/ml</sub>                       | 22.3, 4.48–48.5 (136)    | 21.7, 4.62–43.1 (21)    | 13.9, 9.65–21.2 (21)         | 9.81, 0.02–229 (176)                  | 13.5, 1.08–226.9 (176)                | 11.7, 1.26–134 (85)                  | 16.9, 1.08–226.9 (90)        |  |  |
| #SG                                         | 1,016, 1,002–1,035 (136) | 1,015, 1,002–1,026 (21) | 1,013, 1,003–1,025 (21)      | 1,012, 1,002–1,032 (174) <sup>a</sup> | 1,017; 1,002–1,035 (175) <sup>b</sup> | 1,016; 1,002–1,032 (85) <sup>c</sup> | 1,018; 1,002–1,035 (90)      |  |  |

<sup>a</sup>Data is presented as  $\bar{x}$ , min-max (N); <sup>b</sup> different units are due to the different measurement methods used (see 'Material and methods' section). <sup>c</sup> significant difference between groups (p < 0.05); <sup>d</sup> non-pregnant (B) vs. pregnant women (A), <sup>e</sup> children (C) vs. their mothers (B), <sup>f</sup> girls (C), <sup>g</sup> vs. girls (C), <sup>h</sup> subgroups A" vs A<sub>1</sub>, \* for marginally significant results (p < 0.01); LOD, limit of detection; U-As (total As), cAsB, calculated level of AsB based on subtraction of U-As from U-As; U-ΣAs<sub>sum</sub> sum of AsIII, MMA and DMA; U-ΣAs<sub>4%</sub>%, percentages calculated from U-As.

known exposure to inorganic arsenic the sum of biologically active metabolites in urine is reported to be generally below 10 ng/mL in European countries (International Programme on Chemical Safety, 2004). Indeed, the sums were much lower than total urine As and below 10 ng/mL (GMs 1.87–3.23 ng/mL<sub>SG</sub>) (Table 2). Only a few individuals exceeded 10 ng/mL (13 pregnant women, 2 non-pregnant women and 1 child). However, as in the case of total As, the sum of metabolites was significantly higher among pregnant women, perhaps due to the possible presence of minor amounts of DMA derived from (sea)food items (Taylor et al., 2017). Seafood can contain As in the form of DMA and/or As-sugars that are further metabolized into DMA (Taylor et al., 2017; Thomas and Bradham, 2016). Therefore, in some more recent studies, exposure to iAs is estimated by the sum of AsIII, AsV, and MMA only, and DMA is excluded (Hata et al., 2016). Upon excluding DMA, the differences between our groups lost statistical significance (Table 2). Therefore, in general, in all groups the exposure to iAs was almost equal and very low, as the greatest amounts of urine As were found in the fraction of calculated AsB. Regardless of the group, the calculated As was 0–99% with arithmetic means: 53% for all, 78% for pregnant women, 47% for non-pregnant, and 42% for their children. In participants with urine As > 50 ng/mL (N = 40), the percentage range was 73–99% with consequently higher arithmetic mean of 94%. The presence of undefined As species was tested by mass balance analyses for a few samples (n = 31). We observed some differences between total As levels and the sum of measured metabolites; however, these were within the ranges attributed to analytical reasons, as described in the material and methods section. Skróder et al. (2016) observed a similar phenomenon with mass balance in a study on Bangladeshi children exposed to high iAs levels. They reported a large difference between all analysed species and total As for a subset of samples within the highest quartile of U-Se levels (18–55 ngSe/mL<sub>SG</sub>) and speculated on the possible elimination of the As-Se complex with urine. However, our tested cases with lower iAs and even higher urine Se levels did not confirm such trends (51–229 ngSe/mL<sub>SG</sub>).

### 3.2. Selenium levels

Selenium can substantially influence arsenic metabolic pathways and toxicity as mentioned in introduction (Alexander, 2015; Falnoga et al., 2014; George et al., 2013; Sun et al., 2014; Zeng et al., 2005). Selenium deficiency and variability in its metabolic pathways can decrease the formation of arsenic-selenium complexes and, consequently, As elimination. Blood and plasma selenium can be used as rough estimates of Se nutritional status and general antioxidant status (Alexander, 2015). In the present study, GMs for Se in blood (plasma) and urine for the main investigated groups were respectively 94.1 ng/g (54.8 ng/mL) and 20.8 ng/mL<sub>SG</sub> for pregnant women; 105 ng/mL (82.3 ng/mL) and 9.33 ng/mL<sub>SG</sub> for non-pregnant women; and 82.3 ng/mL (65.8 ng/mL) and 13.8 ng/mL<sub>SG</sub> for children (Table 2). They are within or close to the normal reference intervals as set for the world-wide general population by Roberts et al. (2012): 58–234 ng/mL for blood (group not specified), 55–134 ng/mL for plasma of 4–16-year-old children, and 63–160 ng/mL for plasma of adults. Plasma levels are within the range reported for similar European study groups in terms of age and pregnancy: 34–63 ng/mL for pregnant women (3<sup>rd</sup> trimester), 66–97 ng/mL for non-pregnant women, and 53–75 ng/mL for children (Stoffaneller and Morse, 2015; Zachara, 2016). They are also above or close to the level required for sufficient activity of glutathione peroxidase (63.1 ng/mL) commonly used as a better marker for Se adequacy (Stoffaneller and Morse, 2015).

Decrease in blood/plasma Se during pregnancy is attributed to many factors like Se transportation through the placenta to the developing foetus (Zachara, 2016) and to a pregnancy-related increase in blood and stroke volume, heart rates and plasma volume, faster metabolism, and increased urine elimination (increase in renal plasma flow, glomerular filtration rate up to 85%, and decreased tubular



**Table 3**Distribution of selected SNPs alleles (minor and major) and *AS3MT* haplotypes ('protective' and 'neutral') in study groups.

| SNPs/haplotype         | Allele/haplotype    | Pregnant women (A) % | Non-pregnant women (B) % | Children (C) |                           |                          |
|------------------------|---------------------|----------------------|--------------------------|--------------|---------------------------|--------------------------|
|                        |                     |                      |                          | All %        | Girls (C <sub>1</sub> ) % | Boys (C <sub>2</sub> ) % |
| <b>AS3MT</b>           |                     |                      |                          |              |                           |                          |
| SNP1: rs7085104        | A                   | 62.2                 | 63.6                     | 57.2         | 50.0                      | 64.0                     |
|                        | G                   | 37.7                 | 36.4                     | 42.8         | 50.0*                     | 36.0**                   |
| SNP2: rs3740400        | T                   | 60.3                 | 62.2                     | 55.5         | 49.4                      | 61.2                     |
|                        | G                   | 39.7                 | 37.8                     | 44.5         | 50.6*                     | 38.8**                   |
| SNP3: rs3740393        | G                   | 81.9                 | 81.0                     | 78.6         | 79.5                      | 77.8                     |
|                        | C                   | 18.1                 | 19.0                     | 21.4         | 20.5                      | 22.2                     |
| SNP4: rs3740390        | C                   | 90.7                 | 90.5                     | 86.4         | 85.5                      | 87.2                     |
|                        | T                   | 9.3                  | 9.5                      | 13.6         | 14.5                      | 12.8                     |
| SNP5: rs11191439       | T                   | 86.0                 | 90.3                     | 88.8         | 86.0                      | 85.8                     |
|                        | C                   | 14.0                 | 9.7                      | 11.2         | 14.0                      | 14.2                     |
| SNP6: rs10748835       | G                   | 55.1                 | 56.8                     | 52.1         | 47.5                      | 56.2                     |
|                        | A                   | 44.9                 | 43.2                     | 47.9         | 52.5                      | 43.8                     |
| SNP7: rs1046778        | T                   | 68.4                 | 66.5                     | 62.8         | 61.4                      | 64.0                     |
|                        | C                   | 31.6                 | 33.5                     | 37.2         | 38.6                      | 36.0                     |
| 'protective' haplotype | GGCTAC <sup>a</sup> | 9.6                  | 9.5                      | 11.6         | 12.2                      | 11.7                     |
| 'neutral' haplotype    | ATGCGT <sup>b</sup> | 51.5                 | 55.6                     | 49.7         | 46.2                      | 53.1                     |
| <b>MTHFR</b>           |                     |                      |                          |              |                           |                          |
| rs1801131              | T                   | 73.9                 | 73.0                     | 74.3         | 76.2                      | 72.5                     |
|                        | G                   | 26.1                 | 27.0                     | 25.7         | 23.8                      | 27.5                     |
| rs1801133              | G                   | 63.6                 | 64.8                     | 61.3         | 60.7                      | 61.8                     |
|                        | A                   | 36.4                 | 35.2                     | 38.7         | 39.3                      | 38.2                     |
| <b>AQP4</b>            |                     |                      |                          |              |                           |                          |
| rs9951307              | A                   | 69.8                 | 66.0                     | 66.3         | 62.8                      | 69.5                     |
|                        | G                   | 30.2                 | 34.0                     | 33.7         | 37.2                      | 30.5                     |
| <b>AQP9</b>            |                     |                      |                          |              |                           |                          |
| rs2414539              | A                   | 82.0                 | 82.1                     | 79.6         | 81.0                      | 78.3                     |
|                        | T                   | 18.0                 | 17.9                     | 20.4         | 19.0                      | 21.7                     |
| <b>SELENOP</b>         |                     |                      |                          |              |                           |                          |
| rs7579                 | C                   | 70.8                 | 69.0                     | 69.7         | 69.0                      | 70.3                     |
|                        | T                   | 29.0                 | 31.0                     | 30.3         | 31.0                      | 29.7                     |
| rs3877899              | C                   | 73.2                 | 76.7                     | 80.2         | 81.5                      | 79.0                     |
|                        | T                   | 26.8                 | 23.3                     | 19.8         | 18.5                      | 21.0                     |
| <b>INMT</b>            |                     |                      |                          |              |                           |                          |
| rs6970396              | G                   | 89.7                 | 88.6                     | 87.3         | 89.8                      | 84.8                     |
|                        | A                   | 10.3                 | 11.4                     | 12.7         | 10.2                      | 15.2                     |
| <b>MT2A</b>            |                     |                      |                          |              |                           |                          |
| rs28366003             | A                   | 93.0                 | 95.0                     | 98.0         | 99.0                      | 96.6                     |
|                        | G                   | 7.0                  | 5.0                      | 2.0          | 1.0                       | 3.4                      |

haplotype consisting of SNPs (allele) in 5'–3' order: <sup>a</sup> rs7085104(G), rs3740400(G), rs3740393(C), rs3740390(T), rs10748835(A), and rs1046778(C) and <sup>b</sup> rs7085104(A), rs3740400(T), rs3740393(G), rs3740390(C), rs10748835(G), and rs1046778(T); \* sig difference between groups A and C<sub>1</sub>, \*\*sig difference between groups C<sub>1</sub> and C<sub>2</sub>.

reabsorption) (Cheung and Lafayette, 2013; Gardner et al., 2011; Soma-Pillay et al., 2016). For children, the situation may be related to their faster metabolism at this age (Hall et al., 2009). Owing to the small number of samples, the gender differences were noticed for blood Se only. Levels were slightly but significantly higher for girls than for boys (GM = 85.0 ng/mL for girls and 79.8 ng/mL for boys), as shown in Table 2. Similarly, in a study on British children (174 boys and 132 girls of age 7–10 years) Bates et al. (2002) reported significantly higher erythrocyte Se levels among girls than boys (GMs = 1.53 vs 1.41 μmol/L, respectively).

Concomitant higher urine Se levels observed in pregnant women and children are less known but are obviously related to similar metabolic reasons and relatively good nourishment with selenium. In case of deficiency, its urine excretion would be diminished (Alexander, 2015).

### 3.3. As methylation efficiency

As in studies with higher iAs exposure (Gardner et al., 2011; Hopenhayn et al., 2003), the most efficient As methylation was observed for pregnant women. They had significantly lower AsIII% and MMA%, higher DMA%, and, accordingly, lower PMI with higher SMI and TMI than the non-pregnant group (Table 2). The same differences,

although not significant for each parameter, were confirmed for the 21 selected pregnant women by comparing their status during pregnancy and non-pregnancy, although they were from a different region with higher absolute levels of blood As and higher pre-pregnancy blood (plasma) (Table 2). Some of the various factors (seafood intake, BMI, age, selenium levels, etc.) which can influence arsenic metabolic pathways were considered during association analysis between SNPs and arsenic (and selenium) parameters for each group separately. As discussed in the previous section, pregnancy is accompanied by various anatomical and physiological changes, and some of them are at their peak during the 3<sup>rd</sup> trimester (Cheung and Lafayette, 2013; Soma-Pillay et al., 2016). They influence the pathways and concentrations of essential elements like selenium, and similarly, they can influence As metabolism on more levels (Engström et al., 2011; Gardner et al., 2011; Li et al., 2008; Tseng, 2009).

More efficient As methylation was also observed among children when compared to their mothers; children had lower MMA% and higher DMI% with higher secondary and total methylation index. Literature differences in As methylation capacity between children and adults from regions with high iAs exposure are inconsistent (Bangladesh, Argentina, Chile) (Chowdhury et al., 2003; Schröder Löveborn et al., 2016; Shen et al., 2016), and no studies with low As exposure are reported. According to Hall et al. (2009), children at this

young age (7–8 years) are in a period of rapid growth and increased physical activity that requires upregulation of various metabolic pathways, this can also contribute to more efficient As methylation, increased elimination, but also to higher retention of protein-bound As in body tissues (Hall et al., 2009). Differences between girls and boys are evident although statistically insignificant (Table 2). Literature data are again inconsistent (Recio-Vega et al., 2016; Skräder Löveborn et al., 2016; Torres-Sánchez et al., 2016).

#### 3.4. AS3MT, MTHFR, AQP4, AQP9, SELENOP, INMT, and MT2A polymorphisms and their relationship with As and Se (regression models)

The influence of genetic polymorphisms (SNPs) of selected genes on urine As/As metabolites Se was studied for each study group separately. All genotyped SNPs—seven in AS3MT, two in MTHFR and SELENOP; and one each in INMT, AQP4, AQP9, and MT2A—were in Hardy-Weinberg equilibrium ( $p > 0.05$ ).

In comparison with similar association studies, we were dealing with very low levels of iAs metabolites in urine (median range: 1.76–3.03 ng/mL<sub>SG</sub>,  $n = 488$ ). For example, these were at least 30–60 times lower than those reported for Argentina (median: 200 ng/mL<sub>SG</sub>,  $n = 172$ ) and Bangladesh (median: 100 ng/mL<sub>SG</sub>,  $n = 361$ ) (Engström et al., 2011) and around two times lower than those reported for European countries such as Hungary, Romania, and Slovakia with known hotspots of iAs in drinking water (median: 8 ng/mL<sub>SG</sub>,  $n = 415$ ) (Lindberg et al., 2007). Only Balakrishnan et al. (2018) have recently reported ethnicity and genetic influences on As methylation in six US communities exposed to similarly low levels of iAs (median range: 2.3–4.9 ng/mL<sub>SG</sub>,  $n = 264$ ).

##### 3.4.1. Allele frequencies of study groups

Table 3 shows minor and common alleles frequencies of all studied SNPs and AS3MT haplotypes ('protective' and 'neutral'); the frequencies of SNPs' genotypes are given in Table SP2 in the supplements. Allele frequencies are relatively close to those reported for populations with European ancestry (HapMap, CEU, TSI) (National Center for Biotechnology Information, 2018) (Table SP1), and as expected, there were no significant differences in allele frequencies between the main study groups. The only difference observed for AS3MT SNPs (rs7085104 and rs3740400) was significantly higher MAF for girls than boys (Table 3). It could be coincidental due to low number of subjects in each subgroup.

Our central focus was on AS3MT as the best-studied gene in relation with As metabolism. Fig. 3 shows AS3MT SNPs minor allele frequencies compared with those for populations from other countries having similar or higher exposure to iAs. Our frequencies are significantly lower than those reported for populations known to be genetically adapted to high iAs exposure through many generations, such as Argentina (SAC, ~2x higher for all SNPs and even ~8x for SNP4) (Schlebusch et al., 2015a, 2015b, 2013) and Chile (differences are smaller) (Apata et al., 2017). They are also lower than those reported for the populations of China and Japan that could have been adapted to As through their historical dietary habits, with higher iAs intake through dietary sources like rice and algae (Thomas and Bradham, 2016). Less evident are differences with Bangladesh, having iAs-contaminated water throughout the country during the last century; frequencies were significantly higher for only two AS3MT SNPs and even lower for one. For the Nigerian population (Africa), all frequencies were lower than ours.

The frequencies of the 'protective' haplotype are reported less often. They were between 9% and 12%; these are close to those reported for European countries (Romania, Slovakia, and Hungary: 12%) (Engström et al., 2015) and considerably lower than those found in countries with known (or supposed) historical exposure to high iAs from drinking water (Argentina: 70%, Chile: 48–68%, and Peru: 50%) (Apata et al., 2017; De la Rosa et al., 2017; Engström et al., 2011; Schlebusch et al., 2013). The supplement shows frequency comparisons between various

'protective' haplotypes reported in literatures and corresponding minor haplotypes in our population; we constructed our complimentary sets of haplotypes based on the combination and number of SNPs (Fig. SP1).

##### 3.4.2. Associations between studied SNPs and selected As parameters

Table 4 summarizes the significant differences in the data distribution of various urine As and Se parameters between the carriers and non-carriers of minor alleles for each SNP (or haplotype) in each group (pregnant – A, non-pregnant – B, children – C). Results that were significant in multiple regression models adjusted for various confounders are indicated in bold. Obviously, the influence of various SNPs on the levels of urine As/As metabolites and Se was minimal although significant. The correspondingly low strength of these associations, presented by the coefficient  $\beta$ , is shown in Fig. 4. The influences of confounders (beta coefficients) are summarised in a generalized way in Table 7. Data (ranges) are based on all individual linear regression models for SNPs of genes that were found to have a significant effect on at least one As or Se parameter (specified in Fig. 4).

Associations between SNPs and As(Se) parameters were the most significant among non-pregnant women, less so among pregnant women, and almost absent among children. They are discussed separately for each gene below; all observed influences are commented on in relation to the effects of minor alleles.

**3.4.2.1. AS3MT.** Despite the very low levels of biologically active As metabolites (Table 2), we observed the 'protective' effect of six individual SNPs (minor alleles) and their haplotype in non-pregnant women only (lower MMA% and higher SMD) (Table 4). Associations were completely absent among pregnant women and almost absent among children (an exception was the influence of SNP4). The possible negative effect of 'neutral' haplotypes (common alleles) was not observed. In general, regression models confirmed the results obtained from bivariate comparisons.

Table 5 shows comparisons with existing studies conducted on women (Drobná et al., 2016; Engström et al., 2011, 2007), children (Recio-Vega et al., 2016), and mixed populations (Balakrishnan et al., 2018; Chen et al., 2017; Lindberg et al., 2007; Xu et al., 2016). In general, these individual SNPs and their 'protective' haplotypes were found to be strongly linked with more efficient As methylation in the populations with high iAs exposure. In several studies, they consistently show associations with lower levels of urine AsIII% and MMA% and higher DMA% accompanied with higher secondary and total methylation efficiency index in general populations and pregnant women.

As in the study by Xu et al. (2016) for a Mexican population, we observed the strongest influence for rs3740393 that was significantly associated with almost all As methylation parameters (AsIII%, MMA%, DMA%, SMI, and TMD). The association of rs11191439 (SNP5) with poorer As methylation as found in populations with high iAs exposure levels (Bangladesh and Mexico) (Engström et al., 2011; Recio-Vega et al., 2016) (Table 5) was not observed in our study (Table 4 and Fig. 4). Interestingly, in a study on Central European populations (Romania, Hungary, and Slovakia) exposed to low levels of iAs, Lindberg et al. (2007) observed an association among men but, as in our study, not among women ( $n = 415$ , 225 men; median U-ΣAs<sub>M</sub>: 8 ng/mL<sub>SG</sub>).

Regarding our pregnant women and children, it seems that at low iAs exposure, the altered and more variable physiology during pregnancy and children's growth period (Hall et al., 2009; Soma-Pillay et al., 2016) dominates over the genetic influences on iAs metabolism. This is also in agreement with Gardner et al. (2012), who found a stronger effect of pregnancy than of the AS3MT haplotype on the As metabolite pattern of pregnant Bangladeshi women ( $n = 303$ ; median U-ΣAs<sub>M</sub>: 90 ng/mL<sub>SG</sub>; GW 30). However, unlike in our study, they and some other research groups studying pregnant women in Argentina (Engström et al., 2007) and Mexico (Drobná et al., 2016) observed significant influences of SNPs on As metabolite patterns (Table 5), most probably owing to the much higher iAs exposure levels in their studies.



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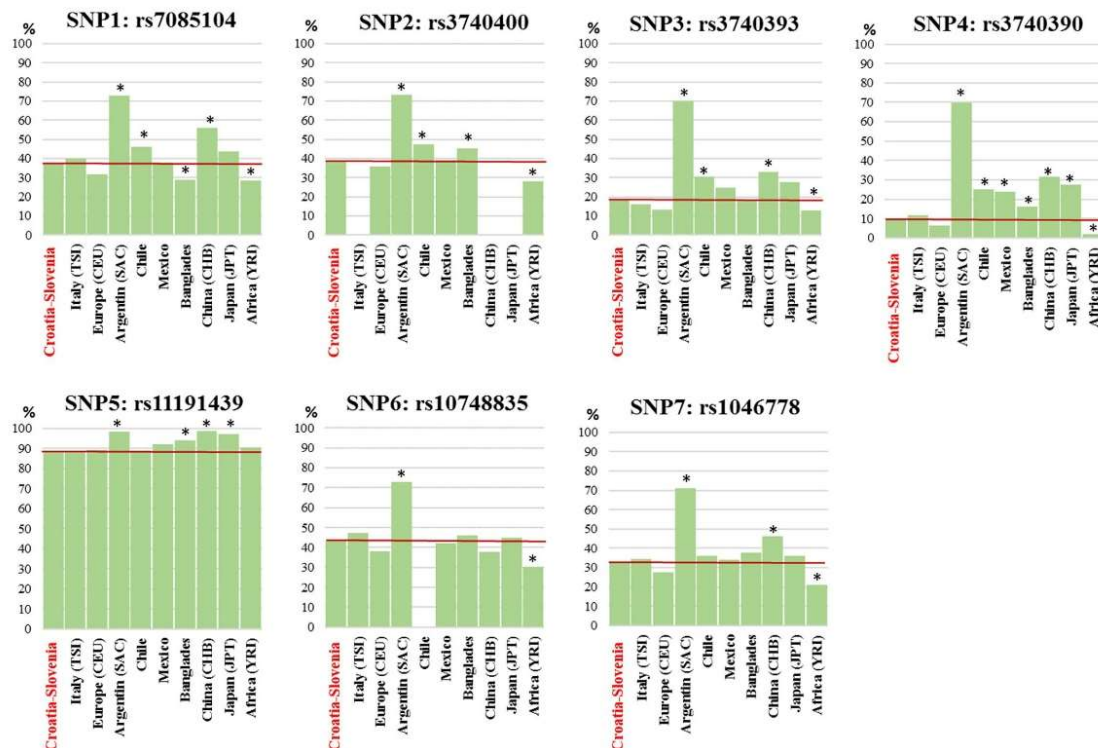


Fig. 3. Comparison of six minor and one major (SNP5) *AS3MT* allele frequencies between present Croatian-Slovenian population (group A + B only, n = 308–312) with those reported for other populations. Italy, Tuscany (TSI; n = 172–176), Utah residents with Northern and Western European ancestry (CEU; n = 120–226), China, Beijing (CHB; n = 80–82), Africa, Nigeria (YRI; n = 118–226), Japan, Tokyo (JPT; n = 120–172), (National Center for Biotechnology Information, 2018); San Antonio de los Cobros, Argentina (SAC; n = 169–171) (Engström et al., 2011), Chile (n = 491–493) (De la Rosa et al., 2017), Mexico (n = 196–197) (Drobná et al., 2016), Bangladesh (n = 351–360) (Engström et al., 2011); \*observed significant differences in allele frequency with our population (red reference line) as assessed using Pearson's chi-squared test, p < 0.05).

The influence of SNPs or their magnitude can differ at different As exposure levels (Xu et al., 2016).

**3.4.2.2. MTHFR.** MTHFR is a coding folate-metabolizing enzyme, and it has been studied frequently in relation to As metabolism (Broberg et al., 2015). We tested two *MTHFR* SNPs, rs1801131 and rs1801133, whose minor alleles are related to a 20–35% decrease in the MTHFR activity of the general population (Vidmar et al., 2016). Lower activity leads to lower levels of folate and consequently, lower SAM (main methyl donor for As methylation) and poorer As methylation (Broberg et al., 2015). However, this effect is probably seen only at higher As exposure levels in the general population (Spratlen et al., 2017). Our results obtained from simple bivariate comparisons did show a significant difference for both SNPs among pregnant women. rs1801131 was associated with lower AsIII% and DMA% and higher TMI and rs1801133, with higher AsIII% and lower DMA%, SMI, TMI, and U-ΣAs<sub>M</sub> (Table 4). As such, trends for the second SNP are consistent with the observations from the literature; Table 6 shows a detailed comparison with literature data. However, after adjusting for confounders, no association was significant (Fig. 4). This could be attributable to low levels of As and sufficient levels of folate, which is regularly prescribed during pregnancy as a supplement by gynaecologists. For clearer interpretation, folate levels should be measured.

**3.4.2.3. AQP4 and AQP9.** Human aquaporins (AQP0–AQP12) are membrane proteins that are important for the transport of water, small solutes, and gases across cellular membranes in various organs (Day et al., 2014). Some aquaporins like AQP3, AQP4, AQP7, AQP9, and AQP10 have been previously recognized to play an important role in the cellular uptake of AsIII in various organisms including humans (Calatayud and Laparra Llopis, 2015; Calatayud et al., 2012; Liu et al., 2002; Matsuzaki et al., 2004; Mukhopadhyay et al., 2014; Roggenbeck et al., 2016). In human As metabolism, they have been mainly studied on the basis of their gene expression; As<sub>2</sub>O<sub>3</sub> uptake was associated with expression of AQP9 (in leukemic cells) (Khairul et al., 2017) and AQP4 (in Caco-2 cell lines as model for intestinal epithelium) (Calatayud and Laparra Llopis, 2015; Calatayud et al., 2012). However, no studies have thus far investigated the influence of AQP SNPs on As metabolism. We tested such relations for AQP4 SNP rs9951307 and AQP9 SNP rs2414539.

AQP4 represents the main water channel in the brain, however, it is also expressed in the kidney, gastrointestinal tract, and placenta (Day et al., 2014; Matsuzaki et al., 2004; Spratlen et al., 2017). Bivariate analysis and regression models clearly showed the significant association of AQP4 rs9951307 with poorer As methylation among pregnant women. They had higher concentration and percentage of AsIII and lower PMI and TMI indexes (Table 4 and Fig. 4). The results can be explained by the role played by AQP4 in kidney water flow regulation (better reabsorption of water into blood) (Day et al., 2014) and lower

**Table 4**

Differences in As and Se parameters between carriers and non-carriers of SNPs minor alleles among pregnant women, non-pregnant women, and children (Mann-Whitney test).\*

| Pregnant woman                  |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
|---------------------------------|------------------------|---------|-------|-------|-------|-------|-------|-----------------------------|---------------------------|----------------------------|-------------------------------------------|-----------------------------|
| GENE/SNPs                       | Allele (N)             | AsIII % | MMA % | DMA % | PMI   | SMI   | TMI   | AsIII(ng/mL <sub>SG</sub> ) | MMA(ng/mL <sub>SG</sub> ) | DMA (ng/mL <sub>SG</sub> ) | U-ΣAs <sub>M</sub> (ng/mL <sub>SG</sub> ) | U-Se (ng/mL <sub>SG</sub> ) |
| <b>MTHFR</b>                    |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs1801131                       | G+ (62)                | 2.80    |       | 81.0  | 1.71  |       | 33.4  | 0.10                        |                           |                            |                                           |                             |
|                                 | G- (74)                | 4.96    |       | 84.1  | 1.09  |       | 18.4  | 0.15                        |                           |                            |                                           |                             |
|                                 | p-value                | 0.003   |       | 0.014 | 0.068 |       | 0.003 | 0.031                       |                           |                            |                                           |                             |
| rs1801133                       | A+ (80)                | 4.57    | 5.84  | 80.6  |       | 13.8  | 20.2  |                             |                           | 2.31                       | 2.86                                      |                             |
|                                 | A- (56)                | 2.97    | 4.26  | 85.7  |       | 20.1  | 31.2  |                             |                           | 3.30                       | 3.85                                      |                             |
|                                 | p-value                | 0.015   | 0.059 | 0.016 |       | 0.036 | 0.015 |                             |                           | 0.009                      | 0.018                                     |                             |
| <b>AQP4</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs9951307                       | G+ (68)                | 4.49    |       |       | 1.14  |       | 20.5  | 0.14                        |                           |                            |                                           |                             |
|                                 | G- (66)                | 3.31    |       |       | 1.57  |       | 27.9  | 0.11                        |                           |                            |                                           |                             |
|                                 | p-value                | 0.096   |       |       | ns    |       | 0.097 | ns                          |                           |                            |                                           |                             |
| <b>INMT</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs6970396                       | A+ (27)                |         |       |       |       |       |       |                             |                           | 2.02                       | 2.53                                      | 23.6                        |
|                                 | A- (109)               |         |       |       |       |       |       |                             |                           | 2.86                       | 3.43                                      | 19.6                        |
|                                 | p-value                |         |       |       |       |       |       |                             |                           | 0.088                      | 0.082                                     | 0.023                       |
| <b>MT2A</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs28366003                      | G+ (18)                |         | 3.63  | 68.3  |       | 18.8  |       | 0.18                        |                           |                            | 4.58                                      |                             |
|                                 | G- (118)               |         | 5.41  | 85.1  |       | 15.7  |       | 0.12                        |                           |                            | 3.07                                      |                             |
|                                 | p-value                |         | 0.080 | 0.031 |       | 0.074 |       | ns                          |                           |                            | ns                                        |                             |
| Non-pregnant woman              |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| GENE/SNPs                       | Allele (N)             | AsIII % | MMA % | DMA % | PMI   | SMI   | TMI   | AsIII(ng/mL <sub>SG</sub> ) | MMA(ng/mL <sub>SG</sub> ) | DMA (ng/mL <sub>SG</sub> ) | U-ΣAs <sub>M</sub> (ng/mL <sub>SG</sub> ) | U-Se (ng/mL <sub>SG</sub> ) |
| <b>AS3MT</b>                    |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| SNP1 (rs7085104)                | G+ (105)               |         | 9.74  | 78.6  | 1.79  | 8.07  |       |                             |                           |                            |                                           |                             |
|                                 | G- (71)                |         | 13.0  | 75.0  | 2.06  | 5.80  |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         | 0.024 | 0.073 | 0.077 | 0.029 |       |                             |                           |                            |                                           |                             |
| SNP2 (rs3740400)                | G+ (108)               |         | 9.80  | 78.6  | 1.79  | 8.03  |       |                             |                           |                            |                                           |                             |
|                                 | G- (69)                |         | 13.0  | 74.7  | 2.10  | 5.73  |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         | 0.023 | 0.071 | 0.055 | 0.029 |       |                             |                           |                            |                                           |                             |
| SNP3 (rs3740393)                | C+ (65)                | 4.78    | 9.53  | 80.3  |       | 8.43  | 19.5  |                             |                           |                            |                                           |                             |
|                                 | C- (112)               | 6.38    | 11.8  | 75.4  |       | 6.38  | 14.3  |                             |                           |                            |                                           |                             |
|                                 | p-value                | 0.018   | 0.069 | 0.019 |       | 0.050 | 0.018 |                             |                           |                            |                                           |                             |
| SNP4 (rs3740390)                | C+ (65)                | 4.78    | 9.53  | 80.3  |       | 8.43  | 19.5  |                             |                           |                            |                                           |                             |
|                                 | C- (112)               | 6.38    | 11.8  | 75.4  |       | 6.38  | 14.3  |                             |                           |                            |                                           |                             |
|                                 | p-value                | 0.024   | 0.012 | 0.007 |       | 0.010 | 0.024 |                             |                           |                            |                                           |                             |
| SNP6 (rs10748835)               | A+ (116)               |         | 10.0  | 78.7  |       | 7.87  |       |                             |                           |                            |                                           |                             |
|                                 | A- (60)                |         | 13.0  | 74.0  |       | 5.69  |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         | 0.043 | 0.053 |       | 0.042 |       |                             |                           |                            |                                           |                             |
| SNP7 (rs1046778)                | C+ (95)                |         | 9.89  | 79.2  |       | 8.01  |       |                             |                           |                            |                                           |                             |
|                                 | C- (81)                |         | 12.3  | 74.7  |       | 6.1   |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         | 0.024 | 0.049 |       | 0.025 |       |                             |                           |                            |                                           |                             |
| 'protective' haplotype (GGCTAC) | at least one copy (33) | 4.78    | 8.30  | 82.2  |       | 9.90  | 20.9  |                             |                           |                            |                                           |                             |
|                                 | zero copies (141)      |         | 6.38  | 11.5  | 76.3  | 6.60  | 15.1  |                             |                           |                            |                                           |                             |
|                                 | p-value                |         | 0.024 | 0.014 | 0.008 | 0.011 | 0.026 |                             |                           |                            |                                           |                             |
| <b>MTHFR</b>                    |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs1801131                       | G+ (62, 81, 78)        |         |       |       |       | 2.07  |       |                             |                           |                            |                                           |                             |
|                                 | G- (74, 95, 93)        |         |       |       |       | 1.75  |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         |       |       |       | 0.067 |       |                             |                           |                            |                                           |                             |
| <b>AQP9</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs2414539                       | T+ (60)                | 4.67    |       |       |       | 2.43  | 20.1  |                             | 0.29                      | 2.01                       | 2.53                                      | 11.8                        |
|                                 | T- (117)               | 6.38    |       |       |       | 1.67  | 14.3  |                             | 0.17                      | 1.17                       | 1.55                                      | 8.29                        |
|                                 | p-value                | 0.014   |       |       |       | 0.001 | 0.014 |                             | 0.000                     | 0.007                      | 0.005                                     | 0.039                       |
| <b>SELENOP</b>                  |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs7579                          | T+ (88)                |         |       |       |       | 1.65  |       |                             |                           |                            |                                           |                             |
|                                 | T- (86)                |         |       |       |       | 2.17  |       |                             |                           |                            |                                           |                             |
|                                 | p-value                |         |       |       |       | 0.014 |       |                             |                           |                            |                                           |                             |
| <b>INMT</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs6970396                       | A+ (37)                |         |       |       |       |       |       |                             | 0.31                      | 2.07                       | 2.66                                      | 12.2                        |
|                                 | A- (139)               |         |       |       |       |       |       |                             | 0.18                      | 1.28                       | 1.66                                      | 8.73                        |
|                                 | p-value                |         |       |       |       |       |       |                             | 0.046                     | 0.085                      | 0.066                                     | 0.045                       |
| <b>MT2A</b>                     |                        |         |       |       |       |       |       |                             |                           |                            |                                           |                             |
| rs28366003                      | G+ (16)                |         |       | 8.67  |       | 1.29  |       | 0.18                        |                           |                            |                                           |                             |
|                                 | G- (160)               |         |       | 11.2  |       | 1.97  |       | 0.10                        |                           |                            |                                           |                             |
|                                 | p-value                |         |       | 0.081 |       | 0.001 |       | ns                          |                           |                            |                                           |                             |

(continued on next page)

Table 4 (continued)

| Children                           |                           |              |       |       |       |              |       |                                 |                               |                                |                                               |                                 |
|------------------------------------|---------------------------|--------------|-------|-------|-------|--------------|-------|---------------------------------|-------------------------------|--------------------------------|-----------------------------------------------|---------------------------------|
| GENE/SNPs                          | Allele (N)                | AsIII %      | MMA % | DMA % | PMI   | SMI          | TMI   | AsIII(ng/<br>mL <sub>SG</sub> ) | MMA(ng/<br>mL <sub>SG</sub> ) | DMA (ng/<br>mL <sub>SG</sub> ) | U-ΣAs <sub>M</sub> (ng/<br>mL <sub>SG</sub> ) | U-Se (ng/<br>mL <sub>SG</sub> ) |
| <b>AS3MT</b>                       |                           |              |       |       |       |              |       |                                 |                               |                                |                                               |                                 |
| SNP1 (rs7085104)                   | G + (113)                 |              |       |       |       |              |       |                                 |                               |                                | 1.89                                          |                                 |
|                                    | G- (60)                   |              |       |       |       |              |       |                                 |                               |                                | 2.81                                          |                                 |
|                                    | p-value                   |              |       |       |       |              |       |                                 |                               |                                | 0.032                                         |                                 |
| SNP2 (rs3740400)                   | G + (117)                 |              |       |       |       |              |       |                                 |                               |                                | 1.88                                          |                                 |
|                                    | G- (56)                   |              |       |       |       |              |       |                                 |                               |                                | 2.74                                          |                                 |
|                                    | p-value                   |              |       |       |       |              |       |                                 |                               |                                | 0.017                                         |                                 |
| SNP3 (rs3740393)                   | C + (68)                  |              | 8.37  |       | 1.74  | 9.63         |       |                                 |                               |                                |                                               |                                 |
|                                    | C- (105)                  |              | 10.0  |       | 2.30  | 7.98         |       |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   |              | 0.059 |       | 0.056 | 0.080        |       |                                 |                               |                                |                                               |                                 |
| SNP4 (rs3740390)                   | C + (68)                  | 3.67         | 8.37  | 80.6  |       | <b>9.63</b>  | 25.8  |                                 |                               |                                |                                               |                                 |
|                                    | C- (105)                  | 4.89         | 10.0  | 80.1  |       | <b>7.98</b>  | 19.0  |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   | 0.055        | 0.006 | 0.006 |       | <b>0.005</b> | 0.055 |                                 |                               |                                |                                               |                                 |
| SNP6 (rs10748835)                  | A + (124)                 |              |       |       | 1.93  |              |       |                                 |                               |                                |                                               |                                 |
|                                    | A- (46)                   |              |       |       | 2.31  |              |       |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   |              |       |       | 0.092 |              |       |                                 |                               |                                |                                               |                                 |
| 'protective' haplotype<br>(GGCTAC) | at least one copy<br>(37) |              | 7.33  | 84.1  |       | 11.5         |       |                                 |                               |                                |                                               |                                 |
|                                    | zero copies<br>(123)      |              | 10.2  | 79.1  |       | 7.80         |       |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   |              | 0.016 | 0.024 |       | 0.015        |       |                                 |                               |                                |                                               |                                 |
| <b>SELENOP</b>                     |                           |              |       |       |       |              |       |                                 |                               |                                |                                               |                                 |
| rs7579                             | T + (86)                  | 4.23         | 8.55  | 81.8  |       | 81.8         | 22.1  |                                 |                               |                                |                                               |                                 |
|                                    | T- (81)                   | 5.09         | 10.4  | 78.4  |       | 78.4         | 18.2  |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   | 0.094        | 0.045 | 0.046 |       | 0.046        | 0.094 |                                 |                               |                                |                                               |                                 |
| <b>MT2A</b>                        |                           |              |       |       |       |              |       |                                 |                               |                                |                                               |                                 |
| rs28366003                         | G + (7)                   | <b>2.47</b>  |       |       |       |              | 39.0  |                                 |                               |                                |                                               |                                 |
|                                    | G- (169)                  | <b>4.62</b>  |       |       |       |              | 20.2  |                                 |                               |                                |                                               |                                 |
|                                    | p-value                   | <b>0.076</b> |       |       |       |              | 0.076 |                                 |                               |                                |                                               |                                 |

\*Data with (marginal) significance ( $p < 0.1$ ); bolded are significant differences in the multivariable linear regression models after adjustment for age, pre-pregnancy BMI, child sex, ever smoking, parity, plasma Se and blood As in pregnant women; age, BMI, ever smoking, plasma Se and blood As in non-pregnant women; and gender, height, weight, smoking parents, plasma Se and blood As in children. U-ΣAs, sum of AsIII, MMA, and DMA; PMI, primary methylation index (MMA/AsIII); SMI, secondary methylation index (DMA/MMA); TMI, total methylation index ((MMA + DMA)/AsIII).

*AQP4* expression among rs9951307 minor allele carriers (Kleffner et al., 2008). We suppose that pregnancy-related enhanced water flow and decreased water reabsorption in the kidney (Cheung and Lafayette, 2013; Soma-Pillay et al., 2016) combined with *AQP4* downregulation resulted in increased urine elimination and, consequently, higher urine concentration of AsIII. If downregulation also affects the placenta, its effect on urine AsIII is even higher.

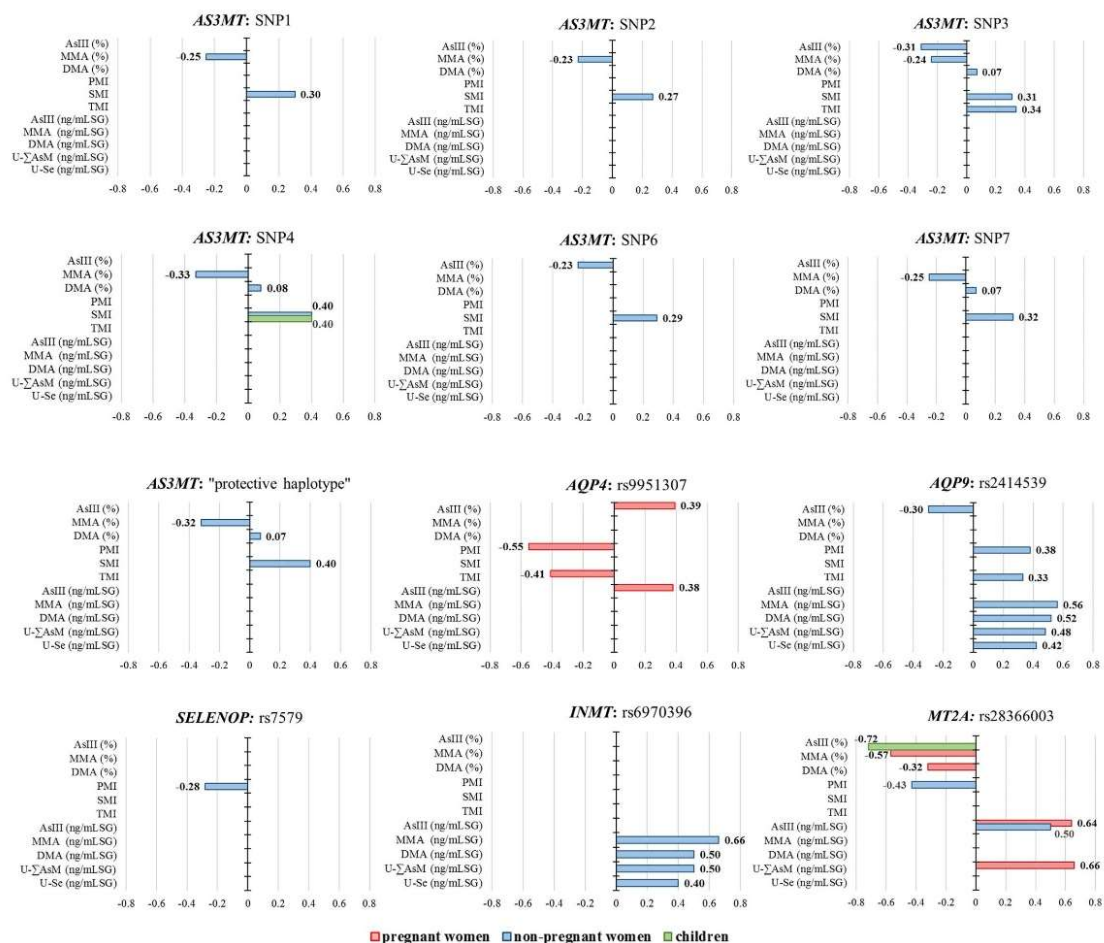
*AQP9* is highly expressed in hepatocytes (primary site of As methylation) where it plays a major role in the cellular uptake of AsIII. Based on experimental studies on rats, it also potentially plays a role in the efflux of As compounds (MMA and DMA) into the blood from where they are eliminated into the urine (Day et al., 2014; Mukhopadhyay et al., 2014; Roggenbeck et al., 2016). Non-pregnant women carrying a minor allele of *AQP9* rs2414539 had higher iAs methylation capacity (lower AsIII% and higher PMI and TMI and higher urine concentrations of MMA, DMA, U-ΣAs<sub>M</sub>, and U-Se (Table 4 and Fig. 4)). These results could suggest higher uptake of AsIII into hepatocytes, leading to its methylation and increased elimination of methylated forms with urine. Of course, at higher exposure levels the same process can be damaging if *AS3MT* cannot cope with (and methylate) all accumulated AsIII. Particularly interesting is the positive association with urine Se (Table 4 and Fig. 4) accompanied by (additionally tested) negative association with blood and plasma Se (data not presented). From the present study, it is impossible to speculate whether the supposed co-elimination of U-ΣAs<sub>M</sub> and U-Se is related to special As-Se complexes (Gailer et al., 2002; La Porte, 2011) or to the coincidental use of the same transport pathways. *AQPs* are generalized metalloids channels (Mukhopadhyay et al., 2014). Therefore, our results could indicate the potential role of *AQPs* (*AQP9*) in the cellular transport of Se, as previously hypothesised by

Misra et al. (2012) in an in vitro study on rainbow trout hepatocytes and enterocytes and by Geng et al. (2017) when studying monomethylselenic acid and selenite in *Xenopus laevis* oocyte after the overexpression of human *AQP9*.

**3.4.2.4. SELENOP.** SELENOP is the major plasma selenoprotein with anti-oxidative, transport, and metal-binding functions (Burk and Hill, 2015). It exists in two isoforms of 60 and 50 kDa, and the latter is considered to lack the cysteine- and selenocysteine-rich C-terminal domain (Burk and Hill, 2015; Méplan et al., 2009). Frequently reported SELENOP SNPs rs7579 and rs3877899 were previously studied mostly in relation to the proportions of SELENOP isoforms and Se levels in blood/plasma (Mao et al., 2016; Méplan et al., 2009). However, ours is the first study to investigate the relation with As metabolism. We did not find any significant associations with Se levels (in urine, blood or plasma). For As, the only significant association was observed for rs7579 in non-pregnant women, specifically, a negative one with PMI (Table 4 and Fig. 4).

SNP rs7579 was previously reported to be associated with higher proportion of the 60 kDa SELENOP isoform containing the cysteine- and histidine-rich domain that is supposed to be involved in the metal(lloid)s binding function (Burk and Hill, 2015; Méplan et al., 2009). According to preliminary results from a study on APL patients treated with As<sub>2</sub>O<sub>3</sub>, a part of AsIII can be bound on SELENOP (Falnoga et al., 2010), especially on the longer 60 kDa isoform (unpublished data Falnoga and Stibilj). Therefore, we could probably speculate on the higher amount of SELENOP-bound As with lower accessibility for methylation among the carriers of the minor allele in the present study. This could explain their lower primary methylation rate.





**Fig. 4.** Significant associations (presented as  $\beta$  coefficients,  $p < 0.05$ ) of SNPs' minor alleles and AS3MT 'protective' haplotype with As methylation parameters (AsIII%, MMA%, DMA%, PMI, SMI, and TMI) and urine elimination parameters of As and Se (AsIII, MMA, DMA, U- $\Sigma$ AsM and U-Se; ng/mL SG) from multiple regression models for pregnant women (adjusted for: age, pre-pregnancy BMI, child sex, ever smoking, parity, P-Se and B-As), non-pregnant women (adjusted for: age, BMI, ever smoking, P-Se and B-As) and children (adjusted for: gender, height, weight, smoking parents, P-Se and B-A).

3.4.2.5. *INMT*. *INMT* is a transmethylation enzyme that has been recently recognized to play an important role in Se elimination (Kuehnelt et al., 2015). Specifically, its gene polymorphism rs6970396 was shown to be a major predictor of the production of the common Se metabolite trimethylselenonium ion (TMSe). Carriers of the minor allele were identified as TMSe producers (high TMSe% in urine) and those of the common allele, as its non-producers (low TMSe% in urine) (Kuehnelt et al., 2015; Skräder et al., 2018). Low *INMT* minor allele frequencies were found for populations with known historical exposure to high iAs through water intake, with 1.5% for Argentinian Andean and 5% for Peruvian populations (Kuehnelt et al., 2015). In combination with high frequencies of 'protective' AS3MT haplotype(s) (Engström et al., 2011), low frequencies of *INMT* are assumed to be beneficial for As detoxification.

In the present study, frequencies of SNP rs6970396 were between 10% and 13% and were significantly associated with higher urine concentrations of As methylated metabolites and/or Se in bivariate comparisons of all groups (Table 4). After adjustment, the associations

for MMA, DMA, and Se were confirmed only in non-pregnant women (Table 4 and Fig. 4).

In a study on Bangladeshi pregnant women ( $n = 226$ ; GW 8), Skräder et al. (2018) reported data that are in accordance with our positive associations for U-Se and absent associations with As methylation efficiency, although their observations were not assessed with adjustment for confounders.

For better interpretation of our results, data on TMSe levels in urine are needed.

3.4.2.6. *MT2A*. Metallothioneins (MTs) are inducible low-molecular (6–7 kDa) cysteine-rich (20) metal-binding proteins with 11 functional (sub)isoforms with various functions (Krešel and Maret, 2017; Ling et al., 2016; Ruttkay-Nedecký et al., 2013; Shen et al., 2013); isoform *MT2A* is the most widely expressed one. *MT2A* and its genetic polymorphisms were frequently studied in relation to metal(loids) levels and diseases owing to their various functions including metal-ion homeostasis (Cu and Zn) redox and anti-oxidative functions and

**Table 5**  
Comparison of observed associations (multiple regression models) between *AS3MT* SNPs and As methylation in present study with those in previous studies exposed to low and high iAs.

| SNP               | Allele | PRESENT STUDY                                      | LITERATURE STUDY                                                                                                                                                           |                                                                                                                                                                                                                            |                                                                                                                                             |
|-------------------|--------|----------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
|                   |        | Association                                        | Association                                                                                                                                                                | Population                                                                                                                                                                                                                 | Reference                                                                                                                                   |
| <b>rs7085104</b>  | minor  | non-pregnant: ↓MMA%, ↑SMI                          | non-pregnant: ↓iAs%, ↓MMA%, ↑DMA%<br>pregnant: ↓MMA%, ↑DMA%<br>ns                                                                                                          | 172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>289 adults; 40% men; China                                                                                            | Engström et al., 2011<br>Drobna et al., 2016<br>Chen et. al., 2017                                                                          |
| <b>rs3740400</b>  | minor  | non-pregnant: ↓MMA%, ↑SMI                          | non-pregnant: ↓MMA%, ↑DMA%<br>pregnant: ↓MMA%, ↑DMA%<br>pregnant: ↓MMA%, ↑DMA%<br>↓MMA%                                                                                    | 172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>289 adults; 40% men; China                                                                                            | Engström et al., 2011<br>Drobna et al., 2016<br>Chen et. al., 2017                                                                          |
| <b>rs3740393</b>  | minor  | non-pregnant: ↓AsII%, ↓MMA%, ↑DMA%, ↑SMI, ↑TMI     | non-pregnant: ↓MMA%, ↑DMA%, ↑SMI<br>pregnant: ↑DMA%<br>non-pregnant: ↓iAs%, ↓MMA%, ↑DMA%<br>pregnant: ↓iAs%, ↑DMA%<br>pregnant: ↓MMA%, ↑DMA%<br>↓MMA%, ↑DMA%, ↑SMI<br>ns   | 111 non-pregnant women and 37 pregnant women; Argentina<br>172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>722 adults; 28% men; Mexico<br>289 adults; 40% men; China  | Engström et al., 2007<br>Engström et al., 2011<br>Drobna et al., 2016<br>Xu et al., 2016<br>Chen et. al., 2017                              |
| <b>rs3740390</b>  | minor  | non-pregnant: ↓MMA%, ↑DMA%, ↑SMI<br>children: ↑SMI | non-pregnant: ↓MMA%, ↑DMA%, ↑SMI<br>pregnant: ↑DMA%<br>non-pregnant: ↓iAs%, ↓MMA%, ↑DMA%<br>pregnant: ↓iAs%, ↑DMA%<br>pregnant: ↓MMA%, ↑DMA%<br>↓MMA%, ↑DMA%, ↑SMI<br>ns   | 111 non-pregnant women and 37 pregnant women; Argentina<br>172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>722 adults; 28% men; Mexico<br>289 adults; 40% men; China  | Balakrishnan et al., 2018<br>Engström et al., 2007<br>Engström et al., 2011<br>Drobna et al., 2016<br>Xu et al., 2016<br>Chen et. al., 2017 |
| <b>rs11191439</b> | major  | ns                                                 | non-pregnant: ns<br>men: ↑MMA%, ↓DMA%,<br>pregnant: ↑iAs%, ↓DMA%<br>pregnant: ns<br>Children: ↑MMA%, ↓SMI                                                                  | 190 non-pregnant women and 225 men; Hungary, Romania, Slovakia<br>172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>332 6–12 years old children (47.6% M); Mexico       | Lindberg et al., 2007<br>Engström et al., 2011<br>Drobna et al., 2016<br>Recio-Vega et al., 2016                                            |
| <b>rs1748835</b>  | minor  | non-pregnant: ↓MMA%, ↑SMI                          | ↓iAs%, ↑MMA%, ↓DMA%, ↓SMI<br>non-pregnant: ↓MMA%, ↑DMA%, ↑SMI<br>pregnant: ↑DMA%<br>non-pregnant: ↓iAs%, ↓MMA%, ↑DMA%<br>pregnant: ↓MMA%<br>pregnant: ↓MMA%<br>↑DMA%, ↑SMI | 722 adults; 28% men; Mexico<br>111 non-pregnant women and 37 pregnant women; Argentina<br>172 non-pregnant women; Argentina<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>722 adults; 28% men; Mexico | Xu et al., 2016<br>Engström et al., 2007<br>Engström et al., 2011<br>Drobna et al., 2016<br>Xu et al., 2016                                 |
| <b>rs1046778</b>  | minor  | non-pregnant: ↓MMA%, ↑SMI                          | non-pregnant: ↓iAs%, ↓MMA%, ↑DMA%<br>pregnant: ↓MMA%<br>pregnant: ↓MMA%, ↑DMA%<br>ns                                                                                       | 172 non-pregnant women; Argentina,<br>361 pregnant women; Bangladesh<br>200 pregnant women; Mexico<br>289 adults; 40% men; China                                                                                           | Engström et al., 2011<br>Drobna et al., 2016<br>Chen et. al., 2017                                                                          |

ns, not significant

**Table 6**  
Comparison of observed associations between *MTHFR* SNPs and As methylation in present study with those in previous studies.

| SNP              | Allele | Present study                                                                 | Literature study                                                             |                                                                                                                            |                                                  |
|------------------|--------|-------------------------------------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|
|                  |        | Association (bivariate analysis)                                              | Association                                                                  | Population                                                                                                                 | Reference                                        |
| <b>rs1801131</b> | Minor  | ns (pregnant: ↓AsII%, ↓DMA%, ↑TMI;<br>ns (pregnant: ↑iAs%, ↓DMA%, ↓SMI, ↓TMI) | Non-pregnant: ns<br>Men: ns                                                  | 190 non-pregnant women and 225 men; Hungary, Romania, Slovakia;                                                            | Lindberg et al. (2007)                           |
| <b>rs1801133</b> | Minor  |                                                                               | Non-pregnant: ↑iAs%, ↓DMA%<br>Men: ns<br>Pregnant: ↑iAs%, ↑MMA%, ↓DMA%, ↓SMI | 190 non-pregnant women and 225 men; Hungary, Romania, Slovakia;<br>111 non-pregnant women and 37 pregnant women; Argentina | Lindberg et al. (2007)<br>Engström et al. (2007) |

ns, not significant.



**Table 7**  
Ranges of selected  $\beta$  coefficients (min/max) <sup>a</sup> for confounders and  $R^2$  values (min/max) which were obtained in multivariable linear regression models for AS3MT, AQP4, AQP9, SELENOP, INMT, and MT2A SNPs associations with urine As and Se parameters.

|                          | Pregnant women       |                |                                      |                        | Non-pregnant women                   |                        |                                      |           | Children                             |            |                                      |                |
|--------------------------|----------------------|----------------|--------------------------------------|------------------------|--------------------------------------|------------------------|--------------------------------------|-----------|--------------------------------------|------------|--------------------------------------|----------------|
|                          | As and Se parameters |                | Confounders ( $\beta$ <sup>a</sup> ) |                        | Confounders ( $\beta$ <sup>a</sup> ) |                        | Confounders ( $\beta$ <sup>a</sup> ) |           | Confounders ( $\beta$ <sup>a</sup> ) |            | Confounders ( $\beta$ <sup>a</sup> ) |                |
|                          | model                | B-As           | P-Se                                 | Parity(1–3 deliveries) | Ever smoking (yes)                   | Parity(1–3 deliveries) | BMI                                  | model     | B-As                                 | P-Se       | model                                | Gender (girls) |
| AsIII <sub>u</sub>       | 0.10/0.14            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.06/0.10 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.06/0.10                            | 0.27/0.34      |
| MMA <sub>u</sub>         | 0.07/0.12            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.09/0.15 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.09/0.15                            | 0.27/0.34      |
| DMA <sub>u</sub>         | 0.03/0.10            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.05/0.09 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.05/0.09                            | – 0.32/– 0.37* |
| PMI                      | 0.08/0.18            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.06/0.09 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.06/0.09                            | – 0.32/– 0.37* |
| SMI                      | 0.07/0.09            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.09/0.15 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.09/0.15                            | – 0.32/– 0.37* |
| TMI                      | 0.10/0.14            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.06/0.10 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.06/0.10                            | – 0.32/– 0.37* |
| AsIII <sub>g</sub> /mLsg | 0.19/0.23            | 0.23/0.28*     | – 0.55/– 0.78                        | 0.65/0.76*             | – 0.26/– 0.33                        | – 0.03/– 0.05*         | – 0.03/– 0.05*                       | 0.03/0.08 | 0.14/0.17*                           | 0.33/0.51* | 0.03/0.08                            | 0.33/0.51*     |
| MMA <sub>g</sub> /mLsg   | 0.10/0.12            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.01/0.10 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.01/0.10                            | – 0.32/– 0.37* |
| DMA <sub>g</sub> /mLsg   | 0.24/0.28            | 0.28/0.31*     | – 0.55/– 0.78                        | 0.65/0.76*             | – 0.26/– 0.33                        | – 0.03/– 0.05*         | – 0.03/– 0.05*                       | 0.04/0.12 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.04/0.12                            | – 0.32/– 0.37* |
| ΣAs <sub>g</sub> /mLsg   | 0.25/0.27            | 0.27/0.28*     | – 0.55/– 0.78                        | 0.65/0.76*             | – 0.26/– 0.33                        | – 0.03/– 0.05*         | – 0.03/– 0.05*                       | 0.03/0.11 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.03/0.11                            | – 0.32/– 0.37* |
| Se <sub>g</sub> /mLsg    | 0.04/0.05            | – 0.14/– 0.16* | – 0.45/– 0.69                        | 0.53/0.64              | – 0.31/– 0.41*                       | – 0.14/– 0.16*         | – 0.14/– 0.16*                       | 0.09/0.10 | – 0.14/– 0.16*                       | 0.27/0.34  | 0.09/0.10                            | – 0.32/– 0.37* |

<sup>a</sup>coefficients were selected according to their significance \*  $p < 0.05$  or strength  $\beta \geq 0.2$  with  $p < 0.2$ .  
U-ΣAs<sub>u</sub>, sum of AsIII, MMA, and DMA; PMI, primary methylation index (MMA/AsIII); SMI, secondary methylation index (DMA/MMA); TMI, total methylation index (DMA + MMA)/AsIII).

metal(loid)s detoxification (Cd, Hg, Pb, and As). However, only a few studies have investigated rs28366003 polymorphisms in MT2A in relation to As (Akyüz et al., 2013; Eom et al., 2016; González-Martínez et al., 2018). In the present study, we observed its influence on As concentration.

Significant associations of SNP were observed in all study groups, but with different As parameters (Table 4 and Fig. 4). Adjusting for confounders resulted in significant associations of MT2A with higher AsIII urine concentrations and (consequently) lower As methylation capacity in pregnant and non-pregnant women (Table 4 and Fig. 4).

Akyüz et al. (2013) observed a similar influence on As levels in urine in a study on individuals who are occupationally exposed to As (n = 95, arithmetic mean: B-As, 21.2 ng/mL and U-As, 6.4 ng/mL). By contrast, Gonzales-Martínez et al. (2018) found no significant association in a recent study on a Colombian population (n = 101, median U-As<sub>M</sub>: 5.9 ng/g crea).

The rs28366003 minor allele was reported to be significantly associated with lower MT2A gene expression (Krzeslak et al., 2013; Starska et al., 2014). Therefore, one can hypothesise that lower MT2A expression could lead to reduced transport of MT2A-bound As into the liver and, consequently, lower amount of methylated As and higher elimination of GSH-conjugated AsIII into urine. However, the interpretation of the observed association in the present study is problematic owing to the very low number of individuals having rs28366003 minor allele (Table 3), especially among children, resulting in low statistical power (n = 18, 16, and 7 in pregnant women, non-pregnant women, and children, respectively).

**3.4.2.7. Confounders and  $R^2$  values of models.** All associations were tested using a set of various confounders whose combination was specific for each study group. As in blood (representing general As intake), plasma Se, age, BMI, and ever smoking status were assessed for pregnant and non-pregnant women, and parity and child sex were additionally assessed for pregnant women only. As in blood, plasma Se, gender, weight, height, and smoking of parents was assessed for children. The selection of confounders is explained in details in the material and methods section.

Only a few of the tested confounders modified the effects of gene polymorphisms on the studied As and Se urine parameters (Table 7). Among all, the strongest influence was observed for plasma Se among pregnant and non-pregnant women ( $\beta \pm 0.5$ –0.9). In pregnant women, it was inversely associated with urine AsIII concentration and AsIII% and, correspondingly, positively associated with PMI and TMI indexes. Such results may roughly indicate the possible increase of As hepatobiliary elimination in the form of the proposed As-Se-GSH complex (Roggenbeck et al., 2016). Among non-pregnant women, plasma Se was positively associated with As methylation efficiency, as most evidently seen from the SMI parameter. Pilsner et al. (2011) found similar trends for associations between plasma Se and As metabolite percentages in blood. They suggested that Se adequacy may be involved in the mechanism by which it can indirectly facilitate the methylation of MMA to DMA (Pilsner et al., 2011).

Other confounders with noticeable influence in order based on the strength of their effect were ever smoking and parity among pregnant women, gender among children, and blood As in all study groups and pre-pregnancy BMI among pregnant women. Associations of ever smoking with higher urine AsIII levels and percentages and poorer methylation confirm the observations of previous studies (Shen et al., 2016; Tseng, 2009). Parity (1–3 deliveries) associated with more efficient methylation can be explained by improved placental functions with number of deliveries (Prior et al., 2014). Arsenic methylation also occurs in the placenta (Fei et al., 2013; Winterbottom et al., 2017). Among children, girls show higher AsIII urine concentration but poorer methylation compared to boys; this finding differs from that of some previous studies that reported no gender influence on As methylation in children (Recio-Vega et al., 2016; Skróder Löveborn et al., 2016; Torres-

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Sánchez et al., 2016). In general, blood As was associated with more efficient As methylation, as reflected mostly by the higher urine DMA concentrations and lower MMA%. These results are in accordance with the known fact that at low levels, iAs can increase AS3MT activity, whereas at higher levels, the enzyme is inhibited (Fowler et al., 2015). Pre-pregnancy BMI among pregnant women was weakly associated with lower levels of DMA and  $\Sigma\text{As}_M$ .

SNPs and confounders explained up to 18% of the variation in As methylation parameters in all study groups (Table 7). A similar percentage was reported by Lindberg et al. (2007) in a comparable study on a Central European population. In the case of As urine parameters, the percentage of variation explained was below 28% for pregnant women and below 12% for non-pregnant women and their children. Less than 10% of the variation in Se urine concentration was explained (Table 7).

#### 4. Conclusions

Polymorphisms of various genes showed distinct impacts on As and Se parameters in study groups with estimated low iAs exposure.

In non-pregnant women we confirmed the positive influence of SNPs (and 'protective' haplotype) of *AS3MT* on iAs methylation efficiency but without a significant change in the urine concentration of metabolites. At the same time, various influences were observed for SNPs of genes which can affect As metabolites transport pathways (cellular uptake or efflux, reuptake in kidney cells). They were associated with increased urine concentration of methylated As metabolites (*AQP9* rs2414539 and *INMT* rs6970396) or *AsIII* only (*MT2A* rs28366003). Consequently, they indirectly influenced methylation capacity in a positive (*AQP9*) or negative (*MT2A*) way through various mechanisms, as discussed above.

Among pregnant women and children, the associations were scarce. A few of them were similar to those observed among non-pregnant women: SNP4 of *AS3MT* (rs3740390) in children and *MT2A* in pregnant women. The influence of *AQP4* (rs9951307) was observed exclusively among pregnant women. Carriers of the minor allele had higher urine *AsIII* (percentages and concentration) and diminished primary and total methylation index.

Selenium levels were used in two ways: as a confounder (plasma Se) and as observing parameter (urine Se). Among confounders it showed a stronger influence on arsenic parameters (like decrease of *AsIII*%). Its urine concentrations were positively affected by *INMT* or *AQP9* SNPs; both effects could be linked to the amounts of the Se metabolite TMSe, which should be analysed in the next step for the proper interpretation of its possible co-excretion with As metabolites.

The assessment showed that at low levels of iAs exposure, the impact of physiological states (pregnancy or childhood) obviously dominated over the genetic effect on methylation (*AS3MT*) or emphasised the genetic effects on As (*AsIII*) levels (*APQ4*). The novel associations found for *AQPs* with As and Se parameters are intriguing and need further study. In the kidney, a whole set of *AQPs* is active and they can even change their function(s) during pregnancy (Nielsen et al., 2002). Therefore, it is needed to check SNPs for all of them as well as their interactive effects.

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#### Conflict of interest

The authors have no conflict of interest.

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Our study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The research protocol of the study was approved by the National Medical Ethics Committee (NMEC) of the Republic of Slovenia (number of accordance: 98/05/06 and 65/09/14) and Ethics Committee of the University Hospital Centre Rijeka, Croatia (number of accordance: 2170-29-02/1-07-1). All participants provided signed informed consent.

#### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2018.11.045.

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## SUPPLEMENTAL MATERIALS

### **Arsenic metabolites; selenium; and *AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *SELENOP*, *INMT*, and *MT2A* polymorphisms in Croatian-Slovenian population from PHIME-CROME study**

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**Table SP1:** Information on studied SNPs

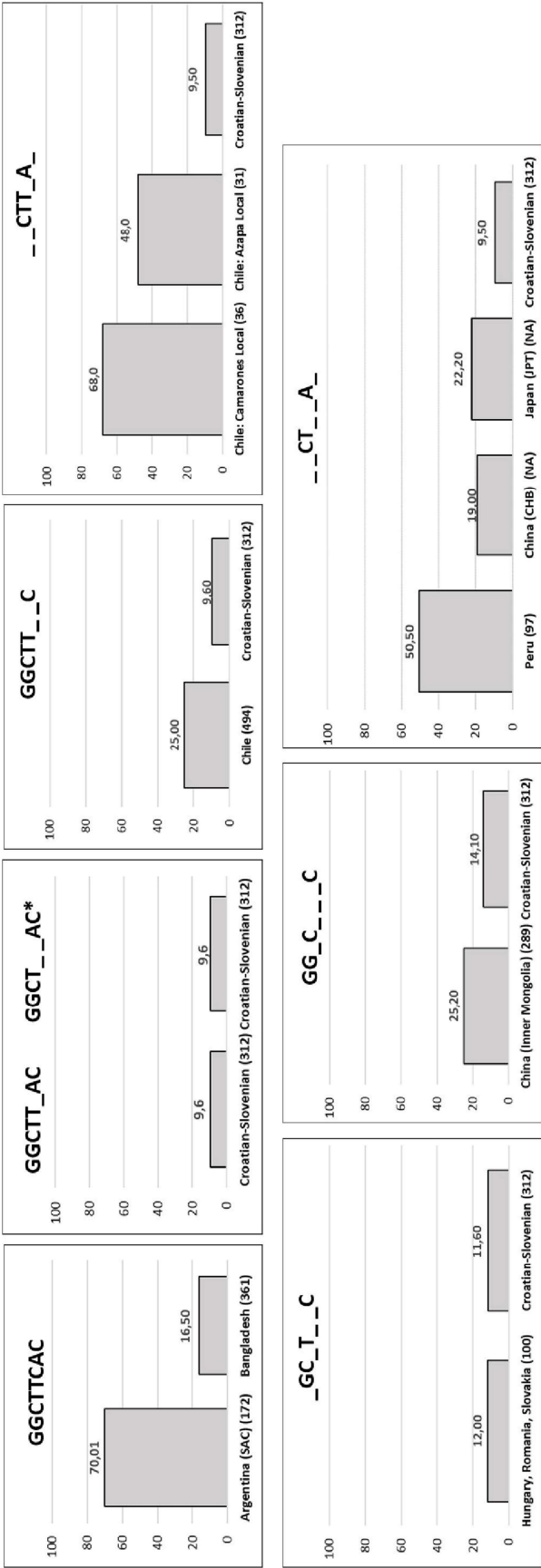
| GENE NAME                            | GENE ID      | dbSNP NCBI ID           | chromosome | location      | Nucleotide change | Amino acid change | MAF <sup>a</sup> CEU; TSI (%) | TaqMan assay ID |
|--------------------------------------|--------------|-------------------------|------------|---------------|-------------------|-------------------|-------------------------------|-----------------|
| Arsenic (3+) methyltransferase       | AS3MT        | rs7085104               | 10         | 5' terminal   | A > G             |                   | 31.9; 39.8                    | C_3284563_10    |
|                                      |              | rs3740400               | 10         | Intron        | T > G             |                   | 35.8; NA                      | C_27510174_10   |
|                                      |              | rs3740393               | 10         | Intron        | G > C             |                   | 13.3; 16.4                    | C_25804287_10   |
|                                      |              | rs3740390               | 10         | Intron        | C > T             |                   | 6.7; 11.6                     | C_27510172_10   |
|                                      |              | rs11191439              | 10         | Exon          | T > C             | Met > Thr         | 10.6; 11.9                    | C_31979150_10   |
|                                      |              | rs10748835              | 10         | Intron        | G > A             |                   | 38.1; 34.5                    | C_2852804_10    |
|                                      |              | rs1046778               | 10         | Exon (3' UTR) | T > C             |                   | 27.4; 34.5                    | C_9596558_10    |
|                                      |              | rs7897654 <sup>b</sup>  | 10         | Intron        | T > C             |                   | 27.4; 35.6                    | C_31979105_20   |
| Methylene tetrahydrofolate reductase | <i>MTHFR</i> | rs1801131               | 1          | Exon          | T > G             | Glu > Ala         | 34.1; 31.8                    | C_850486_20     |
| Aquaporin 4                          | <i>AQP4</i>  | rs1801133               | 1          | Exon          | G > A             | Ala > Val         | 31.0; 46.0                    | C_1202883_20    |
| Aquaporin 9                          | <i>AQP9</i>  | rs9951307               | 18         | 3' terminal   | A > G             |                   | 35.8; 33.0                    | C_29992536_20   |
|                                      |              | rs2414539               | 15         | Intron        | A > T             |                   | 13.8; 9.1                     | C_15799601_10   |
| Selenoprotein P1                     | <i>SEPP1</i> | rs17240643 <sup>b</sup> | 15         | 3' terminal   | C > T             |                   | 14.3; 7.5                     | C_33423683_10   |
|                                      |              | rs7579                  | 5          | Exon (3' UTR) | C > T             |                   | 29.6; 28.4                    | C_8806056_10    |
| Indolethylamine N-methyltransferase  | <i>INMT</i>  | rs3877899               | 5          | Exon          | C > T             | Ala > Thr         | 26.1; 20.4                    | C_2841533_10    |
|                                      |              | rs6970396               | 7          | Exon (3' UTR) | G > A             |                   | 12.3; 9.4                     | C_29402264_10   |
| Metallothionein 2A                   | <i>MT2A</i>  | rs28366003              | 16         | 5' UTR        | A > G             |                   | 3.9; NA                       | C_60284591_10   |

<sup>a</sup>MAF: minor allele frequency based on dbSNP-NCBI data for European population from HapMap project: CEU (Utah residents with ancestry from northern and western Europe), TSI (Toscani, Italy); <sup>b</sup>SNPs: rs7897654 in AS3MT and rs17240643 in AQP9 were genotyped only on a subset of participants and were due to their complete LD with other two SNPs rs1046778 and rs2414539 in the corresponding gene, respectively, excluded from the further analysis.

**Table SP2:** Genotype frequencies of selected SNPs stratified by study groups (N (%)).

| SNP                 | genotype | pregnant women | non-pregnant women | children (all) | girls     | boys         |
|---------------------|----------|----------------|--------------------|----------------|-----------|--------------|
| <i>AS3MT</i>        |          |                |                    |                |           |              |
| SNP1:<br>rs7085104  | AA       | 50 (37.0)      | 71 (40.3)          | 60 (34.5)      | 21 (25)   | 39 (43.8)    |
|                     | AG       | 68 (50.4)      | 82 (46.6)          | 78 (45.1)      | 42 (50)   | 36 (40.4)    |
|                     | GG       | 17 (12.6)      | 23 (13.1)          | 35 (20.4)      | 21 (25)   | 14 (15.7) ** |
| SNP2:<br>rs3740400  | TT       | 47 (34.6)      | 68 (38.6)          | 56 (32.4)      | 21 (25.0) | 35 (39.3)    |
|                     | TG       | 70 (51.5)      | 83 (47.2)          | 80 (46.2)      | 41 (48.8) | 39 (43.8)    |
|                     | GG       | 19 (13.9)      | 25 (14.2)          | 37 (21.4)      | 22 (26.2) | 15 (16.9)    |
| SNP3:<br>rs3740393  | GG       | 92 (68.1)      | 112 (63.6)         | 105 (60.7)     | 52 (62.7) | 53 (58.9)    |
|                     | GC       | 37 (27.4)      | 61 (34.7)          | 62 (35.8)      | 28 (33.7) | 34 (37.8)    |
|                     | CC       | 6 (4.4)        | 3 (1.7)            | 6 (3.5)        | 3 (3.6)   | 3 (3.3)      |
| SNP4:<br>rs3740390  | CC       | 110 (82.1)     | 141 (81.0)         | 117 (74.0)     | 56 (73.7) | 61 (74.3)    |
|                     | CT       | 23 (17.2)      | 33 (19)            | 39 (24.7)      | 18 (23.7) | 21 (25.6)    |
|                     | TT       | 1 (0.7)        | -                  | 2 (1.3)        | 2 (2.6)   | -            |
| SNP5:<br>rs11191439 | TT       | 101 (74.3)     | 142 (81.1)         | 135 (79.4)     | 62 (75.6) | 73 (83.0)    |
|                     | TC       | 32 (23.5)      | 32 (18.3)          | 32 (18.8)      | 17 (20.7) | 15 (17.0)    |
|                     | CC       | 3 (2.2)        | 1 (0.6)            | 3 (1.8)        | 3 (3.7)   | -            |
| SNP6:<br>rs10748835 | GG       | 41 (30.1)      | 60 (34.1)          | 46 (27.1)      | 18 (22.2) | 28 (31.5)    |
|                     | GA       | 68 (50.0)      | 80 (45.5)          | 85 (50)        | 41 (50.6) | 44 (49.4)    |
|                     | AA       | 27 (19.9)      | 36 (20.4)          | 39 (22.9)      | 22 (27.2) | 17 (19.1)    |
| SNP7:<br>rs1046778  | TT       | 65 (47.8)      | 81 (46.0)          | 65 (37.8)      | 30 (36.1) | 35 (39.3)    |
|                     | TC       | 56 (41.2)      | 72 (40.9)          | 86 (50.0)      | 42 (50.6) | 44 (49.4)    |
|                     | CC       | 15 (11.0)      | 23 (13.1)          | 21 (12.2)      | 11 (13.3) | 10 (11.3)    |
| <i>MTHFR</i>        |          |                |                    |                |           |              |
| rs1801131           | TT       | 74 (54.4)      | 95 (53.9)          | 93 (54.4)      | 48 (58.8) | 45 (50.6)    |
|                     | TG       | 53 (39.0)      | 67 (38.1)          | 68 (39.8)      | 29 (35.4) | 39 (43.8)    |
|                     | GG       | 9 (6.6)        | 14 (8.0)           | 10 (5.8)       | 5 (6.1)   | 5 (5.6)      |
| rs1801133           | GG       | 56 (41.2)      | 74 (42.0)          | 62 (35.8)      | 29 (34.5) | 33 (37.1)    |
|                     | GA       | 61 (44.9)      | 80 (45.5)          | 88 (50.9)      | 44 (52.4) | 44 (49.4)    |
|                     | AA       | 19 (13.9)      | 22 (12.5)          | 23 (13.3)      | 11 (13.1) | 12 (13.5)    |
| <i>AQP4</i>         |          |                |                    |                |           |              |
| rs9951307           | AA       | 66 (49.2)      | 77 (44.0)          | 73 (43.0)      | 32 (39.0) | 41 (47.0)    |
|                     | AG       | 55 (41.0)      | 77 (44.0)          | 78 (46.0)      | 39 (48.0) | 39 (45.0)    |
|                     | GG       | 13 (9.8)       | 21 (12.0)          | 18 (11.0)      | 11 (13)   | 7 (8.05)     |
| <i>AQP9</i>         |          |                |                    |                |           |              |
| rs2414539           | AA       | 93 (68.4)      | 117 (66.5)         | 111 (63.8)     | 54 (64.3) | 57 (63.3)    |
|                     | AT       | 37 (27.2)      | 55 (31.3)          | 55 (31.6)      | 28 (33.3) | 27 (30.0)    |
|                     | TT       | 6 (4.4)        | 4 (2.2)            | 8 (4.6)        | 2 (2.4)   | 6 (6.7)      |
| <i>SEPP1</i>        |          |                |                    |                |           |              |
| rs7579              | CC       | 66 (48.5)      | 86 (49.4)          | 82 (48.2)      | 42 (50.0) | 40 (46.5)    |
|                     | CT       | 61 (44.9)      | 68 (39.1)          | 73 (42.9)      | 32 (38.1) | 41 (47.7)    |
|                     | TT       | 9 (6.6)        | 20 (11.5)          | 15 (8.82)      | 10 (11.9) | 5 (5.81)     |
| rs3877899           | CC       | 71 (52.2)      | 105 (59.3)         | 109 (63.4)     | 56 (66.7) | 53 (60.2)    |
|                     | CT       | 57 (41.9)      | 63 (35.6)          | 58 (33.7)      | 25 (29.8) | 33 (37.5)    |
|                     | TT       | 8 (5.9)        | 10 (5.1)           | 5 (2.9)        | 3 (3.6)   | 2 (2.3)      |
| <i>INMT</i>         |          |                |                    |                |           |              |
| rs6970396           | GG       | 109 (80.1)     | 139 (79.0)         | 133 (76.9)     | 67 (79.8) | 66 (74.2)    |
|                     | GA       | 26 (19.1)      | 34 (19.3)          | 36 (20.8)      | 17 (20.2) | 19 (21.3)    |
|                     | AA       | 1 (0.8)        | 3 (1.7)            | 4 (2.3)        | -         | 4 (4.5)      |
| <i>MT2A</i>         |          |                |                    |                |           |              |
| rs28366003          | AA       | 118 (86.8)     | 160 (90)           | 169 (96)       | 85 (99)   | 84 (93)      |
|                     | GA       | 17 (12.5)      | 16 (9)             | 7 (4.0)        | 1(1)      | 6 (7)        |
|                     | GG       | 1 (0.74)       | -                  | -              | -         | -            |

\*\* significant difference between girls and boy



**Figure SP1:** Frequencies of AS3MT “protective” haplotypes, constructed from different number and combination of SNPs, reported for different populations and their comparison with those constructed for present Croatian-Slovenian population. For the purpose of more precise comparisons we constructed corresponding haplotypes (based on combination of reported SNPs). “Protective” haplotype sequences: variant alleles of SNPs in 5’-3’ order, based on their position in the gene: rs7085104 (G), rs3740400(G), rs3740393(C), rs3740390(T), rs11191439(T; common allele), rs11191453(C) rs10748835(A), rs1046778(C); \_ , SNP at this position was not genotyped in the study; Based on the number of SNPs: Engstrom et al., 2011, SAC, San Antonio de los Cobres, Argentina and Bangladesh; De la Rosa et al., 2017, Chile; Apata et al., 2017: Chile (Camarones Local, Azapa Local); Engstrom et al., 2015: Hungary, Romania Slovakia; Chen et al., 2017: China (Inner Mongolia); Schlebush et al., 2013: China (Beijing, CHB), Japan (Tokyo, JPT) Japan and Peru; \* Haplotype sequence, which was used in statistics of the present study for the estimation of influences on As metabolism.

### 3.3 Manuscript 3: Human Biomonitoring Study at Three Uranium Legacy Sites in Kyrgyzstan

Under review: Stajanko A., Tuhvatshin R., Suranova G., Mazej D., Šlejkovec Z., Falnoga I., Krušič Ž., Lespukh E., Stegnar P. (2019), *Science of the Total Environment*

$\delta$ -Aminolevulinate dehydratase (ALAD) – enzyme involved in heme synthesis – is the main binding protein of Pb in blood and tissues. Various studies reported the influence of *ALAD* genetic variations on enzyme's activity and consequently variability in blood Pb levels (Broberg et al., 2015). As such, SNPs rs1805313 and rs1800435 were previously associated with Pb levels; the former in genome-wide association study conducted on occupationally unexposed population (Warrington et al., 2015) and the latter in studies on occupationally exposed populations (Broberg et al., 2015). Both *ALAD* SNPs with the addition of rs818708 were tested in Slovenian-Croatian women – after the published study of As levels affected by SNPs of genes related to As metabolism (Manuscript 2, Section 3.2) – in relation with low Pb exposures (GM,  $\leq 12$  ng/g in blood) as presented in App. A.4. Similarly, as observed in the second manuscript (Section 3.2), the influence of physiological changes (e.g. pregnancy) and other non-genetic factors (e.g. status of essential elements and smoking) were stronger than genetic factors at such low exposure.

Accordingly, in the present manuscript we tested the above-mentioned *ALAD* SNPs in Kyrgyzstan population with moderate Pb exposure (GM, 21.0 ng/g in blood). Population consisted of residents from three uranium legacy sampling sites in Kyrgyzstan, namely Kadji Sai ( $n=46$ ), Mailuu-Suu ( $n=24$ ) and Sumsar ( $n=53$ ), correspondingly representing three individual study groups in the manuscript. Beside blood Pb, we determined also blood levels of Cd, Hg, As, U, Se, Cu, Mn, and Fe, and compared them for differences between the study groups. Associations between above-mentioned SNPs and blood Pb were tested on a whole population and according to different stratifications (age, gender, and region). We also tested for possible confounding by age, gender, region, status of essential trace elements and co-exposure to non-essential elements.

For all studied *ALAD* SNPs we presented minor allele frequencies determined for Kyrgyzstan population and compared them with those of other worldwide populations. Regardless of the small sample size we compared frequencies also between the Kyrgyzstan study groups.

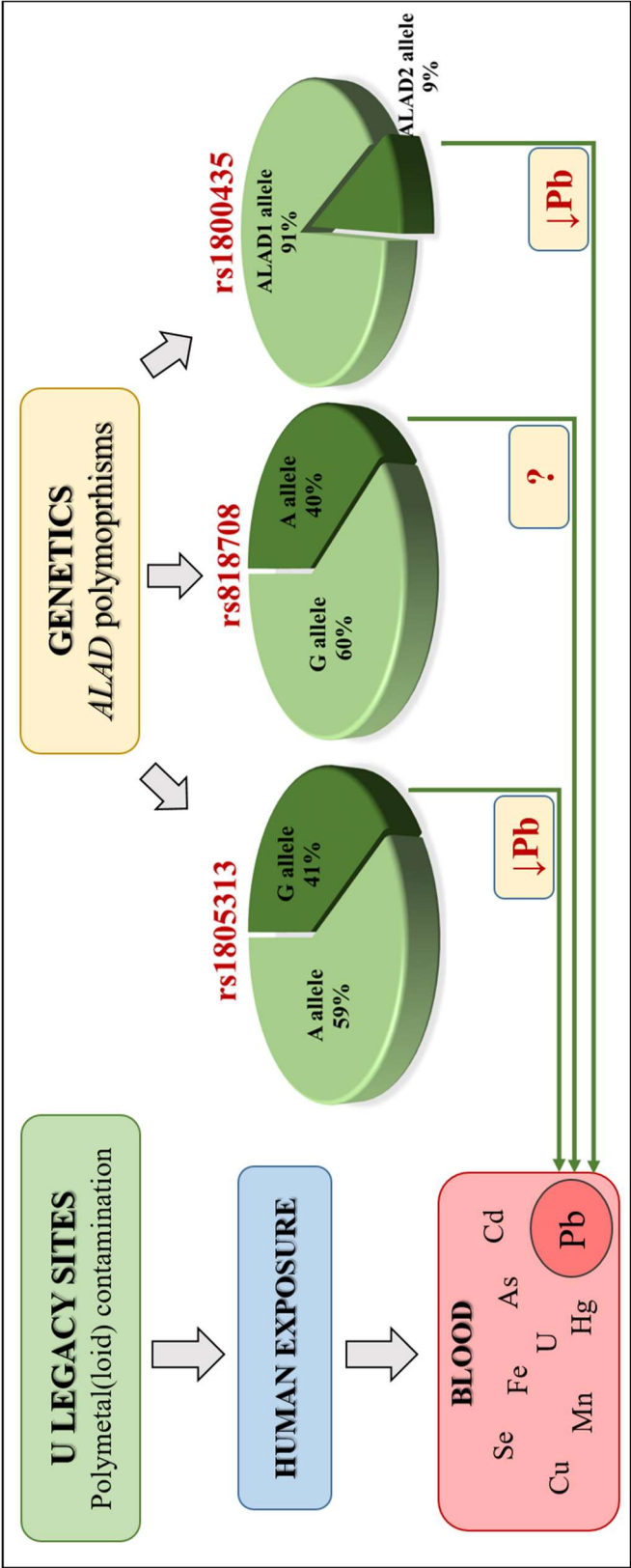
In the manuscript, we:

- i) identified significantly different blood levels for most trace elements between study groups indicating different environmental conditions at three industrial areas,
- ii) found elevated Pb exposure in Sumsar and Mailuu Suu study groups according to literature levels for environmentally unexposed populations;
- iii) confirmed the influence of two *ALAD* SNPs (rs1805313 and rs1800435) on blood Pb levels and their potential role as susceptibility biomarkers;
- iv) observed significant geographical distribution of *ALAD* SNPs (rs1805313 and rs1800435) within Kyrgyzstan, which should be further confirmed on a larger population.

My contribution to the present manuscript was helping with the DNA isolations, preparing samples for multielemental analysis, performing all statistical analyses, preparing tables and figures and in writing related to the assessment of gene-environment interaction.



GRAPHIC ABSTRACT



**TRACE ELEMENTS AND *ALAD* GENE POLYMORPHISMS IN GENERAL POPULATION FROM THREE URANIUM LEGACY SITES – A CASE STUDY IN KYRGYZSTAN**

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## ABSTRACT

At three uranium (U) legacy sites in Kyrgyzstan, namely, Kadji Sai, Mailuu-Suu and Sumsar, an initial human bio-monitoring programme was introduced as a complementary activity to environmental impact studies in these areas. The aim was to assess trace element (TE) contents in blood and genetic susceptibility for Pb as one of the contaminants.

The programme included the determination of 9 TE in blood samples from 123 residents living permanently in this environment. The analyses included U and the potentially toxic TE, lead (Pb), cadmium, mercury (Hg), and arsenic (As), together with essential elements iron (Fe), copper, selenium (Se) and manganese (Mn). TE were analysed by inductively coupled plasma mass spectrometry (ICPMS) and genetic background effect by three single nucleotide polymorphisms (SNPs) of delta-aminolevulinic acid dehydratase (ALAD) (rs1805313, rs818708, rs1800435) genotyped by quantitative polymerase chain reaction (qPCR).

The obtained results were generally similar to literature reference values obtained from the U non-exposed environments. However, some significant findings indicated elevated levels of certain contaminants typical of the studied environment (U, Pb). Several essential (Se, Mn) and toxic TE (Pb, Hg, As, U) in blood showed statistically significant differences among the studied areas. All areas showed diminished Fe blood levels. Altogether, this indicated specific and different environmental conditions at three industrial legacy sites for U milling and processing along with the accompanying chemical (pollutant) elements. Blood U concentrations were slightly higher at Mailuu-Suu, known for elevated technogenic and naturally occurring U. At Sumsar, the distribution of elevated blood Pb concentrations indicated an airborne source of pollution that was different from the anticipated aqueous exposure pathway. Pb blood variability was found associated with ALAD polymorphisms (SNPs rs1805313, rs1800435).

Results are confirming that human data will be a useful and scientifically important additional tool for environmental impact assessment studies at industrial legacy sites in Kyrgyzstan.

## KEY WORDS

Uranium legacy, trace elements, human blood, biological markers, ALAD gene polymorphism, Pb

Blood samples were collected from the residents of Kadji Sai, Mailuu-Suu, and Sumsar based on the framework for basic cardiovascular biomedical research on people living in the environment of selected U legacy sites at high-altitude areas in Kyrgyzstan (study approved by national Ethical Committee in Bishkek, Kyrgyzstan, No. 01-2/367).

The study was supported in part by the Slovenian Research Agency through the Jožef Stefan Institute programme group P1 0143, Jožef Stefan International Postgraduate School (IPS), the 'Health and Environmental wide Association based on Large population Surveys' international project (HEALS; EU FP7, grant agreement No. 603946), and FSD (Swiss Foundation for Mine Action), Geneva, Switzerland.

## 1. INTRODUCTION

Uranium (U) legacy sites in the central Asian republics of Kazakhstan, Kyrgyzstan, Uzbekistan, and Tajikistan have recently been extensively studied by national and international scientists. These studies have focused on environmental impact assessments of radioactive and chemical contaminants, and their results are being used for adopting efficient countermeasures against environmental pollution. Considerable analytical data about radioactive and chemical pollutants have been obtained, and radiation doses and related health risks for resident populations as well as pollution levels of chemical contaminants in the living environment have been assessed (**Stegnar et al., 2013; Salbu et al., 2013; Lind et al., 2013; Skipperud et al., 2013**). Individual doses of ionizing radiation were slightly to moderately elevated in comparison with those in a U clean environment; however, these did not pose health-related radiation risks that would require interventions. Health assessment of chemical contaminants is more complicated and is based on environmental levels of various elements including toxic heavy metals in different environmental compartments. Human bio-monitoring studies should be used for relevant health assessments of resident populations living at U and other industrial legacy sites. These studies include analyses of human body fluids and tissues for chemical contaminants with identification of residents' susceptibility or adaptability characteristics, followed by assessments of potential or actual harmful effects of contaminants. In Kyrgyzstan, several studies have included analyses of human hair and nail tissues as potential indicators of the impact of chemical contamination in the environment (**Aparin et al., 2012; Brázdová et al., 2014**). In our case study, we have added whole blood as an indicator of chronic exposure for the local population covering wide age range at three legacy sites in Kyrgyzstan, namely, Kadji Sai and Mailuu-Suu with U mining legacy wastes, and Sumsar with Pb-Zn mining legacy wastes, but located also in the close vicinity of Shekaftar with mining legacies of U deposits (former Sumsar-Shekaftar mining district) (**Figure 1**). First site is located in Issyk-Kul Province and other two in Jalal-Abad Province. All three sites can introduce unpredictable contamination load into the underground waters and nearby surface waters by chronic or accidental releases of toxic metals, metalloids and U from the legacy wastes as was already reported - by **Lind et al. (2013) and Kulenbekov (2013)** for Kadji Sai, **Aparin et al. (2011)** for Mailuu-Su and **Turgoev and Jakubick (2015)** for Sumsar-Shekaftar. Our aim was to evaluate potentially toxic nonessential and essential chemical elements in whole blood of 123 inhabitants and to assess the association of lead (Pb) content, as one of the main contaminants, with susceptibility biological markers (genetic polymorphisms in delta-aminolevulinic acid dehydratase - ALAD). ALAD or porphobilinogen synthase is a cytoplasmic zinc containing enzyme that plays an important role in haem biosynthesis and a well-known role in Pb toxicity (**Skerfving and Bergdahl, 2015; Fowler, 2010**). ALAD enzymatic activity is inhibited by Pb and this inhibited activity is a direct measure of the physiological response to occupational Pb exposure. In the blood, the majority of Pb is bound in erythrocytes and inside erythrocytes on ALAD. The type and strength of binding depends on ALAD polymorphisms on protein



and/or gene level. This genetic variability may cause different internal exposures at the same occupational or environmental exposure levels. Nevertheless, although there are several studies investigating how the polymorphisms in the *ALAD* gene may affect individual responses to Pb toxicity and blood Pb levels, the results are in many cases inconclusive, particularly at low levels of exposure (**Broberg et al., 2015, Skerfving and Bergdahl 2015**). In this study, we try to assess the genetic cause of blood Pb level variations in the general population of industrially contaminated regions using three *ALAD* single nucleotide polymorphism: rs1805313 (SNP1), rs818708 (SNP2) and rs1800435 (SNP3). First SNP was only recently shown to be significantly associated with Pb levels in cohorts from Australia and UK (genome-wide association analysis on occupationally unexposed population) (**Warrington et al., 2015**) and the third one is the most studied SNP due to its known relations with Pb levels at occupational exposure (**Broberg et al., 2015, Skerfving and Bergdahl, 2015**). However, literature results on its relation with blood Pb variability for occupationally unexposed population are very inconsistent regarding either their presence or their positive or negative associations (**Broberg et al., 2015**). The second one is highly prevalent in Asian population and recently its influence on *ALAD* expression through miRNA binding was observed in Pb occupational exposure study (**Li et al., 2017**). The main objective of the study was to assess the significance of blood trace element contents and genetic susceptibility for Pb - as one of the main contaminants - in residents of three U legacy sites of Kyrgyzstan.

## 2. SAMPLING and ANALYSIS

### 2.1 Participants and sampling

Blood samples were collected from the residents of Kadji Sai, Mailuu-Suu, and Sumsar (**Figure 1**) based on the framework for basic cardiovascular biomedical research on people living in the environment of selected U legacy sites at high-altitude areas in Kyrgyzstan (study approved by national Ethical Committee in Bishkek, Kyrgyzstan, No. 01-2/367). Sampling was performed by trained medical personnel in June and August 2016. Altogether, whole blood was collected from 123 residents covering men and women who voluntarily participated in the event: 32 men and 91 women with average age  $43 \pm 16$  years and eleven of them below 20 years old. Basic participants' data related to each studied settlement are included in **Table 2**. For Kadji Sai there was no male participants. Blood was taken from the forearm vein into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent blood clotting (BD Vacutainer® K2E for Kadji Sai and Sumsar; Ampul Lab K3EDTA Soyagreentec for Mailuu-Suu and 9 samples of Sumsar). The samples were placed in an ice box, transferred to the analytical laboratory at the Jožef Stefan Institute, Ljubljana, Slovenia, and stored at  $-30^{\circ}\text{C}$  until further processing (trace element analysis, DNA extraction and genotyping).



**Figure 1:** Geographical distribution of study areas in Kyrgyzstan.

### 2.2 Environmental (radiological and chemical) conditions at investigated sites

#### *KADJI SAI (Figure 2A)*

Kadji Sai consists of two settlements, lower and upper Kadji Sai, with a population of 7,000 people; it is located ~2.5 km from the southern shore of Lake Issyk-Kul and ~180 m above the water level. A U processing plant operated there until 1967, and U was extracted from coal ash to leave behind ~400,000

m<sup>3</sup> of radioactive residues (**Gavshin et al., 2004, 2005**). The tailings pile was initially remediated and later destroyed. Thus, it could still impact the environment through airborne and aqueous pollution with radioactive and chemical contaminants.

Radiation doses are mainly attributed to indoor radon concentrations and gamma radiation; they ranged from 2.0 to 12.2 mSv per year, thus posing low radiological risk (**Lespukh et al., 2013**) to the local population. Naturally occurring radionuclides in the drinking water sources in the Kadji Sai area have not exceeded individual radiation dose of 0.1 mSv per year.

Trace element concentrations in drinking water were below both internationally recommended values and national standards (maximum permissible concentration, MPC). Relatively high U concentrations were found in the waters of Lake Issyk-Kul (50–60 µg/L) (**Kulenbekov, 2013; Lind et al., 2013**), which exceeded internationally recommended values for drinking water (**WHO, 2011**). Elevated arsenic (As) concentrations were detected in the meat (muscles/fillet) of some fish species from Lake Issyk-Kul (**Lind et al., 2013**), which may increase As uptake in people who eat fish.

#### *MAILUU-SUU (Figure 2B)*

Mailuu-Suu is in the southwestern part of Kyrgyzstan and contains 23 U tailing sites (including mill tailings (13)) with a total of ~2.5 million tonnes (**Vandenhove et al., 2006**) of deposited radioactive residues from the processing of U ore. Most tailings sites and 11 waste rock dumps are located within city limits (**Vandenhove et al., 2006; Kunze et al., 2007**), which has ~20,000 inhabitants. The whole area is prone to natural disasters such as flooding and landslides; these have occurred several times and severely damaged the landscape by transporting contaminated radioactive materials (**Vandenhove et al., 2006**). These events have also affected local surface waters and numerous groundwater sources with radioactive and chemical contaminants (**BGR, 2008**). Previous and current studies have reported elevated levels of U, As, molybdenum, strontium, and sulphates (SO<sub>4</sub><sup>2-</sup>) in the aqueous environment, which pose a risk to human health through drinking water. The radiation dose via drinking water consumption did not exceed 0.1 mSv per year; therefore, no health impact can be expected. However, exposure to chemical elements, especially toxic trace elements, might be significantly elevated, thus potentially impacting the local population. Mailuu-Suu is also known for the elevated levels of technogenic and naturally occurring U and selenium in the environment (**Aparin et al., 2012**). In many water sources, the contents of U and several other trace elements exceeded internationally recommended values for drinking water (**BGR, 2008; Corcho Alvarado et al., 2014; WHO, 2011**) as well as national MPCs, thus making the water unfit for consumption.

#### *SUMSAR (Figure 2C)*

Sumsar is located in the south-western part of Kyrgyzstan in the foothills of the southern spurs of the Chatkal ridge and in the middle stream of the Sumsar transboundary river valley, the right-hand-side

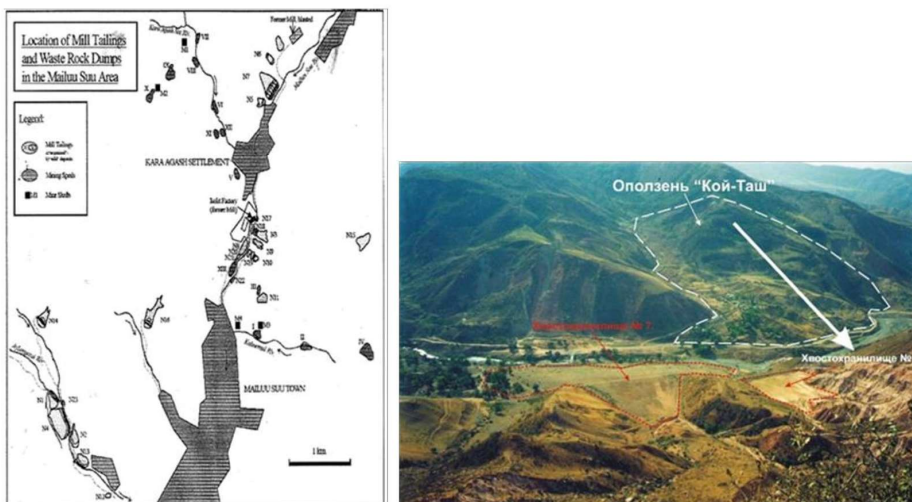
tributary of the Syrdarya River. Sumsar is known for its polymetallic ore deposits, and the Sumsar valley is situated next to the Shekaftar valley, where seven U waste rock dumps are deposited (Stegnar et al., 2013).

A lead-zinc (Pb-Zn) ore extraction and processing complex operated in Sumsar from 1951 to 1978. Initially, it produced only Pb concentrate; from 1963, it also started producing Zn concentrate. Furthermore, in 1961, experimental works for copper extraction started at the Sumsar processing plant. The main environmental pollutants are Pb and Zn contained in industrial wastes from a non-remediated tailings site with area of 14 hectares and containing ~1.8 million m<sup>3</sup> of waste with a tenth of percentage concentration of each metal (**FSD, 2017**). Both contaminants are present in dust particulates that spread easily over the Sumsar valley in windy conditions.

All three tailings sites in the Sumsar area could impact the Sumsar River, the only source of drinking water for the local population. A recent study showed that levels of trace elements in water have not exceeded (**FSD, 2017**) recommended limits (MPCs) for drinking water (**WHO, 2011**), although the drainage and seepage waters from the tailings contained elevated levels of several trace elements and sulphates (**FSD, 2017**).



A) Kadji Sai



B) Mailuu-Suu



C) Sumsar

**Figure 2.** Environmental conditions at investigated sites:

A) **Kadji Sai** - U extraction plant (left), and U tailing pile near Lake Issyk-Kul (right); B) **Mailuu-Suu** - location of mill tailings and waste rock dumps (left), and “Koi-Tash” landslide threatening tailing piles at Mailuu-Suu River (right); C) **Sumsar** - locations of three industrial tailing sites (left), and open surface of tailing #3 located on left bank of the river and ~100 m above the Sumsar valley (right). (Photo: P. Stegnar and I. Torgoev)



### 2.3 Mn, Fe, Cu, Zn, As, Se, Cd, Hg, Pb, and U determination by ICP-MS

Complete chemical decomposition assisted by microwave digestion of the blood samples was performed prior to the multi-element determination of selected trace elements via inductively coupled plasma mass spectrometry (ICP-MS).

Blood (0.3 mL) was digested in 0.5 mL 65% nitric acid (Suprapur) using a microwave system (ULTRAWAVE, MILESTONE Srl, Sorisole, Italy). After appropriate dilution, selected elements were measured using ICP-MS (8800 Agilent, Tokyo, Japan) equipped with an ASX-510 Autosampler (Cetac). The instrument conditions were as follows: Micro Mist nebuliser, Scott-type spray chamber, spray chamber temperature of 5°C, plasma gas flow rate of 15 L/min, carrier gas flow rate of 0.8 L/min, make-up gas flow rate of 0.1 L/min, sample solution uptake flow rate of 1 mL/min, RF power of 1500 W, reaction cell gases (hydrogen or helium) of 4 mL/min, and isotopes monitored —  $^{55}\text{Mn}$ ,  $^{56,57}\text{Fe}$ ,  $^{63}\text{Cu}$ ,  $^{66}\text{Zn}$ ,  $^{75}\text{As}$ ,  $^{78}\text{Se}$ ,  $^{111,114}\text{Cd}$ ,  $^{202}\text{Hg}$ ,  $^{206-208}\text{Pb}$ , and  $^{238}\text{U}$ . Instruments were tuned daily. Blank samples, control samples, and reference materials were tested together with the samples on a daily basis. The limits of detection (LOD) were calculated as three times the standard deviation of the blank sample and were 0.7 (Mn), 80 (Fe), 1 (Cu), 40 (Zn), 0.2 (As), 0.5 (Se), 0.04 (Cd), 0.1 (Hg), 1 (Pb), and 0.03 (U) ng/g. The quality of the results was checked via the regular use of the reference material Seronorm™ Whole Blood Level 1 (SERO, Billingstad, Norway) and for Cd, Hg, and Pb, also by participation in the German External Quality Assessment Scheme (G-EQUAS, Erlangen-Nuremberg, Germany). Analytical and quality control procedures for the elemental analysis used are described in detail in **Miklavčič et al. (2013)** and **Polanska et al. (2018)**. ICP-MS measurements of multiple elements in commercial reference material (CRM) Seronorm™ Trace Elements Whole Blood L-1, ref. 210105, LOT 1702821 with provided recommended/analytical values or acceptable ranges are given in **Table 1**.

Results were in a good agreement with provided Seronorm levels. Different type of tubes for blood collection did not influenced elements determination, except for Zn. Samples from Ampul Lab K3EDTA tubes, which were accidentally used in 33 cases, were contaminated with Zn. Accordingly, all Zn results were excluded from all the assessments. Results for uncontaminated samples were inside reference levels.

**Table 1.** ICP-MS measurements of multiple elements in CRM Seronorm™ Trace Elements Whole Blood L-1, ref. 210105, LOT 1702821.

| Element                | Recommended/analytical value<br>(acceptable range) | Measured value $\pm$ SD*<br>(n=7) |
|------------------------|----------------------------------------------------|-----------------------------------|
| Mn ( $\mu\text{g/L}$ ) | 19.7 (18.1-21.3)                                   | 19.9 $\pm$ 2.6                    |
| Fe ( $\text{mg/L}$ )   | 357                                                | 340 $\pm$ 25                      |
| Cu ( $\text{mg/L}$ )   | 0.64 (0.59-0.70)                                   | 0.61 $\pm$ 0.05                   |
| Zn ( $\text{mg/L}$ )   | 4.6 (3.8-5.3)                                      | 4.7 $\pm$ 0.3                     |
| As ( $\mu\text{g/L}$ ) | 2.1 (1.7-2.5)                                      | 2.0 $\pm$ 0.1                     |
| Se ( $\mu\text{g/L}$ ) | 69 (54-84)                                         | 74 $\pm$ 10                       |
| Cd ( $\mu\text{g/L}$ ) | 0.28 (0.23-0.34)                                   | 0.28 $\pm$ 0.03                   |
| Hg ( $\mu\text{g/L}$ ) | 1.57 (1.25-1.88)                                   | 1.29 $\pm$ 0.06                   |
| Pb ( $\mu\text{g/L}$ ) | 10 (7.9-12.0)                                      | 10.1 $\pm$ 0.6                    |
| U ( $\text{ng/L}$ )    | 0.13                                               | 0.14 $\pm$ 0.02                   |

\*SD – standard deviation of measurements

#### 2.4 DNA extraction and *ALAD* SNPs genotyping

Blood (0.5 mL) was used for DNA extraction using the FlexiGene® DNA Kit (Qiagen, Hilden, Germany). The quantity and quality of extracted DNA was determined by NanoDrop 2000c UV-VIS spectrophotometer (ThermoFisher Scientific, USA). Extracted DNA samples (n = 120) were stored at -80°C and later genotyped for two intron and one exon SNP of the *ALAD* gene on chromosome 9: rs1805313 (A > G), rs818708 (G > A) and rs1800435 (C > G; Lys > Asn). For the latter, in the literature the variant allele (*ALAD2*) is commonly referred as C allele. However, SNP alleles could be reported regarding the choice of DNA strand (Nelson et al., 2012). In the present study, *ALAD2* is given as G allele accordingly to dbSNP, NCBI (NCBI, 2019). Genotyping was performed using pre-designed TaqMan® SNP Genotyping Assays (C\_\_11495186\_1, C\_\_1632155\_20, and C\_\_11495146\_10) (Applied Biosystems, Foster City, CA, USA). The reaction volume was 5  $\mu\text{L}$ : 2.5  $\mu\text{L}$  of TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA, USA), 1.875  $\mu\text{L}$  of ultrapure nuclease-free water (Life Technologies, CA, USA), 0.125  $\mu\text{L}$  of 44X TaqMan probe/primer mix, and 0.5  $\mu\text{L}$  of genomic DNA. Amplification and fluorescence detection were performed by LightCycler® 480 Instrument II and LightCycler® 480 Software version 1.5.1 (Roche). PCR cycling steps were: pre-PCR (1 cycle: 50°C, 2 min), activation (1 cycle: 95°C, 10 min), annealing with amplification (50 cycles: 95°C, 15 s and 61°C, 1 min), and post-PCR (1 cycle: 40°C, 30 s). Subsets of randomly selected samples were repeated as a control (~10%).

## 2.5 Statistics

### *Trace element levels*

Descriptive statistics was used to assess metal(loid) concentrations: geometric mean (GM), 95% confidence interval (95CI); age was expressed in terms of arithmetic mean and minimum - maximum ( $\bar{x}$ , min-max). Values <LOD were substituted with LOD/2; for U, zero values were replaced with the measured minimum value divided by two - (0.001)/2. Comparisons between groups (Kadji Sai, Mailuu-Suu, and Sumsar) were performed by multivariable linear regression analyses with the age and gender adjustment for all residents and with age adjustment for women only.

### *ALAD SNPs and their influence on blood Pb levels*

Pearson's chi squared test was used to test all three ALAD SNPs for deviation from Hardy-Weinberg Equilibrium (HWE;  $p > 0.05$ ). Minor allele frequencies of each SNP were calculated for all participants together and for each group separately. Pearson's chi squared test was then used for comparing the SNP minor allele frequencies (in %) between three Kyrgyzstan groups and to compare minor allele frequency (in %) calculated from data for all Kyrgyzstan participants with those for worldwide population groups. Comparisons of Pb levels between carriers and non-carriers of SNPs variant allele and between genotypes were assessed by the non-parametric Mann-Whitney U test and Kruskal-Wallis rank test, respectively. Comparisons were performed on a group of all participants together, on all those older than 20 years and on all women older than 20 years. Selections were used to gradually exclude physiological differences (adulthood/childhood, women/men). In the same way, we then compared participants from Sumsar region known to be additionally polluted by Pb-Zn mining activities. Associations were further assessed by adjustment for confounders using a multivariable linear regression models. Confounders, age and blood trace elements (Se, Mn, Cu, and Fe) were used as continuous variables while sex and geographical region were used as a categorical variable. Variance inflation factor test was used to assess the collinearity between available confounders (independent variables). We took well-known confounders like age, gender and region for the crude, basic model (model 1), and added the essential elements status to get more sophisticated model (model 2). Particularly valuable was iron representing a substitute for missing haemoglobin levels and as known modifier of Pb uptake. Additional impact of non-essential elements As and Hg was tested by model 3 given in supplements. Models were used for all residents, for women and men older than 20 years and for women older than 20 years; regardless the region and/or for each region separately. In models R ( $R^2$ ) squared represents the proportion of variance in Pb blood levels that is predictable from the combination of all independent variables; beta coefficient with 95 confidence interval ( $\beta$ , 95 CI) represents the percentage change in blood Pb (dependent variable) due to presence of SNP's variant allele, gender (women), region, or one unit change in continuous variables like age and trace element concentrations.

We used **STATA12/SE** statistical software. In multi-variable linear regression analysis, ln-transformed data were used to approximate the normal distribution. The level of statistical significance was set at  $p < 0.05$  (two-sided). **OriginPro 2017** software (OriginLab, Northampton, USA) was used for visualizing the results (box plots, histograms).

### 3. RESULTS AND DISCUSSION

#### 3.1 Trace elements

**Table 2** summarizes data on trace elements in whole blood of Kadji Sai, Mailuu-Suu, and Sumsar residents. Geometric means (GM) exceeding the literature reference data for adults are in bold. Statistically significant differences of data distribution for several trace elements between the investigated areas, adjusted for age and gender, are marked with the letters A, B, and C. Because data for women didn't differ a lot from those of all participants, our comments below are related to all participants. For all participants data distributions is also presented by box plots, separately for a group of non-essential (Figure 3A) and essential trace (Figure 3B) elements in the study areas. Estimated influence of age on trace elements levels was observed particularly for Pb (positive) and Hg (supplements, Table SP1).

##### *Lead (Pb)*

Blood Pb levels (BLL) in Sumsar and Mailuu-Suu were statistically significantly higher than in Kadji Sai (47 % and 44% higher, respectively). They were also above the reference value for adults, in Sumsar for 8% and in Mailuu-Suu for 2%. Although Pb concentrations were only slightly higher than the reference values obtained from a non-exposed environment (<25 ng/g), the results may indicate chronic exposure to Pb through several pathways. This includes air and dust contamination, which is linked to inhalation of Pb-containing dust particles from tailing No.3 with ~0.2% Pb content in the tailings waste (FSD, 2017). Pb intake from other sources like food and drinking water is much less evident.

The obtained BLLs in all three investigated areas are probably still too low to result in Pb intoxication among residents of Sumsar and Mailuu-Suu. Results suggest that in absence of specific physiological (pregnancy, childhood) or patho-physiological conditions there is no reason for serious health concerns for this population, and no symptoms of Pb intoxication are expected. However, symptoms may appear with Pb concentrations exceeding 100 ng/g in blood (Skerfving and Bergdahl, 2016). Considering the data and Pb's wide range of potentially toxic effects, the situation should not be ignored; remediation of the tailing sites and the living environment should be prioritized.

##### *Cadmium (Cd)*

For Cd, another toxic trace element with very low concentrations in blood, statistically significant differences were not observed between study areas. All concentrations were within the reference interval for adults. The primary environmental source of Cd is supposedly water; however, data on Cd as a water pollutant in both areas is scarce. In Kadji Sai, evidence suggests that Cd is leached from the



tailings pile (**Lind et al., 2013**) and can enter groundwater (potentially, also drinking water) sources. It is also important to stress out Cd exposure through tobacco smoke, which can be reflected in higher blood Cd levels depending on Cd content in the tobacco. In present study the smoking of participants was not available, but it could be assumed that, due to the cultural background, at least for women population the effect of smoking was less important. According to WHO Tobacco Control Fact Sheet for Kyrgyzstan the estimated smoking prevalence of adult smokers (25 – 64 years) in 2013 was 48.2% for men and 2.7 % for women (**WHO, 2017**).

#### *Mercury (Hg)*

For Hg, one of the most toxic heavy metals present in blood in very low concentrations, all values were below the reference level. Nevertheless, statistically significant differences were found among all three study areas. The highest concentrations observed in Kadji Sai were 37% and 51% higher than in Mailuu-Suu and Sumsar, respectively. A reliable link could not be found between environmental Hg levels and human blood. Fish could be a cause of Hg intake if they were an important part of the human diet; however, this was not the case at Lake Issyk-Kul. Total Hg concentrations in the muscles of fish from the lake ranged from 0.03 to 0.07 mg/kg dry weight (**Lind et al., 2013**) and were comparable to those at other investigated areas in the CA region (**Skipperud et al., 2013**).

#### *Arsenic (As)*

For As, no relationship was observed between environmental sources of As and blood levels, despite relatively high As concentrations in various groundwater and surface water sources used for drinking water. Recent data (**Lind et al., 2013, Kulenbekov, 2013**) reported As concentrations exceeding internationally recommended values of 10 µg/L (**WHO, 2011**) for drinking water in several water sources. Another possible environmental source may be the somewhat higher concentrations of As in the muscles (fillet) of three fish species, ranging from 0.3 to 0.9 mg/kg dry weight (**Lind et al., 2013**), although fish from Lake Issyk-Kul are not an important part of the diet for Kadji Sai residents. A similar environmental situation is present in Mailuu-Suu, where As concentrations in many water sources exceed MPCs, thus making the water unfit for drinking (**BGR, 2008**). In Sumsar, As is contained in industrial waste tailings (**FSD, 2017**), drainage waters from the tailings, and in the Sumsar river (**FSD, 2017**). Sumsar had statistically significant higher levels of As than areas of Kadji Sai and Mailuu-Suu - 44% and 26% higher, respectively. Anyway, geometrical means were below reference level in all three areas.

#### *Uranium (U)*

U levels in individual blood samples from all three study areas exceeded the reference value of 0.010 ng/g (**Byrne and Benedik, 1991**). U, a highly mobile heavy metal in the environment, is present in many compartments of the Kadji Sai environment. U is present in a wide range of concentrations,

ranging from a few to several hundred micrograms per litre of water, in potential drinking water supplies including Lake Issyk-Kul (**Kulenbekov, 2013, Lind et al., 2013**). Along with drinking water, residents could be exposed to U through airborne pollution from (fly) ash material contained in U mill tailings pile at the upper Kadji Sai settlement (**Kulenbekov, 2013**).

Blood U concentrations exceeded the reference value for more than 60% of adults in Mailuu-Suu; GM exceeded reference level for 9%. This indicates a positive response of human exposure to U in the environment of Mailuu-Suu, mainly through aqueous environment. Aqueous environment is affected by the inflow (e.g. leaching, migration) of U and other chemicals (e.g.  $\text{SO}_4^{2-}$ ) from contaminated surface water to groundwater, which is unfit for drinking at more than 20 locations (**BGR, 2008**) in the area. U levels in several water sources (**BGR, 2008**) greatly exceeded (by orders of magnitude) internationally recommended values of 15–30  $\mu\text{g/L}$  for drinking water (**WHO, 2011; USEPA**). U in blood presumably indicates long-term human exposure and a possible link between increased technogenic U in the environment and blood levels of Mailuu-Suu residents. Residents of Mailuu-Suu had for 82% higher levels of U from residents of Kadji Sai or Sumsar. However, U levels remain much lower than those at which health effects can be expected (**Byrne and Benedik, 1991**).

#### *Selenium (Se)*

In all blood samples, Se was homogeneously distributed with most values within narrow 95% confidence intervals (**Table 2**). Se showed statistically significant differences between all three study areas. Mailuu-Suu is known for the relatively high Se content in soil and vegetation (**Aparin et al., 2012**), as indicated by its elevated blood Se levels compared to Kadji Sai and Sumsar (34% and 17% higher, respectively). The highest GM value in Mailuu-Suu corresponded with higher U concentrations. This may indicate a protective role of Se in the human body. Se, in past considered as toxic, is now classified as an essential element with narrow window between toxicity and deficiency. Its intake is needed for regular synthesis of selenoproteins implicated in redox homeostasis (e.g. thioredoxin reductases - TrxRs), antioxidant processes (e.g. glutathionperoxidases - GPXs, selenoprotein P - SelP), thyroid hormone regulation (e.g. iodothyronine deiodonases – DOIs) (Alexander, 2015). In such way optimal Se status can have important role in endogenous defence system against toxicity of excessive reactive oxygen and nitrogen species (ROS, NOS) produced by ionizing radiation (**Weiss and Landauer, 2009**) or by metals (**Alexander, 2015**). Besides, its metabolic intermediate selenide ( $\text{Se}^{2-}$ ) is believed to be responsible for direct metal-Se interactions by formation of insoluble various Se-metal complexes with modifying effects on toxicokinetic of both elements involved (**Naganuma et al., 1983; Alexander, 2016**).

#### *Copper (Cu), manganese (Mn), and iron (Fe)*

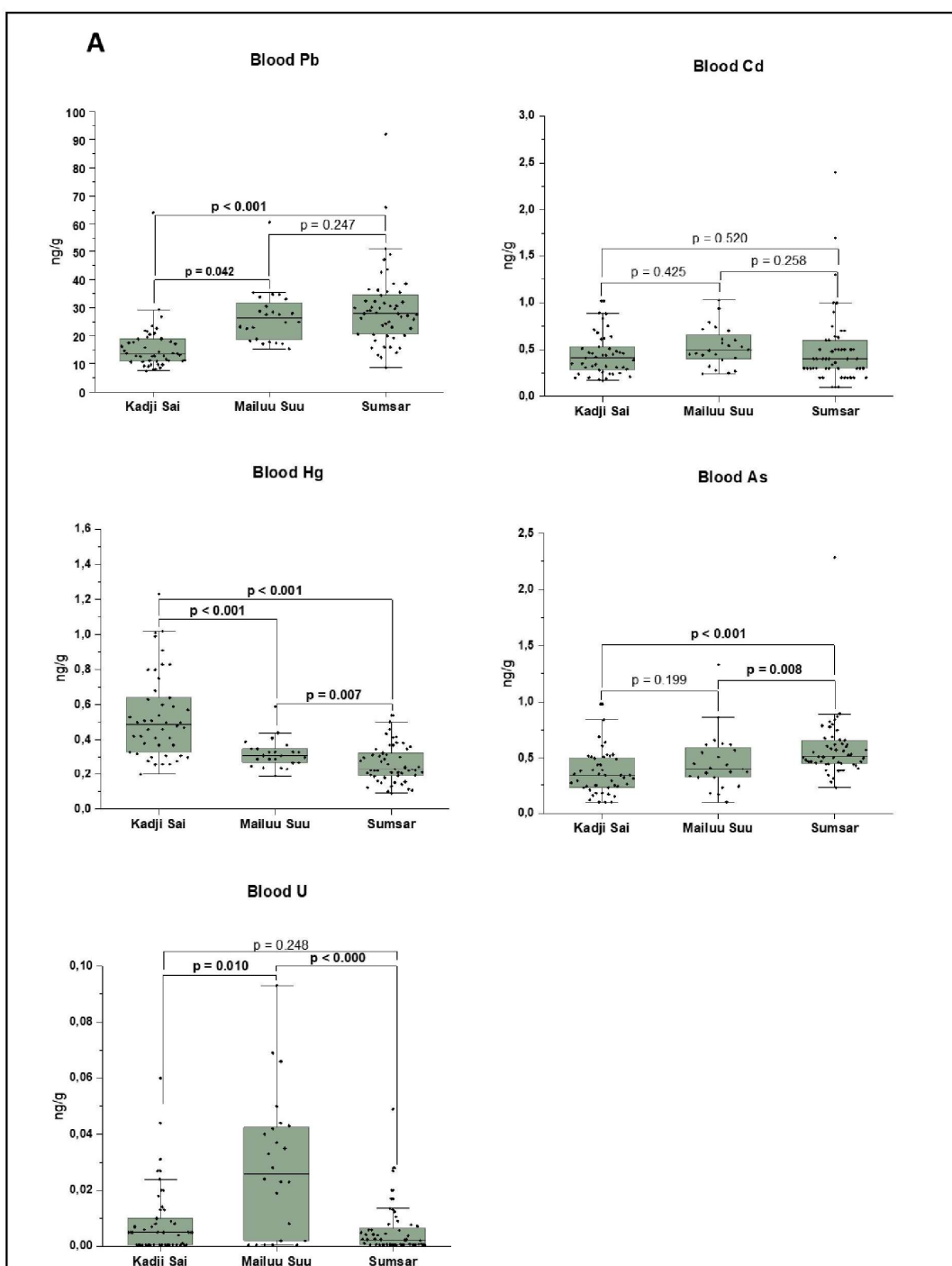
The blood levels of Cu were generally comparable to literature reference values for non-polluted environment, while the variations in Mn were due to specific environmental conditions in the studied

areas. Mn in Kadji Sai exceeded the upper reference level for 12% and exceeded the levels of other two areas for 25% (in Mailuu Suu) and 38 % (in Sumsar). GMs were still below suggested minimum risk level ( $< 18$  ng/mL) (**Schofield et al 2017**). In a few surveys the elevated levels of Mn, together with some other metals, were observed in the sediments of Lake Issyk – Kul; the runoff from tailing dumps from Kadji Sai was suggested as the most likely source (TA 7228-KGZ, 2009; Lind 2013). For Fe, the mean values were slightly lower (2-9%) than the reference interval regardless of the investigated area; the lowest level was observed in Kadji Sai. Results seems to be in agreement with known high prevalence of iron deficiency anaemia (IDA) reported for Central Asia, including Kyrgyzstan.

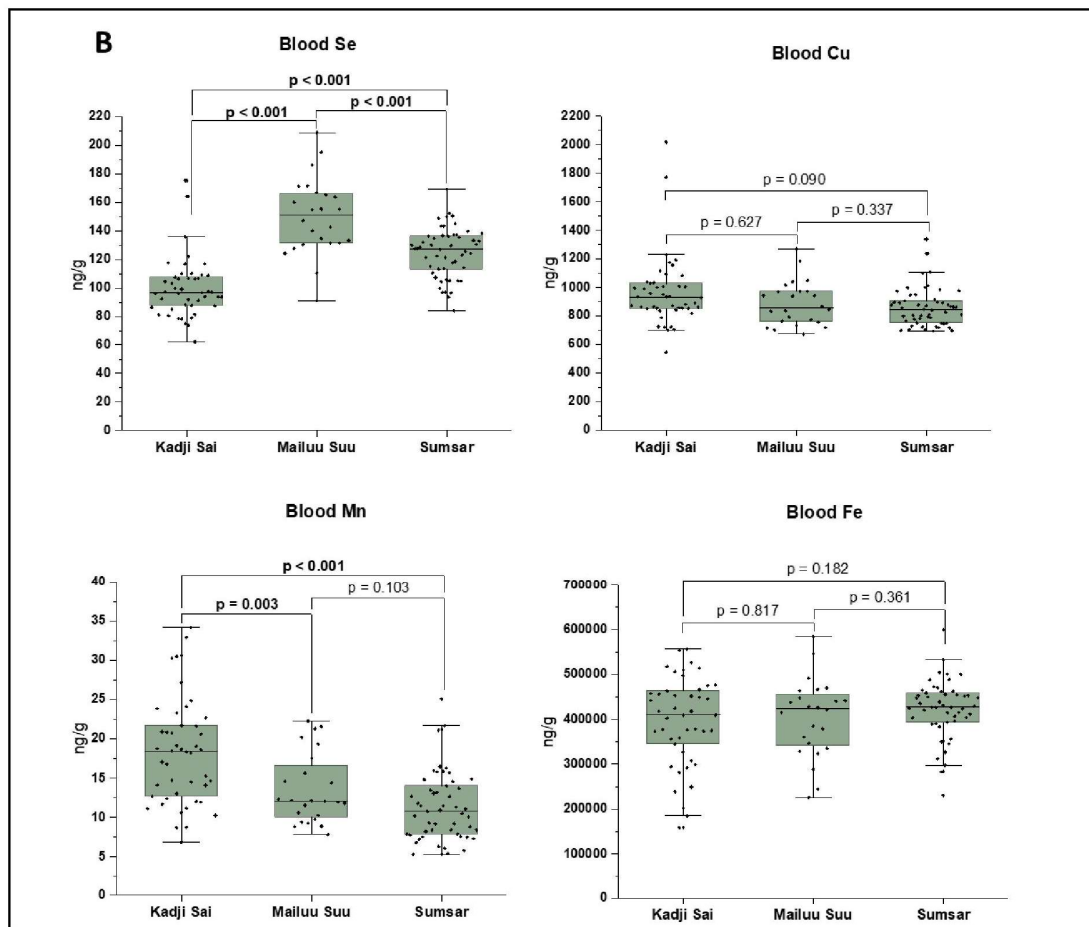
**Table 2.** Trace elements in whole blood of Kadji Sai, Mailuu-Suu, and Sumsar residents.

| Participants                                                       | KADJI SAI                              | MAILUU SUU                                | SUMSAR                                | RI                      |
|--------------------------------------------------------------------|----------------------------------------|-------------------------------------------|---------------------------------------|-------------------------|
| <b>NUMBER</b>                                                      |                                        |                                           |                                       |                         |
| All                                                                | 46                                     | 24                                        | 53                                    |                         |
| W/M                                                                | 46/-                                   | 12/12                                     | 33/20                                 |                         |
| W/M > 20y                                                          | 45/ -                                  | 12/10                                     | 26/19                                 |                         |
| <b>AGE</b> in years, $\bar{x}$ (min-max)                           |                                        |                                           |                                       |                         |
| A-I                                                                | 38 (19 -68) <sup>AB</sup>              | 47 (16 - 83) <sup>A</sup>                 | 43 (10 - 82) <sup>B</sup>             |                         |
| W/M                                                                | 38 (19 - 68)/ -                        | 54 (42 - 83)/41 (16 - 72)                 | 38 (10 - 58)/56 (17 - 82)             |                         |
| W/M > 20y                                                          | 39 (22 - 68)/-                         | 54 (42 -83)/46 (22 - 72)                  | 44 (27 - 63)/58 (32 - 82)             |                         |
| <b>TRACE ELEMENTS</b> in ng/g; $\mu\text{g/g}$ for Fe, GM (95% CI) |                                        |                                           |                                       |                         |
| All                                                                |                                        |                                           |                                       |                         |
| <b>Pb</b>                                                          | 14.3 (12.7 -18.1) <sup>AB</sup>        | <b>25.4 (22.4 - 29.1)<sup>A</sup></b>     | <b>27.2 (24.2 - 30.6)<sup>B</sup></b> | <25 <sup>1</sup>        |
| Cd                                                                 | 0.40 (0.35 - 0.46)                     | 0.49 (0.41 - 0.58)                        | 0.40 (0.33 - 0.48)                    | 0.3 - 1.2 <sup>1</sup>  |
| Hg                                                                 | 0.49 (0.34 - 0.55) <sup>AB</sup>       | 0.31 (0.28 - 0.34) <sup>A</sup>           | 0.24 (0.21 - 0.27) <sup>B</sup>       | <5.0 <sup>1</sup>       |
| As*                                                                | 0.30 (0.24 - 0.37) <sup>B</sup>        | 0.40 (0.31 - 0.51) <sup>C</sup>           | 0.54 (0.49 - 0.60) <sup>BC</sup>      | <1.7 <sup>1</sup>       |
| <b>U*</b>                                                          | 0.002 (0.001 - 0.004) <sup>A</sup>     | <b>0.011 (0.005 - 0.025)<sup>AC</sup></b> | 0.002 (0.002 - 0.003) <sup>C</sup>    | <0.010 <sup>2</sup>     |
| Se                                                                 | 98 (92 - 103) <sup>AB</sup>            | 148 (136 - 159) <sup>AC</sup>             | 123 (118 - 128) <sup>BC</sup>         | 58 - 234 <sup>1</sup>   |
| Cu                                                                 | 940 (881 -1003)                        | 876 (815 - 940)                           | 849 (816 - 884)                       | 800 - 1100 <sup>1</sup> |
| <b>Mn</b>                                                          | <b>17.1 (15.3 - 19.2)<sup>AB</sup></b> | 12.9 (11.3 - 14.8) <sup>A</sup>           | 10.6 (9.52 - 11.8) <sup>B</sup>       | 4 - 15 <sup>1</sup>     |
| <b>Fe*</b>                                                         | <b>385 (353 - 419)</b>                 | <b>395 (358 - 435)</b>                    | <b>418 (399- 538)</b>                 | 425 - 500 <sup>3</sup>  |
| Women                                                              |                                        |                                           |                                       |                         |
| Pb                                                                 | 14.3 (12.7 -18.1) <sup>AB</sup>        | 23.0 (17.1 - 29.1) <sup>A</sup>           | 24.3 (21.5 - 29) <sup>B</sup>         | <25 <sup>1</sup>        |
| Cd                                                                 | 0.40 (0.35 - 0.46)                     | 0.55 (0.46 - 0.65)                        | 0.39 (0.31 -0.50)                     | 0.3 - 1.2 <sup>1</sup>  |
| Hg                                                                 | 0.49 (0.34 - 0.55) <sup>AB</sup>       | 0.34 (0.29 - 0.40) <sup>A</sup>           | 0.24 (0.21 - 0.28) <sup>B</sup>       | <5.0 <sup>1</sup>       |
| As*                                                                | 0.30 (0.24 - 0.37) <sup>B</sup>        | 0.42 (0.26 - 0.67) <sup>C</sup>           | 0.50 (0.46 - 0.56) <sup>BC</sup>      | <1.7 <sup>1</sup>       |
| <b>U*</b>                                                          | 0.002 (0.001 - 0.004) <sup>A</sup>     | <b>0.011 (0.003 - 0.04)<sup>AC</sup></b>  | 0.002 (0.001 - 0.003) <sup>C</sup>    | <0.010 <sup>2</sup>     |
| Se                                                                 | 98 (92 - 103) <sup>AB</sup>            | 142 (123 - 163) <sup>AC</sup>             | 126 (120 - 131) <sup>BC</sup>         | 58 - 234 <sup>1</sup>   |
| Cu                                                                 | 940 (881 -1003)                        | 964 (875 - 1063)                          | 878 (831 - 928)                       | 800 - 1100 <sup>1</sup> |
| <b>Mn</b>                                                          | <b>17.1 (15.3 - 19.2)<sup>AB</sup></b> | 12.7 (10.1 - 16.1) <sup>A</sup>           | 10.8 (9.58 - 12.4) <sup>B</sup>       | 4 - 15 <sup>1</sup>     |
| <b>Fe*</b>                                                         | <b>385 (353 - 419)</b>                 | <b>388 (335 - 450)</b>                    | <b>419 (399 - 439)</b>                | 425 - 500 <sup>3</sup>  |

W – women, M – men; y – years; RI – reference interval;  $\bar{x}$  – arithmetic mean; GM – geometric mean; 95% CI – confidence interval; \* – in SUMSAR samples, As, U and Fe measurements are missing for two cases; A, B, C – statistically significant difference ( $p < 0.05$ ), with adjustment for age and gender, between: KADJI SAI and MAILUU-SUU (A); KADJI SAI and SUMSAR (B); MAILUU-SUU and SUMSAR (C); In bold - levels below or above reference values. 1 - TIETZ textbook (Roberts et al., 2012); 2 - Iyengar (1998) and Byrne and Benedik (1991), 3- Iyengar (1998).







**Figure 3.** Data distribution of trace elements (A - non-essential and B - essential) in Kadji Sai, Mailuu-Suu, and Sumsar. (Box plots show individual values, median, 25<sup>th</sup> and 75<sup>th</sup> percentiles, and outliers; added are p-values (adjusted for age and gender) for comparisons between groups).

### 3.2 *ALAD* polymorphisms and Pb

Blood DNA was genotyped for three SNPs of *ALAD* gene: SNP1 - rs1805313 (A>G), SNP2 - rs818708 (G>A) and SNP3 - rs1800435 (C>G; Lys>Asn; G allele referred to as *ALAD2*). Due to known binding of Pb on *ALAD* it is expected that some *ALAD* gene polymorphisms can influence the type of Pb-*ALAD* interactions and consequently the blood Pb levels. In **Table 3** are presented simple comparisons of blood Pb levels between variant allele carriers and non-carriers of SNPs - for whole and stratified population (age over 20 y, women gender, Sumsar region). We observed the influence of the first and third SNP while no significant associations were found for the second one. For SNP1 and 3, the variant allele was related to lower Pb levels - the first regarding combined participants of all three regions and the third at Sumsar only. Regarding the first one, rs1805313 (G+), our results confirmed very recent literature data (**Warrington et al., 2015; Szymańska-Chabowska et al., 2015**) obtained on general or occupationally exposed population. For third one, rs1800435 (G+ = *ALAD2*), the literature data are less clear and less consistent; anyway mostly is variably associated with higher Pb, particularly at occupationally exposed population (Scinciarillo et al 2007, **Broberg et al, 2015**). So it is probably not surprising that for this SNP, the relation with Pb was observed at Sumsar residents (or at Sumsar + Mailuu Suu, data not shown) with the highest internal Pb exposure (**Table 2**), although in opposite direction. Such negative relations are known and were already analysed and commented (**Zhao et al, 2007; Scinciarillo et al 2007, Broberg et al 2015**). They are rare, but present in cases with occupational and non-occupational Pb exposure; some of them are given in **Table 4**. According to meta-analysis of the effects of *ALAD* polymorphism on blood and bone lead levels, and on indicators of target organ toxicity (**Zhao et al 2007, Scinciarillo et al 2007**), and regarding the opinions in review articles (**Broberg et al, 2015, Skerfving and Bergdahl, 2015**) it is concluded that ‘*ALAD2* carriers generally show higher BLL levels in adults at increased levels of lead exposure, and appear to be protected against hemopoietic effects’ of Pb.

**Table 4.** Studies with observed association of rs1800435 *ALAD2* allele with lower blood Pb.

| Pb exposure                                                                           | Population                       |                                 | No. of<br><i>ALAD2/ALAD1</i><br>carriers | <i>ALAD2</i><br>MAF<br>(%) | B-Pb levels<br><i>ALAD2/ALAD1</i><br>(ng/mL) | Reference                  |
|---------------------------------------------------------------------------------------|----------------------------------|---------------------------------|------------------------------------------|----------------------------|----------------------------------------------|----------------------------|
|                                                                                       | Nationality                      | Age, gender(N)                  |                                          |                            |                                              |                            |
| Occupational:<br>Battery plants                                                       | Korean                           | 22-51y, M (307)                 | 21/44                                    | 16                         | 263 <sup>a</sup> /314                        | Sithisarankul et al., 1997 |
| Occupational: lead-acid<br>battery and electric-flex<br>m plants                      | Chinese                          | 22 – 59y,<br>M (51) and W 105   | 10/146                                   | 3                          | 178 <sup>b</sup> /271                        | Yang et al., 2012          |
| Occupational: factory of<br>lead stabiliser and<br>battery factory                    | Singaporean<br>and<br>Vietnamese | Mean 39y; M<br>(384), 75 W( 75) | 40/364                                   | 5                          | 130 <sup>f</sup> /170                        | Chia et al., 2006          |
| Occupational:<br>brass, ceramic, dyeing,<br>glaze, painting and<br>smelting factories | Indian                           | 25 – 35y,                       | 41/160                                   | 13                         | 136 <sup>g</sup> /181                        | Nariya et al., 2017        |
| Occupational:<br>ammunition factory                                                   | Egyptian                         | Mean 49y; M (50)                | 12/38                                    | 12                         | 270 <sup>b</sup> /330                        | Siha et al., 2019          |
| Non-occupational                                                                      | American                         | 47 -81y; M (726)                | 118/608                                  | 8                          | 57 <sup>b</sup> /63                          | Hu et al. 2001             |
| Non-occupational                                                                      | American                         | 20-59y; (?)                     | 161/1835                                 | 4                          | 25 <sup>g</sup> /28                          | Krieg et al., 2009         |
|                                                                                       |                                  | 12-16y; (?)                     | 67/751                                   | 4                          | 13 <sup>g</sup> /20                          |                            |

No.- number, y, years; M – men; W – women; <sup>a,b,c</sup> - difference in B-Pb levels between *ALAD2* and *ALAD1* allele carriers: <sup>a</sup> – difference observed based on mean value, <sup>b</sup> statistically significant difference observed based on simple group comparison and, <sup>c</sup> statistically significant difference observed based on multiple linear regression analyses.

To exclude possible errors due to impact of various individual factors we tested observed relations by multivariable models presented in **Table 5A** for all residents and in **5B** for Sumsar region; possible influence of age, gender and region on Pb levels was estimated by Model 1 and additional influence of essential trace elements by Model 2. The associations of SNP1 and SNP3 with Pb were confirmed and the assessments were mostly better with the inclusion of essential trace element (**Table 5A** - SNP1, Model 2; **Table 5B** – SNP3, Model 2). Variant allele carriers had 20 to 39% lower Pb levels. In addition to SNP allele, a statistically significant influence on Pb levels was observed for gender (negative; women vs. men), Se (positive), Mn (negative) and Fe (positive), but not for all in all cases. Lower Pb for women may be related to their supposed lower outdoor exposure. Unfortunately, we did not have data on smoking and drinking habits, which can represent an assessment bias. With model 2 we explained between 26 and 42% ( $R^2$ ) of Pb blood variability. Main results for combined Sumsar and Mailuu Suu residents were in agreement with those of Sumsar (Table SP2).

If we summarised it's evident that variant allele carriers of SNP3 in Sumsar had lower Pb levels, regardless the type of analysis (simple comparison or multiple regression analysis) and also regardless the type of stratification (all / participants over 20 y old / women over 20 y) (**Table 3 and 5B**); its effect on all residents was not observed (Sumsar + Mailuu Suu + Kadji Sai). For SNP1, the effect of variant allele on lower Pb levels was confirmed, regardless the type analysis (simple comparison or multiple regression analysis), only for women from all regions older than 20y. (**Table 3 and 5A**).

Co-exposure to some other non-essential elements like As and Hg, both statistically different among regions, was low (**Table 2**). Anyway, if we included them as additional confounding variables (Model 3), they slightly modify *ALAD* SNPs effects on Pb although without a change in the basic trends (**Table**

**SP3 in supplements**). According to obtained results, it seems that at low Pb exposure levels the co-exposure to other toxic elements and the status of essential elements could play important role in association studies. As ALAD is supposed to interact with other metals beside Pb (**Bernard and Lauwerys, 1987; Rocha et al, 2012**), the relations between *ALAD* SNPs variant alleles and other measured trace elements was also tested by simple comparison (**Table SP4 in supplements**). A slight influence was observed on Hg (SNP1), As (SNP2 and 3) and Se (SNP2 and 3); *ALAD2* (SNP3) was in positive relation to As and Se and non-significantly also with Hg.

In **Figure 4** are presented obtained frequencies of variant alleles of studied SNPs with comparison between Kyrgyzstan groups and between Kyrgyzstan population and other worldwide populations. Surprisingly, for SNP1 and 3 the frequencies of variant allele inside Kyrgyzstan varied a lot between observation regions. The frequency of SNP1 (rs1805313) variant allele for Kadji Sai residents (51%) considerably differs from that for Mailuu-Suu and Sumsar residents (35%; 35%). In general, the worldwide frequencies of this variant allele are relatively high although still quite variable. Frequencies of SNP3 (rs1800435) variant allele are much lower worldwide and in Kyrgyzstan (**Figure 4**). Anyway, interesting difference was observed inside Kyrgyzstan: Sumsar (16%) represents exception, as the frequency was considerably higher from other two regions (2%; 6%) and from dbSNP – NCBI populations. Observed differences in frequency distributions must be confirmed by higher number of participants per region. According to the results of present study, then they may be used to predict different internal Pb exposure at the same external Pb exposure.

Regarding Pb effects, the most susceptible groups are children, pregnant women and the elderly: children because of their higher intake, uptake and vulnerability than adults, and the other two groups because of heightened bone turnover during pregnancy/lactation and menopause/ageing, respectively. Over 90% of the Pb found in the adult human body is stored in the bones, with an average half-life of about 10 years (**Skerfving and Bergdahl, 2015**) ‘the bones’ lead stores may be mobilised during the occasions mentioned above. Bone Pb accumulation and its release in the bones both follow the general physiology of bone calcium metabolism. To find out if pregnant women (and new-borns) are at risk, maternal Pb levels can be followed by collecting blood samples from mothers and their new-borns during regular medical examinations at the beginning of pregnancy or during the third trimester, when Pb levels may be the highest. In future *ALAD* SNPs genotype assessments could represent additional information about possible higher or lower vulnerability of pregnant women, new-borns, young children and elderly. However, as stated by **Warrington et al. (2015)**, at present ‘we cannot be sure whether rs1805131 and other variants of *ALAD* affect whole-body lead burden and lead related risk’. Obtained results for present participants should be compared (or combined) with their general health status including biological parameters like blood pressure, ferritin, vitamin D status, haemoglobin levels etc. In that way, it could be possible to confirm, how *ALAD* SNPs are influencing not only Pb blood levels

but also the effects of Pb on health issues. With such wider association analyses (Pb blood concentration – *ALAD* polymorphism – specific health data related to Pb toxicity) it would be possible to identify (for rs1805131) or confirm (for *ALAD2*) if the carriers of specific *ALAD* allele(s) are more or less sensitive for Pb harmful effects at relatively low exposure levels. As noted above, the haematopoietic system is important target for lead toxicity (**Skerfving and Bergdahl, 2016**) even at low-dose levels (**Fowler, 2010**). Besides, the binding of Pb to *ALAD* enzyme is partially related to Pb induced hypertension and related cardiovascular diseases (**Skerfving and Bergdahl, 2016; Skoczynska and Skoczynska, 2012**).



**Table 3.** Data distribution of B-Pb (GM (P5-P95) between carriers and non-carriers of *ALAD* SNPs variant alleles and genotypes in all regions and in Sumsar.

| All regions      |      |                    |       | Sumsar             |       |                    |      |                     |      |                    |      |                      |
|------------------|------|--------------------|-------|--------------------|-------|--------------------|------|---------------------|------|--------------------|------|----------------------|
| All              |      | All > 20 years     |       | Women > 20 years   |       | All                |      | All > 20 years      |      | Women > 20 years   |      |                      |
| N                | ng/g | N                  | ng/g  | N                  | ng/g  | N                  | ng/g | N                   | ng/g | N                  | ng/g |                      |
| SNP 1: rs1805313 |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| Allele           |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| G-               | 40   | 24.3 (11.3 – 41.0) | 36    | 24.2 (10.9 – 43.6) | 25    | 22.2 (12.9 – 36.5) | 20   | 27.1 (11.3 – 54.7)  | 19   | 26.8 (8.60 – 65.9) | 12   | 26.1 (8.60 – 65.9) – |
| G+               | 79   | 20.1 (9.05 – 51.0) | 72    | 20.1 (9.05 – 50.9) | 55    | 17.3 (8.58 – 49.1) | 33   | 27.8 (13.1 – 51.0)  | 25   | 30.0 (16.0 – 51.0) | 14   | 25.1 (16.0 – 51.0)   |
| p-value          |      |                    | 0.022 |                    | 0.028 | 0.009              |      | 0.925               |      | 0.515              |      | 0.777                |
| Genotype         |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| AA               | 40   | 24.3 (11.3 – 41.0) | 36    | 24.2 (10.9 – 43.6) | 25    | 22.2 (12.9 – 36.5) | 20   | 27.1 (11.3 – 54.7)  | 19   | 26.8 (8.60 – 65.9) | 12   | 26.1 (8.60 – 65.9)   |
| AG               | 61   | 20.3 (9.73 – 47.1) | 56    | 20.6 (9.70 – 49.1) | 40    | 17.7 (9.38 – 39.5) | 29   | 25.9 (13.1 – 49.1)  | 23   | 28.8 (16.0 – 49.1) | 13   | 24.4 (16.0 – 49.1)   |
| GG               | 18   | 19.6 (7.44 – 91.9) | 16    | 18.2 (7.44 – 64.1) | 15    | 16.3 (7.44 – 64.1) | 4    | 45.5 (30.6 – 91.9)  | 2    | 57.2 (35.7 – 91.9) | 1    |                      |
| p-value          |      |                    | 0.066 |                    | 0.045 | 0.027              |      | 0.116               |      | 0.169              |      | 0.390                |
| SNP2: rs818708   |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| Allele           |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| A-               | 40   | 20.3 (17.5 – 23.6) | 38    | 20.1 (17.3 – 23.4) | 32    | 18.2 (15.9 – 20.8) | 16   | 28.1 (21.9 – 35.8)  | 14   | 28.4 (21.9 – 36.8) | 11   | 24.1 (20.1 – 28.7)   |
| A+               | 79   | 22.0 (19.7 – 24.7) | 70    | 22.0 (19.5 – 25.0) | 48    | 19.0 (16.4 – 22.1) | 36   | 27.3 (23.7 – 31.4)  | 30   | 26.7 (22.2 – 32.2) | 15   | 26.7 (22.2 – 32.2)   |
| p-value          |      |                    | 0.184 |                    | 0.161 | 0.724              |      | 0.606               |      | 0.383              |      | 0.243                |
| Genotype         |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| GG               | 40   | 20.3 (17.5 – 23.6) | 38    | 20.1 (17.3 – 23.4) | 32    | 18.2 (15.9 – 20.8) | 16   | 28.1 (21.9 – 35.8)  | 14   | 28.4 (21.9 – 36.8) | 11   | 24.1 (20.1 – 28.7) – |
| GA               | 63   | 23.2 (20.6 – 26.3) | 56    | 23.2 (20.4 – 26.6) | 37    | 19.6 (16.7 – 23.1) | 30   | 28.9 (25.3 – 33.1)  | 26   | 30.3 (26.4 – 34.6) | 13   | 26.9 (21.8 – 33.2)   |
| AA               | 16   | 17.9 (13.2 – 24.3) | 14    | 17.9 (12.5 – 24.8) | 11    | 17.2 (11.5 – 25.6) | 6    | 20.2 (11.4 – 35.88) | 4    | 20.3 (7.68 – 53.7) | 2    | 25.5 (1.25 – 518.8)  |
| p-value          |      |                    | 0.120 |                    | 0.113 | 0.469              |      | 0.372               |      | 0.302              |      | 0.496                |
| SNP 3: rs1800435 |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| Allele*          |      |                    |       |                    |       |                    |      |                     |      |                    |      |                      |
| G- (ALAD1)       | 98   | 21.3 (9.05 – 49.1) | 90    | 21.2 (9.05 – 49.1) | 68    | 18.4 (8.59 – 36.5) | 36   | 30.5 (15.6 – 65.9)  | 30   | 32.2 (20.5 – 62.0) | 17   | 28.9 (20.4 – 49.1)   |
| G+ (ALAD2)       | 21   | 21.9 (13.6 – 43.6) | 19    | 21.1 (8.60 – 51.0) | 13    | 19.0 (13.6 – 43.5) | 16   | 23.0 (8.60 – 51.0)  | 14   | 22.1 (8.60 – 51.0) | 9    | 20.3 (14.0 – 43.6)   |
| p-value          |      |                    | 0.859 |                    | 0.832 | 0.989              |      | 0.038               |      | 0.005              |      | 0.002                |

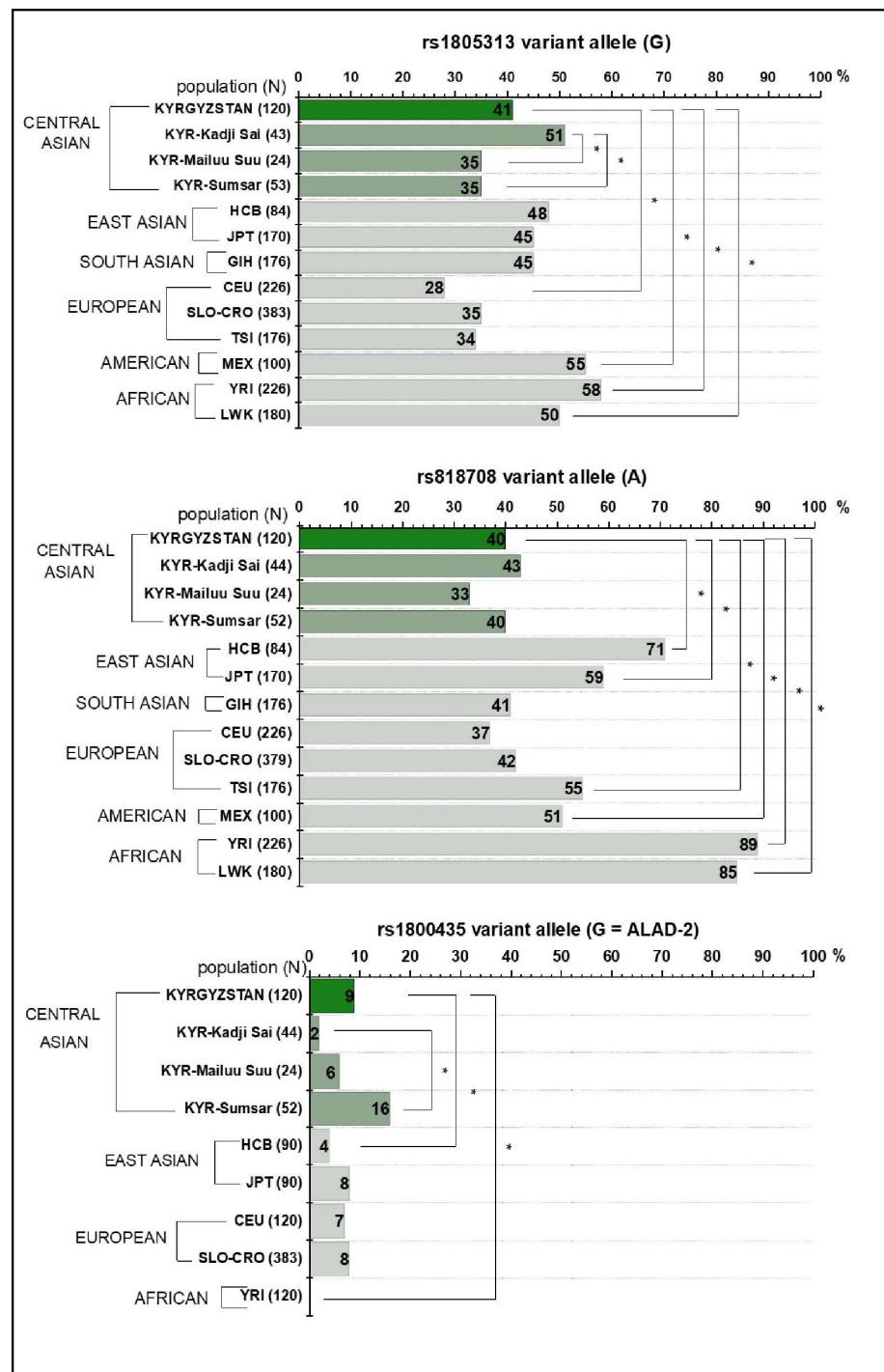
Significant results indicated in bold ( $p < 0.05$ ), marginally significant in bold italics ( $p < 0.1$ ); \* genotype distribution not given due to only one homozygote (GG) for the variant allele

**Table 5.** Influence of *ALAD* rs1805313 and rs1800435 variant alleles (G) on blood Pb in residents of all regions (A) and Sumsar (B), assessed by multi-variable linear regression models (model 1 and 2).

| A – All regions |                              |                       |         |                             |                       |         |                             |                       |         |                             |
|-----------------|------------------------------|-----------------------|---------|-----------------------------|-----------------------|---------|-----------------------------|-----------------------|---------|-----------------------------|
| B-Pb            | Independent Variable         | All                   |         |                             | All > 20 years        |         |                             | Women >20 years       |         |                             |
|                 |                              | β (95% CI)            | p-value | N, R <sup>2</sup> (p-value) | β (95% CI)            | p-value | N, R <sup>2</sup> (p-value) | β (95% CI)            | p-value | N, R <sup>2</sup> (p-value) |
| Model 1         | SNP1: rs1805313 (G+)         |                       |         |                             |                       |         |                             |                       |         |                             |
|                 | Age                          | -0.12 (-0.29, 0.05)   | 0.162   | 119                         | -0.14 (-0.31, 0.04)   | 0.131   | 108                         | -0.22 (-0.43, -0.01)  | 0.038   | 80                          |
|                 | Region <sup>a</sup>          | 0.004 (-0.001, 0.01)  | 0.101   | 0.27 (<0.001)               | 0.007 (0.000, 0.01)   | 0.037   | 0.29 (<0.001)               | 0.01 (0.003, 0.017)   | 0.006   | 0.18 (0.001)                |
|                 | Gender (women)               | 0.13 (0.03, 0.23)     | 0.015   |                             | 0.12 (0.01, 0.023)    | 0.034   |                             | 0.16 (0.02, 0.31)     | 0.029   |                             |
|                 |                              | -0.45 (-0.64, -0.26)  | <0.00   |                             | 0.41 (-0.61, -0.21)   | <0.001  |                             |                       |         |                             |
|                 | SNP3: rs1800435 (G+ = ALAD2) |                       |         |                             |                       |         |                             |                       |         |                             |
|                 | Age                          | -0.09 (-0.31, 0.12)   | 0.400   | 119                         | -0.12 (-0.35, 0.11)   | 0.296   | 109                         | -0.10 (-0.37, 0.17)   | 0.469   | 81                          |
|                 | Region <sup>a</sup>          | 0.004 (-0.001, 0.001) | 0.195   | 0.26 (<0.001)               | 0.006 (-0.000, 0.01)  | 0.054   | 0.28 (<0.001)               | 0.01 (0.002, 0.02)    | 0.012   | 0.13 (0.012)                |
|                 | Gender (women)               | 0.15 (0.04, 0.20)     | 0.007   |                             | 0.15 (0.03, 0.26)     | 0.013   |                             | 0.21 (0.05, -0.36)    | 0.009   |                             |
|                 |                              | -0.49 (-0.68, -0.29)  | <0.00   |                             | -0.44 (-0.64, -0.23)  | <0.001  |                             |                       |         |                             |
| Model 2         | SNP1: rs1805313 (G+)         |                       |         |                             |                       |         |                             |                       |         |                             |
|                 | Age                          | -0.13 (-0.29, 0.03)   | 0.111   | 116                         | -0.14 (-0.31, 0.03)   | 0.110   | 105                         | -0.20 (-0.39, -0.000) | 0.049   | 79                          |
|                 | Region <sup>a</sup>          | 0.003 (-0.002, 0.007) | 0.267   | 0.39 (<0.001)               | 0.002 (-0.004, 0.009) | 0.418   | 0.30 (<0.001)               | 0.003 (-0.006, 0.01)  | 0.484   | 0.30 (<0.001)               |
|                 | Gender (women)               | 0.10 (0.03, 0.29)     | 0.077   |                             | 0.10 (0.13, 0.40)     | 0.086   |                             | 0.10 (0.05, 0.25)     | 0.197   |                             |
|                 | B-Se                         | -0.37 (-0.57, -0.18)  | 0.001   |                             | -0.36 (-0.57, -0.15)  | 0.001   |                             | 0.41 (-0.02 – 0.83)   | 0.060   |                             |
|                 | B-Mn                         | 0.38 (0.01 – 0.75)    | 0.042   |                             | 0.37 (-0.01 – 0.76)   | 0.058   |                             | -0.31 (-0.55, -0.06)  | 0.014   |                             |
|                 | B-Cu                         | -0.27 (-0.47, -0.07)  | 0.007   |                             | -0.28 (-0.48, -0.08)  | 0.007   |                             | 0.29 (-0.20, 0.77)    | 0.245   |                             |
|                 | B-Fe                         | 0.22 (-0.21, 0.65)    | 0.317   |                             | 0.19 (-0.27, 0.65)    | 0.417   |                             | 0.17 (-0.21, 0.56)    | 0.380   |                             |
|                 |                              | 0.26 (-0.08, 0.59)    | 0.135   |                             | 0.26 (-0.09, 0.60)    | 0.140   |                             |                       |         |                             |
|                 |                              | -0.14 (-0.34, 0.07)   | 0.191   | 116                         | -0.18 (0.39, 0.04)    | 0.110   | 106                         | -0.12 (-0.39, 0.14)   | 0.362   | 80                          |
|                 | SNP3: rs1800435 (G+ = ALAD2) |                       |         |                             |                       |         |                             |                       |         |                             |
|                 | Age                          | 0.002 (-0.003, 0.007) | 0.446   | 0.39 (<0.001)               | 0.002 (-0.004, 0.009) | 0.447   | 0.40 (<0.001)               | 0.002 (-0.006, 0.011) | 0.563   | 0.28 (<0.001)               |
|                 | Region <sup>a</sup>          | 0.13 (0.02, 0.24)     | 0.026   |                             | 0.14 (0.02 – 0.25)    | 0.023   |                             | 0.14 (0.02, 0.29)     | 0.091   |                             |
|                 | Gender (women)               | -0.41 (-0.60, -0.21)  | <0.00   |                             | -0.37 (-0.58, -0.16)  | 0.001   |                             |                       |         |                             |
|                 | B-Se                         | 0.46 (0.08, 0.83)     | 0.017   |                             | 0.46 (0.07, 0.84)     | 0.023   |                             | 0.46 (0.02 – 0.90)    | 0.042   | 80                          |
|                 | B-Mn                         | -0.27 (-0.46, -0.07)  | 0.010   |                             | -0.27 (-0.47, -0.07)  | 0.010   |                             | -0.32 (-0.57, -0.06)  | 0.015   | 0.28 (<0.001)               |
|                 | B-Cu                         | 0.25 (-0.20, 0.69)    | 0.275   |                             | 0.15 (-0.31, 0.61)    | 0.521   |                             | 0.26 (-0.25, 0.76)    | 0.314   |                             |
| B-Fe            | 0.27 (-0.6, 0.61)            | 0.106                 |         | 0.28 (-0.05 – 0.62)         | 0.099                 |         | 0.24 (-0.15, 0.63)          | 0.225                 |         |                             |

<sup>a</sup> Sumsar versus Kadji Sai or Mailuu Suu; in bold are significant ( $p \leq 0.05$ ) results and in bold italic marginally significant ( $p \leq 0.1$ )

| B - Sumsar |                              |                                                                                                                       |                                                  |                             |                                                                                                                       |                                                  |                             |                                                                                                                      |                                                  |                             |
|------------|------------------------------|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-----------------------------|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-----------------------------|----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-----------------------------|
| B-Pb       | Independent Variable         | All                                                                                                                   |                                                  |                             | All > 20 years                                                                                                        |                                                  |                             | Women ≥20 years                                                                                                      |                                                  |                             |
|            |                              | β (95% CI)                                                                                                            | p-value                                          | N, R <sup>2</sup> (p-value) | β (95% CI)                                                                                                            | p-value                                          | N, R <sup>2</sup> (p-value) | β (95% CI)                                                                                                           | p-value                                          | N, R <sup>2</sup> (p-value) |
| Model 1    | SNP1: rs1805313 (G+)         |                                                                                                                       |                                                  |                             |                                                                                                                       |                                                  |                             |                                                                                                                      |                                                  |                             |
|            | Age                          | 0.05 (-0.18, 0.28)<br>0.003 (-0.00, 0.01)<br><i>-0.24 (-0.51, 0.03)</i>                                               | 0.644<br>0.310<br><i>0.088</i>                   | 52<br>0.15<br>(0.049)       | 0.09 (-0.15, 0.33)<br>0.001 (-0.01, 0.01)<br>-0.25 (-0.54, 0.06)                                                      | 0.460<br>0.824<br>0.107                          | 44<br>0.13<br>(0.143)       | -0.03 (-0.30, 0.24)<br>-0.003 (0.01, 0.01)                                                                           | 0.843<br>0.712                                   | 26<br>0.01<br>(0.386)       |
|            | SNP3: rs1800435 (G+ = ALAD2) |                                                                                                                       |                                                  |                             |                                                                                                                       |                                                  |                             |                                                                                                                      |                                                  |                             |
|            | Age                          | -0.28 (-0.50, -0.05)<br>0.002 (-0.005, 0.009)<br><i>-0.25 (-0.51, 0.003)</i>                                          | 0.018<br>0.579<br><i>0.053</i>                   | 51<br>0.22<br>(0.007)       | -0.36 (-0.59, -0.12)<br>0.001 (-0.009, 0.01)<br><i>-0.23 (-0.51, 0.04)</i>                                            | 0.004<br>0.834<br><i>0.095</i>                   | 44<br>0.28<br>(0.004)       | -0.36 (-0.58, -0.14)<br>-0.004 (-0.01, 0.007)<br><i>-0.36 (-0.58, -0.14)</i>                                         | 0.003<br>0.424<br><b>0.003</b>                   | 26<br>0.34<br>(0.009)       |
|            | SNP1: rs1805313 (G+)         |                                                                                                                       |                                                  |                             |                                                                                                                       |                                                  |                             |                                                                                                                      |                                                  |                             |
|            | Age                          | -0.05 (-0.31, 0.21)<br>0.004 (-0.004, 0.012)                                                                          | 0.699<br>0.314                                   |                             | -0.01 (-0.29, 0.27)<br>0.001 (-0.01, 0.02)                                                                            | 0.937<br>0.799                                   |                             | -0.02 (-0.35, 0.30)<br>-0.009 (-0.03, 0.01)                                                                          | 0.887<br>0.342                                   |                             |
|            | Gender (women)               | -0.26 (-0.55, -0.03)<br>-0.21 (-1.18, 0.77)<br>-0.11 (-0.42, 0.21)<br>0.15 (-0.70, 0.99)<br><b>0.84 (0.02 - 1.66)</b> | 0.076<br>0.672<br>0.506<br>0.727<br><b>0.046</b> | 50<br>0.26<br>(0.007)       | -0.26 (-0.61, 0.09)<br>-0.12 (-1.20, 0.96)<br>-0.15 (-0.48, 0.17)<br>0.02 (-1.01, 1.04)<br>0.69 (-0.16, 1.53)         | 0.138<br>0.829<br>0.344<br>0.976<br>0.108        | 42<br>0.24<br>(0.182)       | 0.50 (-0.98, 1.98)<br>-0.20 (-0.67, 0.29)<br>0.49 (-0.65, 1.64)<br>0.19 (-0.97, 1.36)<br><b>-0.36 (-0.53, -0.03)</b> | 0.490<br>0.412<br>0.378<br>0.731<br><b>0.009</b> | 26<br>0.12<br>(0.851)       |
| Model 2    | SNP3: rs1800435 (G+ = ALAD2) |                                                                                                                       |                                                  |                             |                                                                                                                       |                                                  |                             |                                                                                                                      |                                                  |                             |
|            | Age                          | -0.29 (-0.53, -0.05)<br>0.003 (-0.004, 0.10)                                                                          | 0.018<br>0.456                                   |                             | -0.39 (-0.63, -0.15)<br>0.003 (-0.008, 0.01)                                                                          | 0.002<br>0.609                                   |                             | -0.36 (-0.53, -0.03)<br>-0.008 (-0.02, 0.007)                                                                        | 0.009<br>0.285                                   |                             |
|            | Gender (women)               | -0.29 (-0.56, -0.02)<br>-0.06 (-0.92, 0.80)<br>-0.01 (-0.31, 0.29)<br>0.20 (-0.64, 1.05)<br><b>0.82 (0.10 - 1.54)</b> | 0.035<br>0.886<br>0.938<br>0.633<br><b>0.026</b> | 49<br>0.34<br>(0.012)       | -0.21 (-0.52, 0.09)<br>0.05 (-0.85, 0.94)<br>-0.09 (-0.37, 0.20)<br>-0.32 (-1.23, 0.60)<br><i>0.67 (-0.02 - 1.37)</i> | 0.160<br>0.917<br>0.541<br>0.488<br><i>0.057</i> | 42<br>0.42<br>(0.006)       | 0.62 (-0.51, 1.74)<br>-0.16 (-0.55, 0.22)<br>0.001 (-0.98, 0.98)<br>-0.06 (-0.98, 0.86)                              | 0.265<br>0.381<br>0.998<br>0.894                 | 26<br>0.39<br>(0.045)       |



**Figure 4.** Comparison of variant allele frequencies (%) between Kyrgyzstan groups and between Kyrgyzstan population (KYR) and other worldwide populations (dbSNP, NCBI and Stajanko et al., 2018) for SNP1, SNP2 and SNP3. (\* – significant difference  $p < 0.05$ ; HCB – Beijing, China; JPT – Tokyo, Japan; GIH – Gujarati Indian from Houston, Texas; CEU – Utah residents with ancestry from northern and western Europe; SLO-CRO – Ljubljana, Slovenia and Rijeka, Croatia; TSI – Tuscany, Italy; MEX – Mexican Ancestry from Los Angeles, USA; YRI – Yoruba in Ibadan, Nigeria; LWK – Luhya in Webuye, Kenya).

### 3.3. Strengths and limitations of the study

Our study presents first human biomonitoring data on blood trace element levels for Kyrgyzstan population covering three U legacy sites with known polymetallic contamination, namely Sumsar and Mailuu-Suu from the Jalal-Abad Province and Sumsar from Issyk-Kul Province. Screening for 5 potentially toxic non-essential and 4 essential elements was conducted on individuals covering a wide age range in order to represent general population of studied areas. However, the major limitation of the study is the missing information on some general personal characteristics (height, weight and smoking habits) and biochemical data (haemoglobin, haematocrit, and ferritin) which could influence the assessment of trace elements levels and group comparisons.

Additionally, the present study determined allele frequency distributions of three *ALAD* polymorphisms and assessed their associations with blood Pb levels, which were for Kyrgyzstan population to our knowledge assessed for the first time. However, Moldogazieva (2010) reported that populations of studied Kyrgyzstan provinces consist of Kyrgyz and other ethnic groups; in Jalal-Abad Province 44.3% of residents covered other ethnic groups in following order: Uzbek, Russians, Tatars, Ukrainians, Germans, Kazakhs; and in Issyk-Kul Province 22.2% in following order: Russians, Tatars and Ukrainians. As the genetic background can vary among different ethnic groups, missing information on ethnicity of our participants could represent bias in the assessment of *ALAD* allele frequency distribution.

## 4. CONCLUSIONS

The concentrations of selected essential and toxic trace elements in whole human blood of Kyrgyzstan residents from Sumsar, Mailuu Su and Kadji Sai were low and not greatly different from literature level obtained in non-polluted (exposed) areas. Only Pb in Sumsar and Mailuu Su, U in Mailuu Su and Mn in Kadji Sai slightly exceeded the reference intervals (up to 12%). Fe levels were slightly below reference value regardless the study area.

Most essential (Mn, Se) and toxic trace elements (Pb, As, U, Hg) in human blood showed statistically significant differences between the studied areas:

- Pb levels in Sumsar and Mailuu Su were higher from Kadji Sai for 47 and 44%, respectively; and similarly As levels were higher from Kadji Sai and Mailuu-Suu for 44% and 26%, respectively
- U levels in Mailuu Suu were higher from other two regions for 82%, and Se from Kadji Sai for 34% and from Sumsar for 17%;



- Hg and Mn in Kadji Sai were higher from Mailuu Su and Sumsar (Hg for 37 and 51%, respectively, and Mn for 25 and 38% respectively);

This indicated specific and different environmental conditions at industrial legacy sites for U milling and processing along with accompanying chemical (pollutant) elements. In general, no acute harm and related immediate risk to human health can be expected based on the obtained results. Human bio-monitoring results can support environmental data for certain trace elements, although an extended study is not easily justified in the environment with technologically enhanced radioactive and chemical contaminants.

The most significant findings were higher blood Pb concentrations that reliably indicated a pathway of human exposure. Furthermore, U can be used as a potential indicator of specific environmental conditions in technologically or naturally enhanced areas with radioactive and chemical contaminants.

For Pb, we have confirmed genetic influences (*ALAD* SNPs rs1805313 and rs1800435) on its blood variability; according to statistical analysis by regression models, up to 31% of variability in Pb blood was explained by *ALAD* polymorphisms. The frequency of the possibly protective *ALAD* rs1805313 variant allele (G) differed among the study areas (Kadji Sai > Mailuu-Suu and Sumsar); so did, although in different way, the frequency of *ALAD* rs1800435 variant allele (G = *ALAD*-2) (Sumsar > Mailuu-Suu > Kadji Sai).

Human bio-monitoring data can significantly contribute to environmental impact assessments and feasibility studies in areas with high health risks because of environmental contaminants, such as mining and processing. Such data can also contribute to the basic science of biomarkers and to the assessment of the general health status of concerned populations.

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## **SUPPLEMENTAL MATERIALS**

### **TRACE ELEMENTS AND *ALAD* GENE POLYMORPHISMS IN GENERAL POPULATION FROM THREE URANIUM LEGACY SITES – A CASE STUDY IN KYRGYZSTAN**

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**Table SP1:** Correlation between age and trace element levels ( $r_s$ , p-value) for all residents of Kyrgyzstan and for residents of each studied area separately.

|           | Spearman correlation: $r_s$ (p-value) |                       |                 |                |
|-----------|---------------------------------------|-----------------------|-----------------|----------------|
|           | Age-All residents                     | Age – Kadji Sai       | Age- Mailuu-Suu | Age - Sumsar   |
| <b>Pb</b> | <b>0.201 (0.023)</b>                  | 0.202 (0.176)         | 0.290 (0.168)   | 0.197 (0.157)  |
| <b>Cd</b> | <b>0.179 (0.048)</b>                  | 0.166 (0.271)         | 0.143 (0.507)   | 0.147 (0.294)  |
| <b>Hg</b> | -0.023 (0.763)                        | -0.132 (0.379)        | 0.299 (0.155)   | 0.180 (0.195)  |
| <b>As</b> | 0.155 (0.09)                          | 0.124 (0.416)         | 0.055 (0.799)   | 0.220 (0.122)  |
| <b>U</b>  | 0.038 (0.677)                         | -0.059 (0.696)        | 0.050 (0.816)   | 0.042 (0.767)  |
| <b>Se</b> | 0.075 (0.41)                          | 0.149 (0.325)         | -0.280 (0.184)  | -0.206 (0.139) |
| <b>Cu</b> | 0.104 (0.251)                         | <b>0.340 (0.021)</b>  | 0.254 (0.230)   | 0.030 (0.831)  |
| <b>Mn</b> | -0.148 (0.102)                        | <b>-0.349 (0.017)</b> | 0.180 (0.398)   | -0.013 (0.928) |
| <b>Fe</b> | 0.010 (0.909)                         | 0.031 (0.840)         | 0.124 (0.563)   | -0.119 (0.407) |

bolded are significant correlations ( $p < 0.05$ )

**Table SP2:** Influence of *ALAD* rs1805313 and rs1800435 variant alleles (G) on blood Pb in residents of Sumsar and Mailuu-Suu assessed by multi-variable linear regression models (model 1 and 2).

| SUMSAR and MAILUU-SUU |                              |                       |         |                             |                       |                 |                      |                             |                             |
|-----------------------|------------------------------|-----------------------|---------|-----------------------------|-----------------------|-----------------|----------------------|-----------------------------|-----------------------------|
| B-Pb                  | Independent Variable         | All                   |         | All > 20 years              |                       | Women >20 years |                      | N, R <sup>2</sup> (p-value) |                             |
|                       |                              | $\beta$ (95% CI)      | p-value | N, R <sup>2</sup> (p-value) | $\beta$ (95% CI)      | p-value         | $\beta$ (95% CI)     | p-value                     | N, R <sup>2</sup> (p-value) |
| Model 1               | SNP1: rs1805313 (G+)         |                       |         |                             |                       |                 |                      |                             |                             |
|                       | Age                          | -0.04 (-0.22, 0.14)   | 0.679   | 76                          | 0.004 (-0.19, 0.19)   | 0.964           | -0.06 (-0.30, 0.17)  | 0.582                       | 38                          |
|                       | Region <sup>a</sup>          | 0.001 (-0.004, 0.007) | 0.550   | 0.13 (0.037)                | 0.000 (-0.007, 0.007) | 0.975           | -0.001 (-0.01, 0.01) | 0.825                       | 0.036 (0.735)               |
|                       | Gender (women)               | 0.12 (-0.07, 0.30)    | 0.212   |                             | 0.15 (-0.04, 0.35)    | 0.1210          | 0.08 (-0.18, 0.34)   | 0.544                       |                             |
|                       | SNP3: rs1800435 (G+ = ALAD2) | -0.26 (-0.44, -0.07)  | 0.007   |                             | -0.24 (-0.43, -0.04)  | 0.018           | -0.32 (-0.53, -0.09) | 0.007                       |                             |
|                       | Age                          | -0.24 (-0.43, -0.05)  | 0.015   | 75                          | -0.30 (-0.50, -0.10)  | 0.004           | 0.000 (-0.01, 0.01)  | 0.887                       | 38                          |
|                       | Region <sup>a</sup>          | 0.000 (-0.004, 0.004) | 0.820   | 0.19 (0.004)                | 0.000 (-0.007, 0.007) | 0.980           | 0.16 (-0.08, -0.39)  | 0.200                       | 0.22 (0.036)                |
|                       | Gender (women)               | 0.17 (-0.008, 0.35)   | 0.061   |                             | 0.21 (0.02, 0.39)     | 0.029           |                      |                             |                             |
|                       |                              | -0.25 (-0.42, -0.07)  | 0.005   |                             | -0.22 8-0.40, -0.04)  | 0.020           |                      |                             |                             |
|                       | SNP1: rs1805313 (G+)         |                       |         |                             |                       |                 |                      |                             |                             |
| Model 2               | Age                          | -0.08 (-0.27, 0.11)   | 0.406   |                             | -0.04 (-0.24, 0.17)   | 0.713           | -0.08 (-0.34, 0.17)  | 0.523                       |                             |
|                       | Region <sup>a</sup>          | 0.001 (-0.003, 0.008) | 0.498   |                             | 0.000 8-0.008,        | 0.966           | -0.003 (-0.02, 0.01) | 0.611                       |                             |
|                       | Gender (women)               | 0.09 (-0.15, 0.32)    | 0.458   | 74                          | 0.13 (-0.12, 0.37)    | 0.298           | 0.11 (-0.20, 0.43)   | 0.455                       | 38                          |
|                       | B-Se                         | -0.27 (-0.48, -0.07)  | 0.010   | 0.19 (0.084)                | -0.26 (-0.48, -0.04)  | 0.024           | 0.25 (-0.54, 1.05)   | 0.525                       | 0.12 (0.782)                |
|                       | B-Mn                         | 0.07 (-0.56, 0.71)    | 0.819   |                             | 0.13 (-0.54, 0.79)    | 0.706           | -0.17 (-0.51, 0.17)  | 0.316                       |                             |
|                       | B-Cu                         | -0.17 (-0.42, 0.08)   | 0.173   |                             | -0.20 (-0.45, 0.06)   | 0.128           | 0.51 (-0.32, 1.34)   | 0.216                       |                             |
|                       | B-Fe                         | 0.16 (-0.48, 0.79)    | 0.628   |                             | 0.16 (-0.55, 0.88)    | 0.654           | -0.07 (-0.78, 0.64)  | 0.847                       |                             |
|                       |                              | 0.31 (-0.19, 0.81)    | 0.224   |                             | 0.26 (-0.25, 0.77)    | 0.311           |                      |                             |                             |
|                       | SNP3: rs1800435 (G+ = ALAD2) | -0.24 (-0.44, -0.03)  | 0.024   |                             | -0.30 (-0.51, -0.09)  | 0.006           | -0.29 (-0.55, 0.03)  | 0.028                       |                             |
|                       | Age                          | 0.001 (-0.004, 0.007) | 0.733   |                             | 0.000 (-0.007, 0.008) | 0.876           | -0.001 (-0.01, 0.01) | 0.810                       |                             |
|                       | Region <sup>a</sup>          | 0.18 (-0.05, 0.40)    | 0.125   |                             | 0.20 (-0.03, 0.43)    | 0.087           | 0.20 (-0.10, 0.49)   | 0.191                       | 38                          |
|                       | Gender (women)               | -0.28 (-0.47, -0.09)  | 0.006   | 73                          | -0.22 (-0.42, -0.01)  | 0.040           | 0.28 (-0.46, 1.00)   | 0.450                       | 0.24 (0.264)                |
|                       | B-Se                         | 0.14 (-0.45, 0.73)    | 0.642   | 0.24 (0.019)                | 0.16 (-0.45, 0.77)    | 0.603           | -0.07, (-0.40, 0.27) | 0.688                       |                             |
|                       | B-Mn                         | -0.10 (-0.35, 0.14)   | 0.391   |                             | -0.13 (-0.37, 0.11)   | 0.277           | 0.21 (-0.59, 1.00)   | 0.601                       |                             |
|                       | B-Cu                         | 0.19 (-0.45, 0.83)    | 0.548   |                             | -0.03 (-0.72, 0.65)   | 0.916           |                      |                             |                             |
|                       | B-Fe                         | 0.28 (-0.19, 0.74)    | 0.245   |                             | 0.29 (-0.18, 0.75)    | 0.224           | -0.08 (-0.74, 0.58)  | 0.801                       |                             |

<sup>a</sup> Sumsar versus Mailuu-Suu; in bold are significant ( $p \leq 0.05$ ) results and in bold italic marginally significant ( $p \leq 0.1$ )



**Table SP3:** Influence of *ALAD* rs1805313 and rs1800435 variant alleles (G) on blood Pb in residents of all regions (A) and Sumsar (B), assessed by multi-variable linear regression model (model 3)

| Dependent variable (Pb) | Independent variable        | A - All regions        |         |                             |                        |               |                                         |
|-------------------------|-----------------------------|------------------------|---------|-----------------------------|------------------------|---------------|-----------------------------------------|
|                         |                             | All                    |         | All (>20y)                  |                        | Women (>20 y) |                                         |
|                         |                             | $\beta$ (95% CI)       | p-value | N, R <sup>2</sup> (p-value) | $\beta$ (95% CI)       | p-value       | N, R <sup>2</sup> (p-value)             |
| Model 3                 | SNP1: rs1805313 (G)         |                        |         |                             |                        |               |                                         |
|                         | Age                         | -0.12 (-0.28, 0.04)    | 0.150   |                             | -0.12 (-0.29, 0.05)    | 0.159         | -0.15 (-0.34 - 0.04) 0.100              |
|                         | Region <sup>a</sup>         | 0.002 (-0.002, 0.007)  | 0.381   |                             | 0.001 (-0.004, 0.008)  | 0.596         | 0.00 (-0.007, 0.008) 0.912              |
|                         | Gender (women)              | 0.06 (0.05, 0.17)      | 0.288   |                             | 0.08 (-0.04, 0.020)    | 0.180         | -0.05 (-0.19, 0.10) 0.532               |
|                         | B-Se                        | -0.34 (-0.53, -0.15)   | 0.001   | 115 0.44 (<0.001)           | -0.34 (-0.54, -0.14)   | 0.001         | 0.46 (0.05, 0.86) 0.027 78              |
|                         | B-Mn                        | 0.37 (0.008, 0.74)     | 0.045   |                             | 0.41 (0.02, 0.80)      | 0.038         | -0.18 (-0.41, 0.06) 0.138 0.40 (<0.001) |
|                         | B-Cu                        | -0.19 (-0.39, 0.01)    | 0.067   |                             | -0.21 (-0.42, -0.01)   | 0.041         | 0.37 (-0.07, 0.82) 0.102                |
|                         | B-Fe                        | 0.26 (-0.15, 0.68)     | 0.217   |                             | 0.23 (-0.21, 0.67)     | 0.297         | 0.17 (-0.21, 0.54) 0.379                |
|                         | B-As                        | 0.25 (-0.09, 0.59)     | 0.147   |                             | 0.23 (-0.11, 0.58)     | 0.186         | 0.07 (-0.010, 0.24) 0.397               |
|                         | B-Hg                        | 0.09 (-0.06, 0.25)     | 0.227   |                             | 0.06 (-0.10, 0.22)     | 0.475         | -0.24 (-0.45, -0.04) 0.022              |
|                         | B-Hg                        | -0.16 (-0.34, 0.01)    | 0.080   |                             | -0.13 (-0.32, -0.06)   | 0.172         | -0.17 (-0.40, 0.07) 0.151               |
| Model 3                 | SNP3: rs1800435 (G = ALAD2) |                        |         |                             |                        |               |                                         |
|                         | Age                         | -0.17 (-0.37, 0.03)    | 0.086   |                             | -0.20 (-0.41, 0.03)    | 0.050         | 0.000 (-0.01, 0.01) 0.950               |
|                         | Region <sup>a</sup>         | -0.001 (-0.003, 0.007) | 0.496   |                             | -0.001 (-0.004, 0.008) | 0.602         | 0.06 (0.08, 0.21), 0.401                |
|                         | Gender (women)              | 0.08 (0.03, -0.20)     | 0.144   |                             | 0.10 (-0.22, -0.01)    | 0.089         |                                         |
|                         | B-Se                        | -0.36 (-0.5, -0.17)    | <0.001  | 115 0.46 (<0.001)           | -0.33 (-0.54, -0.13)   | 0.002         | 0.50 (0.10, 0.91) 0.016 79              |
|                         | B-Mn                        | 0.44 (0.08, 0.80)      | 0.018   |                             | 0.47 (0.09, 0.85)      | 0.016         | -0.14 (-0.37, 0.10) 0.262 0.41 (<0.001) |
|                         | B-Cu                        | -0.16 (-0.35, 0.04)    | 0.120   |                             | -0.18 (-0.39, 0.03)    | 0.088         | 0.26 (-0.10, 0.61) 0.129                |
|                         | B-Fe                        | 0.28 (-0.14, 0.69)     | 0.191   |                             | 0.20 (-0.22, 0.59)     | 0.358         | 0.07 (-0.09, 0.24) 0.159                |
|                         | B-As                        | 0.29 (-0.04, 0.62)     | 0.087   |                             | 0.29 (-0.04, 0.63)     | 0.086         | 0.08 (-0.08, 0.25) 0.372                |
|                         | B-Hg                        | 0.09 (-0.06, 0.24)     | 0.220   |                             | 0.07 (-0.09, 0.02)     | 0.388         | -0.34 (-0.53, -0.15) 0.001              |
|                         | B-Hg                        | -0.22 (-0.39, -0.05)   | 0.011   |                             | -0.21 (-0.38, -0.03)   | 0.020         |                                         |

<sup>a</sup> Sumsar versus Kadji Sai or Mailuu-Suu;

| B - Sumsar                         |                             |              |                             |                              |              |                             |                             |              |                             |
|------------------------------------|-----------------------------|--------------|-----------------------------|------------------------------|--------------|-----------------------------|-----------------------------|--------------|-----------------------------|
| Dependent variable (Ph)            | All                         |              |                             | All (>20y)                   |              |                             | Women (>20 y)               |              |                             |
|                                    | $\beta$ (95% CI)            | p-value      | N, R <sup>2</sup> (p-value) | $\beta$ (95% CI)             | p-value      | N, R <sup>2</sup> (p-value) | $\beta$ (95% CI)            | p-value      | N, R <sup>2</sup> (p-value) |
| <b>SNP1: rs1805313 (G)</b>         |                             |              |                             |                              |              |                             |                             |              |                             |
| Age                                | -0.03 (-0.30, 0.23)         | 0.792        |                             | -0.06 (-0.35, 0.24)          | 0.704        |                             | 0.02 (-0.31, 0.33)          | 0.910        |                             |
| Gender (women)                     | 0.004 (-0.007, 0.01)        | 0.280        |                             | -0.002 (-0.02, 0.001)        | 0.810        |                             | -0.01 (-0.3, 0.001)         | 0.228        |                             |
|                                    | <b>-0.30 (-0.561, 0.01)</b> | <b>0.058</b> |                             | <b>-0.39 (-0.78, -0.004)</b> | <b>0.048</b> |                             |                             |              |                             |
| B-Se                               | -0.16 (-1.18, 10.85)        | 0.747        | 50                          | -0.13 (-1.23, 0.98)          | 0.817        | 42                          | 0.68 (-0.81, 2.17)          | 0.349        | 26                          |
| B-Mn                               | -0.07 (-0.50, 0.16)         | 0.685        | 0.36                        | -0.13 (-0.47, 0.21)          | 0.438        | 0.29                        | -0.11 (-0.61, 0.39)         | 0.640        | 0.23                        |
| B-Cu                               | 0.20 (-0.67, 1.06)          | 0.644        | (0.135)                     | 0.23 (-0.83, 1.30)           | 0.659        | (0.192)                     | 0.74 (0.44, 1.92)           | 0.205        | (0.724)                     |
| B-Fe                               | <b>1.03 (0.07, 1.98)</b>    | <b>0.035</b> |                             | <b>0.87 (-0.10, 1.82)</b>    | <b>0.076</b> |                             | 0.61 (-0.77, 1.98)          | 0.363        |                             |
| B-As                               | -0.17 (-0.57, 0.23)         | 0.403        |                             | -0.28 (-0.70, 0.14)          | 0.181        |                             | -0.41 (-0.98, 0.15)         | 0.139        |                             |
| B-Hg                               | -0.03 (-0.36, 0.30)         | 0.854        |                             | 0.17 (-0.21, 0.454)          | 0.375        |                             | -0.04 (-0.52, 0.43)         | 0.843        |                             |
| <b>Model 3</b>                     |                             |              |                             |                              |              |                             |                             |              |                             |
| <b>SNP3: rs1800435 (G = ALAD2)</b> |                             |              |                             |                              |              |                             |                             |              |                             |
| Age                                | <b>-0.33 (-0.57, 0.09)</b>  | <b>0.009</b> |                             | <b>-0.43 (-0.51, 0.05)</b>   | <b>0.001</b> |                             | <b>-0.43 (0.65, -0.19)</b>  | <b>0.001</b> |                             |
| Gender (women)                     | 0.002 (-0.01, 0.01)         | 0.464        |                             | 0.000 (-0.01, 0.01)          | 0.924        |                             | <b>-0.01 (-0.03, -0.00)</b> | <b>0.084</b> |                             |
|                                    | <b>-0.38 (-0.67, -0.09)</b> | <b>0.011</b> |                             | <b>-0.37 (-0.69, -0.05)</b>  | <b>0.025</b> |                             |                             |              |                             |
| B-Se                               | -0.03 (-0.88, 0.82)         | 0.941        | 49                          | 0.21 (-0.72, 1.01)           | 0.744        | 42                          | <b>0.85 (0.17, 1.87)</b>    | <b>0.096</b> | 26                          |
| B-Mn                               | 0.06 (-0.25, 0.36)          | 0.721        | 0.46                        | -0.04 (-0.32, 0.25)          | 0.806        | 0.50                        | -0.10 (-0.47, 0.27)         | 0.580        | 0.59                        |
| B-Cu                               | 0.34 (-0.51, 1.21)          | 0.421        | (0.005)                     | -0.08 (-0.98, 0.81)          | 0.853        | (<0.001)                    | 0.24 (-0.65, 1.13)          | 0.572        | (<0.001)                    |
| B-Fe                               | <b>1.913 (0.28, 1.99)</b>   | <b>0.010</b> |                             | <b>0.92 (-0.13, 1.70)</b>    | <b>0.024</b> |                             | 0.30 (-0.71, 1.32)          | 0.538        |                             |
| B-As                               | <b>-0.32 (-0.70, -0.05)</b> | <b>0.088</b> |                             | <b>-0.38 (-0.574, -0.03)</b> | <b>0.036</b> |                             | <b>-0.57 (-0.99, -0.15)</b> | <b>0.011</b> |                             |
| B-Hg                               | 0.03 (-0.27, 0.33)          | 0.844        |                             | 0.15 (-0.14, 0.44)           | 0.288        |                             | 0.07 (-0.24, 0.39)          | 0.8635       |                             |

**Table SP4:** Data distribution of trace elements (GM) between carriers and non-carriers of ALAD SNPs variant alleles in all residents older than 20 years or women older than 20 years in case of all regions (A) or Sumсар only (B).

| A-All regions           |    |              |              |              |              |          |              |              |           |              |
|-------------------------|----|--------------|--------------|--------------|--------------|----------|--------------|--------------|-----------|--------------|
|                         |    | Pb (ng/g)    | Cd (ng/g)    | Hg (ng/g)    | As (ng/g)    | U (ng/g) | Se (ng/g)    | Cu (ng/g)    | Mn (ng/g) | Fe (ng/g)    |
| <b>SNP 1: rs1805313</b> |    |              |              |              |              |          |              |              |           |              |
| All ≥ 20 years          | N  |              |              |              |              |          |              |              |           |              |
| G-                      | 36 | <b>24.2</b>  | 0.41         | <b>0.26</b>  | 0.39         | 0.004    | 120          | <b>871</b>   | 13.2      | 394          |
| G+                      | 72 | <b>20.1</b>  | 0.43         | <b>0.38</b>  | 0.43         | 0.003    | 114          | <b>900</b>   | 12.3      | 404          |
| p-value                 |    | <b>0.028</b> | 0.802        | <b>0.001</b> | 0.755        | 0.635    | 0.233        | <b>0.094</b> | 0.917     | 0.654        |
| Women ≥ 20 years        |    |              |              |              |              |          |              |              |           |              |
| G-                      | 25 | <b>22.2</b>  | 0.40         | <b>0.28</b>  | 0.35         | 0.003    | 115          | 902          | 13.7      | 404          |
| G+                      | 55 | <b>17.3</b>  | 0.43         | <b>0.42</b>  | 0.40         | 0.003    | 111          | 936          | 14.3      | 395          |
| p-value                 |    | <b>0.009</b> | 0.732        | <b>0.002</b> | 0.426        | 0.949    | 0.446        | 0.111        | 0.705     | 0.736        |
| <b>SNP2: rs818708</b>   |    |              |              |              |              |          |              |              |           |              |
| All ≥ 20 years          | N  |              |              |              |              |          |              |              |           |              |
| A-                      | 38 | 20.1         | 0.42         | 0.37         | 0.44         | 0.003    | 121          | 911          | 14.0      | <b>428</b>   |
| A+                      | 70 | 22.0         | 0.43         | 0.31         | 0.40         | 0.004    | 114          | 879          | 12.9      | <b>387</b>   |
| p-value                 |    | 0.161        | 0.842        | 0.116        | 0.251        | 0.510    | 0.165        | 0.364        | 0.373     | <b>0.047</b> |
| Women ≥ 20 years        | N  |              |              |              |              |          |              |              |           |              |
| A-                      | 32 | 18.2         | 0.41         | 0.39         | <b>0.43</b>  | 0.003    | <b>121</b>   | 930          | 14.5      | <b>426</b>   |
| A+                      | 48 | 19.0         | 0.43         | 0.35         | <b>0.35</b>  | 0.004    | <b>107</b>   | 922          | 13.9      | <b>380</b>   |
| p-value                 |    | 0.724        | 0.768        | 0.415        | <b>0.046</b> | 0.509    | <b>0.017</b> | 0.906        | 0.746     | <b>0.058</b> |
| <b>SNP 3: rs1800435</b> |    |              |              |              |              |          |              |              |           |              |
| All ≥ 20 years          | N  |              |              |              |              |          |              |              |           |              |
| G- (ALAD1)              | 90 | 21.2         | 0.44         | 0.35         | <b>0.39</b>  | 0.003    | <b>114</b>   | 901          | 13.5      | 396          |
| G+ (ALAD2)              | 19 | 21.1         | 0.36         | 0.28         | <b>0.54</b>  | 0.006    | <b>126</b>   | 840          | 12.3      | 411          |
| p-value                 |    | 0.832        | 0.109        | 0.105        | <b>0.007</b> | 0.114    | <b>0.085</b> | 0.153        | 0.528     | 0.905        |
| Women ≥ 20 years        |    |              |              |              |              |          |              |              |           |              |
| G- (ALAD1)              | 68 | 18.4         | <b>0.44</b>  | 0.39         | <b>0.36</b>  | 0.003    | <b>110</b>   | 937          | 14.3      | 393          |
| G+ (ALAD2)              | 13 | 19.0         | <b>0.35</b>  | 0.29         | <b>0.51</b>  | 0.006    | <b>126</b>   | 861          | 13.1      | 406          |
| p-value                 |    | 0.989        | <b>0.099</b> | 0.142        | <b>0.031</b> | 0.104    | <b>0.045</b> | 0.165        | 0.585     | 0.943        |

in bold are significant results ( $p < 0.05$ ) and in bold italic marginally significant ( $p < 0.1$ );

| B-Sumsar         |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
|------------------|----|----------------------|----------------------|----------------------|----------------------|---------------------|----------------------|----------------------|----------------------|----------------------|
|                  |    | Pb <sub>(ng/g)</sub> | Cd <sub>(ng/g)</sub> | Hg <sub>(ng/g)</sub> | As <sub>(ng/g)</sub> | U <sub>(ng/g)</sub> | Se <sub>(ng/g)</sub> | Cu <sub>(ng/g)</sub> | Mn <sub>(ng/g)</sub> | Fe <sub>(ng/g)</sub> |
| SNP 1: rs1805313 |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| All ≥ 20 years   | N  |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| G-               | 19 | 26.8                 | 0.38                 | <b>0.19</b>          | 0.52                 | 0.003               | 126                  | 833                  | 11.3                 | 396                  |
| G+               | 25 | 30.0                 | 0.43                 | <b>0.27</b>          | 0.57                 | 0.002               | 118                  | 841                  | 9.88                 | 433                  |
| p-value          |    | 0.515                | 0.472                | <b>0.006</b>         | 0.647                | 0.123               | 0.166                | 0.546                | 0.314                | 0.334                |
| Women ≥ 20 years |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| G-               | 12 | 26.1                 | 0.35                 | <b>0.19</b>          | 0.48                 | 0.003               | 129                  | <b>824</b>           | 10.8                 | 414                  |
| G+               | 14 | 25.1                 | 0.45                 | <b>0.29</b>          | 0.52                 | 0.002               | 122                  | <b>908</b>           | 10.5                 | 423                  |
| p-value          |    | 0.777                | 0.198                | <b>0.009</b>         | 0.472                | 0.201               | 0.237                | <b>0.014</b>         | 0.758                | 0.980                |
| SNP2: rs818708   |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| All ≥ 20 years   | N  |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| A-               | 14 | 28.4                 | 0.43                 | <b>0.28</b>          | 0.54                 | <b>0.001</b>        | 126                  | 847                  | 11.5                 | 440                  |
| A+               | 30 | 28.7                 | 0.40                 | <b>0.22</b>          | 0.55                 | <b>0.003</b>        | 120                  | 833                  | 9.99                 | 405                  |
| p-value          |    | 0.338                | 0.483                | <b>0.062</b>         | 0.894                | <b>0.017</b>        | 0.364                | 0.545                | 0.241                | 0.298                |
| Women ≥ 20 years | N  |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| A-               | 11 | 24.1                 | 0.44                 | <b>0.29</b>          | 0.55                 | <b>0.001</b>        | <b>133</b>           | 881                  | 12.0                 | 435                  |
| A+               | 15 | 26.7                 | 0.37                 | <b>0.20</b>          | 0.47                 | <b>0.003</b>        | <b>120</b>           | 859                  | 9.77                 | 407                  |
| p-value          |    | 0.243                | 0.340                | <b>0.036</b>         | 0.204                | <b>0.040</b>        | <b>0.052</b>         | 0.364                | 0.222                | 0.364                |
| SNP 3: rs1800435 |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| All ≥ 20 years   | N  |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| G- (ALAD1)       | 30 | <b>32.2</b>          | 0.41                 | 0.23                 | 0.56                 | 0.002               | 120                  | 852                  | 10.0                 | 415                  |
| G+ (ALAD2)       | 14 | <b>22.1</b>          | 0.39                 | 0.25                 | 0.51                 | 0.003               | 126                  | 807                  | 11.5                 | 420                  |
| p-value          |    | <b>0.005</b>         | 0.789                | 0.449                | 0.522                | 0.265               | 0.351                | 0.314                | 0.313                | 0.749                |
| Women ≥ 20 years |    |                      |                      |                      |                      |                     |                      |                      |                      |                      |
| G- (ALAD1)       | 19 | <b>28.9</b>          | 0.42                 | 0.23                 | 0.52                 | 0.002               | 124                  | 901                  | 10.1                 | 421                  |
| G+ (ALAD2)       | 9  | <b>20.3</b>          | 0.37                 | 0.24                 | 0.46                 | 0.003               | 128                  | 809                  | 11.7                 | 414                  |
| p-value          |    | <b>0.002</b>         | 0.509                | 0.435                | 0.293                | 0.339               | 0.850                | 0.112                | 0.280                | 0.500                |

in bold are significant results ( $p < 0.05$ ) and in bold italic marginally significant ( $p < 0.1$ );

### 3.4 Manuscript 4: As Urine Metabolites, *AS3MT* Haplotype and *AS3MT* Expression in Cancer Patients Treated with As<sub>2</sub>O<sub>3</sub>: a Case Study

In preparation: Stajniko A., Zupan J., Podgornik H., Šlejkovec Z., Jagodic M., Černelč P., Marc J., Falnoga I. (2019)

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is a promising drug for treatment of acute promyelocytic leukemia (APL). The efficiency and outcome of the treatment can considerably depend on the patient's As methylation capacity. The assessment of As urine metabolite patterns (AsIII, AsV, MMA and DMA) of various patients clearly showed variability in the rate of As methylation between them (Šlejkovec et al., 2016). Such variabilities between patients result due to the influence of various factors such as: genetic variations, nutritional status, antioxidative status, age, BMI, medication etc. Moreover, As<sub>2</sub>O<sub>3</sub> – through interaction with -SH and -SeH groups – influences the performance of various physiological pathways, for example the activity of enzymes or proteins involved in oxidative stress or As metabolism. Such influences vary from patient to patient, leading to their different responses to the treatment.

In the present manuscript, we investigated relations between “protective” *AS3MT* SNPs haplotype, *AS3MT* mRNA expression and urine arsenic metabolites in patients with acute promyelocytic leukemia (APL) treated with As<sub>2</sub>O<sub>3</sub>. As such, we followed urine As metabolites (AsIII, AsV, MMA and DMA) and presence or absence of “protective” *AS3MT* SNPs haplotype – as constructed in Manuscript 2 of the dissertation (see Section 3.2) – for five APL patients, while *AS3MT* mRNA levels were assessed for four APL patients and 14 controls. For two patients we also followed As in serum.

In the manuscript, we observed:

- i) clear variations in As methylation efficiency between patients,
- ii) more efficient As methylation among carriers of “protective” *AS3MT* haplotype,
- iii) *AS3MT* mRNA levels were influenced by *AS3MT* haplotype, treatment and probably by other factors such as BMI and Se status.
- iv) that “protective” *AS3MT* haplotype could be a good predictor for therapeutic efficiency in patients treated with As<sub>2</sub>O<sub>3</sub>.

We have simultaneously studied relations between As<sub>2</sub>O<sub>3</sub> treatment and several other genes' expression in APL patient, such as *SELENOP1*, *GPX1,3* and *4*, *TXNRD1* and *2*, *CAT*, *AQP9* and *MTs* – in one or two APL patients. Results for one patient have been partially presented at Selenium conference 2013 (Falnoga et al., 2013) and recently re-analyzed and combined with new patient and new genes data which are summarized in the Working report presented in App. A.5; in general As<sub>2</sub>O<sub>3</sub> treatment reduced mRNA levels of the studied genes in both patients. Combined genetic influence of various genes or their haplotypes on gene expression and As metabolites in APL patients is our future challenge.

In the present manuscript, my work consisted of AS3MT genotyping, gene expression analyses, statistical analysis, preparation of tables and figures and writing of the manuscript.



## The impact of *AS3MT* haplotype on *AS3MT* expression and As urine metabolite patterns in cancer patients treated with As<sub>2</sub>O<sub>3</sub>

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### Abstract

Genetic variations in arsenic (3) methyltransferase (*AS3MT*) can influence its gene and enzyme expression and enzyme activity, which can result in interindividual differences of As methylation efficiency. In cancer patients treated with As<sub>2</sub>O<sub>3</sub>, this could result in a higher or lower susceptibility to the AsIII side effects and influence the efficiency of the therapy. In studies conducted on As<sub>2</sub>O<sub>3</sub> treated patients, the information on their genetic background is scarce.

In the present study relations between presence or absence of “protective” *AS3MT* haplotype (p*AS3MThap*<sup>+</sup> or <sup>-</sup>), As methylation efficiency through urine As metabolites and *AS3MT* expression were assessed for 5 As<sub>2</sub>O<sub>3</sub> treated acute promyelocytic leukemia (APL) patients (iv infusion 0.15 mg/g/day; 41 – 82 years old). *AS3MT* haplotype constructed of six SNPs (rs7085104, rs3740400, rs3740393, rs3740390, rs10748835 and rs1046778) and As metabolites in urine were determined for all patients, while blood *AS3MT* mRNA levels (normalised by *HPRT1* mRNA levels) for four of them and 14 healthy controls. Relations between p*AS3MThap* and As methylation efficiency were evaluated separately for steady state of the therapy during induction phase (8–25<sup>th</sup> day).

Two APL patients identified as p*AS3MThap*<sup>+</sup> expressed significantly higher levels of urine DMA%, and secondary methylation index (SMI) - indicated higher As methylation efficiency - when compared to p*AS3MThap*<sup>-</sup>. Furthermore, p*AS3MThap*<sup>+</sup> patient showed significantly lower *AS3MT* expression levels when compared to p*AS3MThap*<sup>-</sup> patients (average *AS3MT/HPRT1* mRNA level 8 - 25<sup>th</sup> day: 0.21 vs. 1.12 – 1.30 ng/μL, respectively), similar trends were observed between carriers versus non-carriers among controls. Comparison of patient's basal gene expression levels (without As<sub>2</sub>O<sub>3</sub> treatment) with average expression level of healthy controls showed differences, which were specific for each studied patient. Moreover, comparison of *AS3MT* expression between sample taken prior therapy and sample at the end of the therapy (available for one patient only) showed significantly reduced expression with As<sub>2</sub>O<sub>3</sub> treatment (*AS3MT/HPRT1* mRNA level: 2.26 vs. 0.87, respectively;  $p < 0.001$ ).

However, we believe that beside *AS3MT* genetic variations arsenic methylation efficiency was influenced also by variability of other factors such as BMI and Se status.

As methylation efficiency varied among patients according to the presence or absence of p*AS3MThap*, therefore we believe that p*AS3MThap* genotyping in relation with urine As metabolites, could be used as a predictor for therapeutic efficiency and of patients treated with As<sub>2</sub>O<sub>3</sub>.

**Keywords:** As<sub>2</sub>O<sub>3</sub>, DMA, MMA, acute promyelocytic leukemia, “protective” *AS3MT* haplotype, gene expression

## 1. INTRODUCTION

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>, AsIII in solution; ATO; Trisenox®) is for almost two decades approved for the treatment of acute promyelocytic leukemia (APL) patients, which were unresponsive to or relapsed from conventional treatment (Douer & Tallman, 2005; Miller et al., 2002) and from 2016 as a first choice drug for patients with low- or intermediate-risk APL (Khairul et al., 2017; Lo-Coco, 2016). APL is a rare subtype of acute myeloid leukemia (AML) characterized by specific reciprocal chromosome (15 and 17) translocation of promyelocytic leukemia gene (PML) and retinoic acid receptor gene (RAR- $\alpha$ ) resulting in PML/RAR- $\alpha$  and RAR- $\alpha$ /PML fusion genes; transcription activity of the first, PML/RAR- $\alpha$ , is responsible for deficiency of mature blood cells and excess of immature promyelocytes (Diverio et al., 1995). Such malignant cells are due to their low intracellular antioxidative capacity and low As<sub>2</sub>O<sub>3</sub> efflux capability, more sensitive to AsIII adverse effect than healthy cells (Jing et al., 1999). Their As sensitivity is also related to high expression levels of aquaporin 9 (AQP9; membrane protein forming water channel) responsible for As uptake and accumulation in cells (Iriyama et al., 2013; Khairul et al., 2017; Leung et al., 2007; Roggenbeck et al., 2016). Due to the success in APL treatment, As<sub>2</sub>O<sub>3</sub> has been tested in various cell experiments and clinical trials as a potential drug for other malignancies including multiple myeloma (MM; malignancy of plasma cells in the bone marrow) (Chen et al., 2003; Falnoga et al., 2014; Falnoga et al., 2007; Murgo, 2001; Podgornik et al., 2014).

After treatment, AsIII (As<sub>2</sub>O<sub>3</sub>) is generally metabolized and excreted in urine mainly as mono- and di- methylated metabolites (MMA and DMA), excess of AsIII and in smaller amount as AsV (due to the oxidation of AsIII) (Cullen, 2014; Khairul et al., 2017; Šlejkovec et al., 2016). However, as reported by several authors, urine patterns of As metabolites can considerably vary among similarly treated APL patients and inside individual patients during treatment; such differences in As methylation can influence anticancerogenic effectivity and systemic toxicity of arsenic trioxide (Khairul et al., 2017; Šlejkovec et al., 2016; Wang et al., 2013). Arsenic methylation primarily - although, not exclusively - depends on the expression and activity of arsenic (3+) methyltransferase (AS3MT) (Aposhian et al., 2003; Khairul et al., 2017) and is affected by various factors (Balakrishnan et al., 2018; Broberg et al., 2015; Engström et al., 2011; Xu et al., 2016) among them dose of arsenic, gender, obesity, nutritional status, pregnancy, liver or kidney affected diseases, genetics in general and in particular genetic of AS3MT. The relative strength of each of those factors is hard to predict. Anyway, AS3MT is a polymorphic gene, whose SNPs have been reported to predict expression and activity of AS3MT and to be responsible for inter-individual differences in As methylation (reviewed by Broberg et al., 2015). In studies on excretion of As and its metabolites in As<sub>2</sub>O<sub>3</sub> treated APL or MM patients the information on such genetic background can represent important contribution to individual treatment dose regime. Only one recent study examined and pointed out one AS3MT SNP (rs3740390) as an important predictor of As<sub>2</sub>O<sub>3</sub> therapeutic efficiency and patient's susceptibility to its side effects (Lu et al., 2018). However, mostly several AS3MT SNPs are occurring together as a haplotype in a population specific manner; such haplotypes are usually marked as "protective" due to their beneficial effect on As methylation (higher urine DMA%) (Broberg et al., 2015). They were identified in populations with historical exposure to high inorganic As in water (Agusa et al., 2015; De la Rosa et al., 2017; Engström et al., 2011), and latter distinguished also in historically non-exposed populations (Stajanko et al., 2019).

In present study, we investigated relations between constructed “protective” *AS3MT* SNPs haplotype, *AS3MT* mRNA expression and urine arsenic metabolites in As<sub>2</sub>O<sub>3</sub> treated cancer patients.

## 2. MATERIAL AND METHODS:

### 2.1 Participants and As<sub>2</sub>O<sub>3</sub> treatment protocol

Present study is a part of wider (pre)clinical investigation of As<sub>2</sub>O<sub>3</sub> metabolism ongoing at Jožef Stefan Institute and University Medical Centre Ljubljana (Falnoga et al., 2017). Investigation was approved by the Slovenian National Medical Ethic Committee and all participating patients, resistant to or in relapse after conventional treatment (ATRA-EORTEC), gave their informed consent. In present study, we include five APL patients. They received As<sub>2</sub>O<sub>3</sub> treatment at the University Medical Centre in Ljubljana according to standard protocols: that means 2 h iv infusion of 0.15 mg As<sub>2</sub>O<sub>3</sub>/kg/day for 25 (I - induction of remission phase) + 25 (C - consolidation phase) days with a 7 day break in between (B – break phase). A few timing variations in treatment protocol were introduced due to patients’ health issues or logistic reasons. Individual data of treatment and break days together with personal characteristics (age, weight, height) given, *AS3MT* polymorphism genotyping, *AS3MT* mRNA expression and As analyses in collected samples of serum and urine are summarised in **Table 1**.

For the purpose of genotyping and/or gene expression analysis, we also included 14 healthy controls sampled in years 2012 and 2017. All signed informed consent of participation. Data available for control group of participants are added in **Table 1**, although they are less detailed.

**Table1:** Basic characteristics of participants and their treatment.

|                                       | 1<br>CR                 | 2<br>KB               | 3<br>AN   | 4<br>MVG | 5<br>BH               | 14 CONTROLS |         |
|---------------------------------------|-------------------------|-----------------------|-----------|----------|-----------------------|-------------|---------|
| Gender                                | W                       | W                     | W         | W        | M                     | 7F          | 7M      |
| Age (years)                           | 41                      | 82                    | 43        | 64       | 80                    | 28 - 53     | 35 - 49 |
| Weight (kg)                           | 131                     | 71                    | 94        | 92       | 85                    |             |         |
| Height (cm)                           | 168                     | 165                   | 169       | 175      | 176                   |             |         |
| BMI (kg/m <sup>2</sup> )              | 46.4                    | 26.1                  | 32.9      | 30.1     | 27.4                  |             |         |
| Diagnosis                             | APL                     | APL                   | APL       | APL      | APL                   |             |         |
| Days <sub>(n)</sub> of I, B, C        | 25, 11, 25 <sup>a</sup> | 9 <sup>b</sup> , 0, 0 | 25, 7, 25 | 25, 7, 5 | 4 <sup>b</sup> , 0, 0 |             |         |
| As measurement <sub>(n)</sub> in U, S | 38, 5                   | 9, 0                  | 14, 7     | 24, 0    | 1, 0                  |             |         |
| Genotyping                            | +                       | +                     | +         | +        | +                     | +           | +       |
| mRNA expression                       | +                       | +                     | +         | -        | +                     | +           | +       |

M – men, W- women; APL - acute promyelocytic leukemia; MM – multiple myeloma; I – induction, B-break, C- consolidation; <sup>a</sup> – one week break at 15<sup>th</sup> day of consolidation phase due to infection; <sup>b</sup> – death due to ATRA syndrome; U - urine, S – serum, n- number of collected samples

### 2.2 Collection of spot urine and blood (serum) samples

Urine and blood (serum) samples of patients were collected following standard procedures at the Haematology department of University Medical Centre of Ljubljana. Spot urine samples were collected in sterile vessels before and after As<sub>2</sub>O<sub>3</sub> application on daily basis during the first 7 days and later at least once per week before the infusion only. Blood samples (whole blood, serum) were obtained before and after or at least before infusion once per week using



standard heparin or EDTA containers. For four APL patients' blood was simultaneously collected also into Tempus<sup>TM</sup> Blood RNA tubes for gene expression analysis. The number of obtained samples varied between the patients. All samples were frozen and stored at least at -20°C prior to the analyses. Number of collected samples for each patient/are given in **Table 1**. For *AS3MT* genotyping and/or mRNA expression analysis blood was taken in the same way also from 14 healthy controls.

### **2.3 Determination of serum As and As metabolites in urine (AsIII, AsV, DMA, MMA)**

Serum total As levels were determined at Jožef Stefan Institute, Department of environmental Sciences, Slovenia by inductively coupled plasma mass spectrometer (ICP-MS, 7500ce, Agilent Technologies, Santa Clara, CA, USA) equipped with an ASX-510 Autosampler (Cetac) as described in details in (Miklavčič et al., 2013). The limit of detection (LOD) calculated, as three times the standard deviation of blanks was 0.1 ng/g.

Common urine As metabolites, namely AsIII, AsV, MMA(III+V) and DMA(III+V) were determined using high pressure liquid chromatography (HPLC) combined with an online hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS, PS Analytical, Kent, UK) as described in details by (Šlejkovec et al., 2008). LOD calculated as three times the standard deviation of blanks were 0.3 ng/mL for AsIII and MMA and 0.5 ng/mL for AsV and DMA. Total urinary arsenic was considered as the sum of all four analysed metabolites. Separate metabolites were presented as a percentage of the sum of metabolites. Second methylation index (SMI) calculated as DMA/MMA ratio and total methylation index (TMI) calculated as DMA+MMA/iAs ratio were used for the estimation of As methylation efficiency. In a subset of samples determined was also arsenobetain (AsB). As expected, based on the low seafood intake in Slovenian population as observed before (Stajnko et al., 2019), the levels were very low. As such, we believe that seafood intake should not influence our results.

Calculated urine As levels were normalized for differences in urinary flow rate either by urine levels of creatinine or by specific gravity (SG) - depending on the availability of data. Creatinine was determined by standard Jaffe method (Larsen, 1972) and SG by PAL-10S refractometer (Atago, Japan). In SG normalisation calculations, as a standard SG was used 1.019 for men and 1.012 for women – mean SG previously determined for Slovenian general population (Stajnko et al., 2017, 2019); calculation is described in the literature (Suwazono et al., 2005).

### **2.4 DNA extraction, SNPs genotyping and haplotype construction**

DNA of four patients (2-5) was isolated from 0.5 – 1 mL of peripheral blood using FlexiGene® DNA Kit (Qiagen, Hilden, Germany) and genotyped for 6 *AS3MT* SNPs: rs7085104, rs3740400, rs3740393, rs3740390, rs10748835 and rs1046778 using predesigned TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The reaction volume was 5 µL: 2.5 µL TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA, USA), 1.875 µL ultrapure nuclease-free water (Life Technologies, CA, USA), 0.125 µL of 44X TaqMan probe/primer mix, and 0.5 µL genomic DNA. Amplifications and genotype identification were performed using LightCycler® 480 Instrument II and LightCycler® 480 Software version 1.5.1 (Roche). PCR cycling included: pre-PCR (1 cycle 50°C, 2 min),

activation (1 cycle 95°C, 10 min), annealing and amplification (50 cycles 95°C, 15 s and 61°C, 1 min), and (post-PCR (1 cycle 40°C, 30). All genotyped data was used for the construction of the “protective” *AS3MT* haplotype, with the sequence of variant alleles of SNPs in 5’-3’ order, based on their position in the gene: rs7085104(G), rs3740400(G), rs3740393(C), rs3740390(T), rs10748835(A) and rs1046778(C); described in details by [Stajanko et al., 2019](#).

For patient 1CR only, the DNA was isolated from bone marrow cells, collected by standard clinical method at the Haematology department of University Medical Centre of Ljubljana, with QIAamp DNA Extraction kit (Qiagen, Hilden, Germany) following manufacturer’s protocol.

In the present study, carriers and non-carriers of “protective” *AS3MT* haplotype were referred to as p*AS3MT*hap<sup>+</sup> and p*AS3MT*hap<sup>-</sup>, respectively.

### 2.5 RNA extraction, cDNA synthesis and gene expression

RNA extraction, cDNA synthesis and gene expression were performed for four APL patients (1CR, 2KB, 3AN and 5BH; **Table 1**) and all 14 healthy controls at the laboratory of Faculty of Pharmacy, University of Ljubljana.

RNA was extracted from blood (3mL) using an Ambion® Tempus™ Spin RNA isolation Kit (Applied Biosystems, ZDA). The concentration and purity of RNA were assessed by the NanoDrop® ND-1000 or ND-ONE-W spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Purity was assessed based on the standard absorbance ratio 260nm/280 nm. RNA integrity was evaluated by RNA integrity number (RIN; average 7.2, range 3.6 – 8.7) obtained by Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) and Agilent RNA 6000 Nano Kit (Agilent Technologies, Germany).

A total of 2.5 µg RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA Master Mix Kit (Applied Biosystems Foster City, CA, USA) and Thermocycler PeqStar 348 HPL (PEQLAB, Germany). Samples of cDNA were stored at -80°C prior to further analysis. All above-mentioned procedures were performed following manufacture’s protocols.

Expression levels were followed for 3 genes: arsenic (3+) methyltransferase (*AS3MT*), hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT1*) and ribosomal phosphoprotein P0 (*RPLP0*). The latter two were chosen and used as housekeeping genes based on literature data ([K. Engström et al., 2011](#); [Fei et al., 2013](#)).

Levels of mRNA were measured by quantitative real-time polymerase chain reaction (qPCR) using LightCycler® 480 Instrument II (Roche, Indianapolis, USA). *AS3MT* and *HPRT1* mRNAs were detected by TaqMan Gene expression assay (Applied Biosystems, ZDA) with 5X HOT FIREPol Probe qPCR Mix plus (Solis BioDyne, Estonia) and *RPLP0* mRNA by SYBR Green I assay with 5X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia). IDs for TaqMan probes were Hs00960529\_m1 and Hs02800695\_m1 for *AS3MT* and *HPRT1* gene respectively; and sequences of forward and reverse primers for *RPLP0* gene 5'TCTACAACCCTGAAGTGCTTGAT-3' and 5'-CAATCTGCAGACAGACACTGG-3', respectively.

For *AS3MT*, *HPRT1* and *RPLP0* expression, samples were measured in triplicates including five negative controls to test for possible contamination with genomic DNA. PCR amplifications were performed in 15 µl reactions with two different PCR conditions in respect to either TaqMan or SYBR Green I expression assay. PCR conditions for both are presented in **Table 2**.

**Table 2:** PCR conditions for TaqMan and SYBR Green I expression assay

| PCR Step                             | Temperature (°C) | Time (s) | Number of cycles |
|--------------------------------------|------------------|----------|------------------|
| <b>TaqMan expression assay</b>       |                  |          |                  |
| Activation                           | 95               | 900      | 1                |
| Denaturation                         | 95               | 20       | 50               |
| Annealing, elongation                | 60               | 60       |                  |
| Cooling                              | 4                | 30       | 1                |
| <b>SYBR Green I expression assay</b> |                  |          |                  |
| Activation                           | 95               | 900      | 1                |
| Denaturation                         | 95               | 15       | 45               |
| Annealing                            | 61               | 20       |                  |
| Elongation                           | 72               | 20       |                  |
| Melting curve                        | 95               | 5        | 1                |
|                                      | 65               | 60       |                  |
|                                      | 97               | (5°C/s)  |                  |
| Cooling                              | 4                | 30       | 1                |

qPCR data was analysed using LightCycler® 480 Software version 1.5.1 (Roche, Applied Science, USA). The threshold cycle ( $C_T$ ) of each sample was determined by the 2<sup>nd</sup> derivate maximum method and the level of mRNA by relative quantification. Samples with a standard deviation > 0.3 for  $C_T$ , were excluded from the analysis.

Gene expression of both housekeeping genes was responsive to As<sub>2</sub>O<sub>3</sub> treatment for all patients. However, As exposure related changes were considerably higher for *RPLP0* than *HPRT1* (coefficient of variance for patient 3: 63% and 30%, respectively). Accordingly, *AS3MT* expression levels normalised only to *HPRT1* expression value were chosen as more reliable, although not optimal.

## 2.6 Statistical analysis

Al arsenic parameters – total As concentrations, metabolites percentages, methylation indexes - are presented as arithmetic mean with added minimum and maximum ( $\bar{x}$ , min-max). The distribution of data around mean values were estimated by standard deviation (SD) or coefficient of variance (CV). Statistical differences of As methylation efficiency and differences in gene expression between *AS3MT* haplotype carriers and non-carriers were assessed by Student t-test (two-tailed distribution; two-sample unequal variance), with level of statistical significance set at  $p < 0.05$  (two-sided). For statistics and the visualisation of results, we used Microsoft Excel 2016.



### 3. RESULTS AND DISCUSSION

#### 3.1 Protective AS3MT haplotype

After genotyping and construction of protective AS3MT haplotype (pAS3MThap<sup>+</sup>; rs7085104(G), rs3740400(G), rs3740393(C), rs3740390(T), rs10748835(A) and rs1046778(C)), we identified five carriers of protective haplotype: two among five patients (1CR and 2KB) and three among 14 controls (**Table 3**). The frequency of the pAS3MThap<sup>+</sup> was 14%. On a populational level, we previously reported slightly lower frequencies for Slovenian 176 women with 10% and their 176 children with 12% (Stajniko et al., 2019). Low percentage was found particularly for SNPs rs3740390 (9.5 and 13.6, for mother and children, respectively) and accordingly also for pAS3MThap<sup>+</sup>.

**Table 3:** The presence or absence of AS3MT haplotype (pAS3MThap<sup>+</sup>; pAS3MThap<sup>-</sup>) among patients and controls.

|                        | 1CR | 2KB | 3AN | 4MVG | 5BH | Controls (7W+ 7M) |
|------------------------|-----|-----|-----|------|-----|-------------------|
| pAS3MThap <sup>+</sup> | +   | +   |     |      |     | 3+ (2W, 1M)       |
| pAS3MThap <sup>-</sup> |     |     | +   | +    | +   | 11+ (5W, 6M)      |

Protective AS3MT haplotype (pAS3MThap) sequence: variant alleles of SNPs in 5'-3' order, based on their position in the gene rs7085104 (G), rs3740400 (G), rs3740393(C), rs3740390 (T), rs10748835 (A) and rs1046778(C).

#### 3.2 As variables in patients stratified by the presence of AS3MT haplotype

**Figure 1** presents daily excretion levels of total arsenic ( $\mu\text{g}/\text{L}_{\text{SG}}$  or  $\mu\text{g}/\text{g}_{\text{crea}}$ ) and percentage share of the main metabolites (AsIII, AsV, MMA, DMA) throughout the treatment in all available pre-infusion urine samples of all 5 patients divided in two carrying (1CR, 2KB; **Figure 1a**) and three non-carrying AS3MT haplotype (3AN, 4MVG, 5BH; **Figure 1b**). The most interesting was the difference in DMA% between carriers and non-carriers. DMA is representing the end-stage of methylation and as such is reflecting As methylation efficiency (Cullen et al., 2014, Broberg et al., 2015). Methylation efficiency was obviously higher in both pAS3MThap<sup>+</sup> carriers than in non-carriers throughout the treatment. Few exceptions were only observed during the initial week of treatment when inter-individual variability in As methylation and excretion is known to be the highest (Šlejkovec et al 2016) due to the physiological adaptability to the treatment dose (enzymes induction including AS3MT, As tissue distribution and disposition). Unfortunately, we did not have data covering the whole treatment period for all patients. For all we had data from initial treatment week and for four of them (two carriers and two non-carriers) also available from the second week on, although not for all until the end of the treatment. After the stabilization of percentages of methylated metabolites, which occurred at the beginning of second week, the share of DMA was between 60 and 80% for carriers and between 30 and 50% for non-carriers, if we excluded 1<sup>st</sup> after-break day (destabilization period). Non-methylated part represented around 10% in carriers and between 20 and 30% in non-carriers.

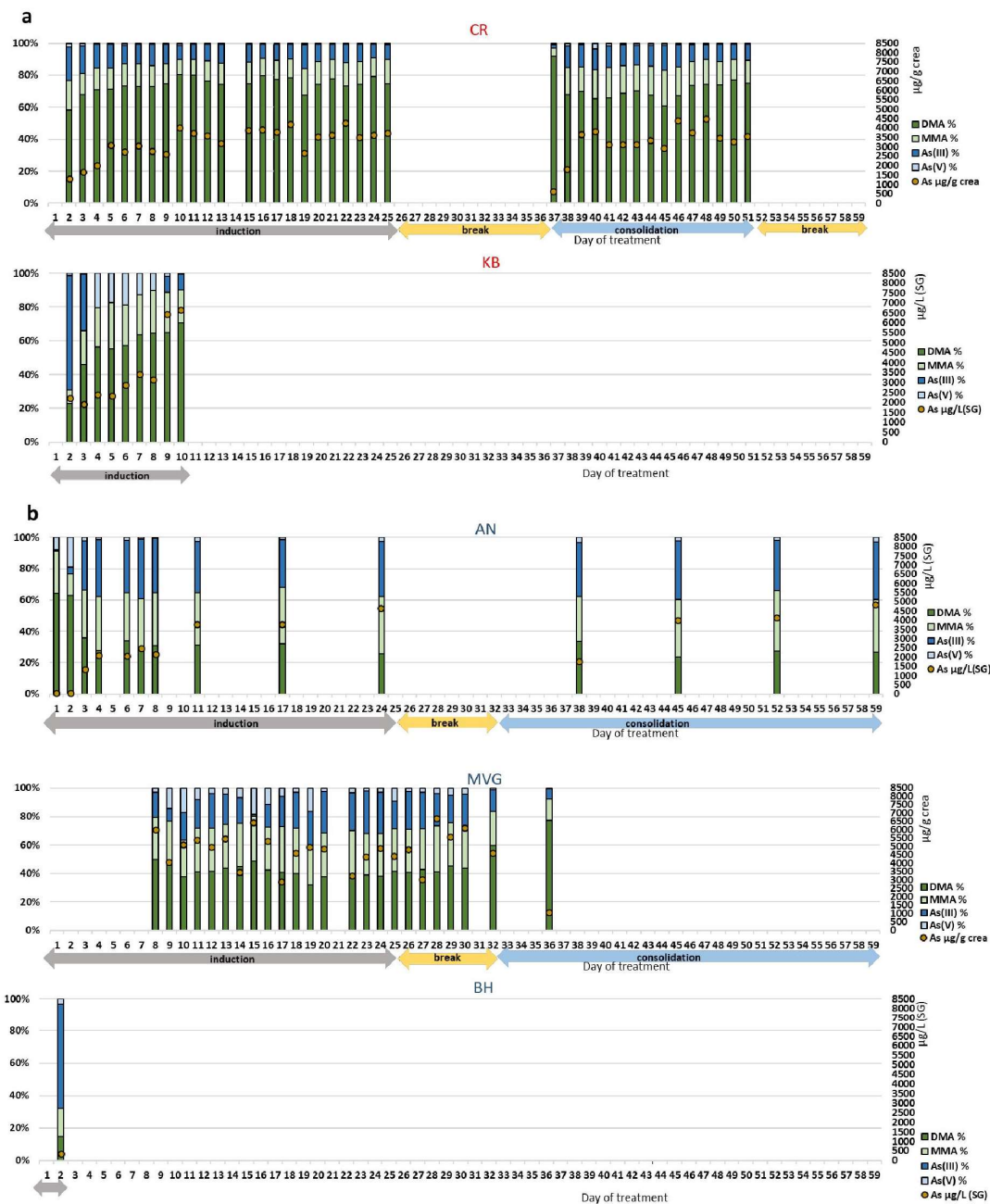
For more elaborate comparison, we chose induction phase, between 8<sup>th</sup> and 25<sup>th</sup> day, and compared average daily values for various As parameters for each of those four patients. That means induction period without first metabolically most variable week. Results are given in

**Table 4** and include percentages DMA and DMA+MMA followed by second (SMI = DMA/MMA) and total (TMI = MMA+DMA/iAs) methylation indexes – all reflecting methylation capacity; added are total As levels in serum for patients 1CR and 3AN. Patients 1CR and 1KB, identified as pAS3MThap<sup>+</sup> carriers, showed higher methylation capacity than pAS3MThap<sup>-</sup> non-carriers (patients 3AN, 4MVG). Difference was observed based on all studied methylation efficiency parameters. For one carrier/non-carrier pair with appropriate sample size the difference was confirmed by statistical analyses (1CR and 4MVG). Moreover, the comparison of total As levels in serum between carrier 1CR and non-carrier 3AN showed that the former one had in average for about 14% lower levels than the latter one, inside induction phase (**Table 4**) and latter (**Figure 2**), which could possibly additionally support the more efficient methylation among pAS3MThap carriers. Nevertheless, it should also be appointed out that at therapeutic exposure blood levels could be influenced by interindividual variability in selenium-mediated As excretion (Ponomarenko et al., 2017). Previously we tested and observed the increased level of both elements in faeces samples of few patients (unpublished data).

Additionally, it is interesting that statistically significant higher SMI was observed for 1CR patient when compared to 5KB patient, both pAS3MThap<sup>+</sup> ( $p < 0.001$ ) during the initial phase of treatment, 1<sup>th</sup> to 7<sup>th</sup> day (data not presented). The same trend was evident also between 8<sup>th</sup> and 25<sup>th</sup> day (**Table 4**) where the number of data was too small for statistical evaluation. This difference is pointing on the influence of factors other than AS3MT haplotype on As methylation efficiency. In our previous publication, dealing with biotransformation variability in APL patients (Šlejko et al. 2016), we already reported the extremely high methylation efficiency in 1CR patient and discussed that it might be related to her high BMI (CR1 46.4 kg/m<sup>2</sup> vs KB 26.1 kg/m<sup>2</sup>; **Table 1**) which falls inside levels indicating obesity. Positive relation between BMI and As methylation efficiency on population level was previously also reported and commented by (Gribble et al., 2013).

Altogether, the results are in accordance with the literature data pointing on positive effect of studied AS3MT SNPs/haplotypes on As methylation observed in various healthy populations (Broberg et al., 2015; Mendez et al., 2016; Stajanko et al., 2019; Xu et al., 2016) and more recently also in APL patients (Lu et al., 2018), as well as on positive association between BMI and As methylation (Gribble et al 2013).

Although AS3MT is the key enzyme in As metabolism, methylation can be carried out also independently of AS3MT (Drobna et al., 2009). Recently, N-6-adenine-specific DNA methyltransferase (N6AMT1) and aquaporin 9 (AQP9) and their genetic variations were reported as associated with As methylation (Chen et al., 2017; Stajanko et al., 2019) and as such could play an important role in the outcome of As<sub>2</sub>O<sub>3</sub> treatment. Furthermore, as the role of Se in As metabolism is well recognised (George et al., 2013; Zeng et al., 2005) also Se status and genetic variations of various selenoproteins in APL patients should be evaluated (Fálnoga, 2013;2014). As such, difference in the initial Se status (serum Se: 80 ng/g for 3AN, 112 ng/g for 1CR, 55 ng/g for 4MVG and 62 ng/g for KB; unpublished data) could also contribute to variable As methylation efficiency among patients during the therapy.

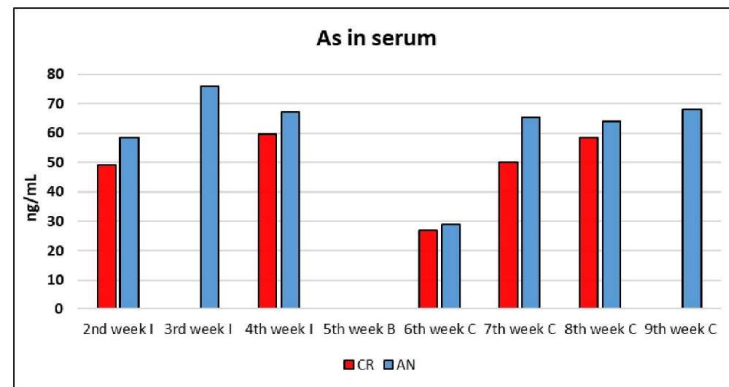


**Figure 1:** As metabolite percentages and total As levels in pre-infusion urines of a) two pAS3MThap<sup>+</sup> patients and b) three pAS3MThap<sup>-</sup> patient, during the induction, break and consolidation phase of the therapy.

**Table 4:** Mean values of As variables in two carriers of protective *AS3MT* haplotype and two non-carriers within 8th – 25<sup>th</sup> day of induction therapy.

| URINE      |      |        |                               |                               |                               |                               |                               | SERUM |                               |  |      |  |
|------------|------|--------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------|-------------------------------|--|------|--|
|            |      |        | DMA %                         |                               | (MMA + DMA) %                 |                               | SMI (DMA/MMA)                 |       | TMI (DMA+MMA/As)              |  | S-As |  |
| Patient    | Days | N      | $\bar{x}$ (min-max)<br>CV (%) | $\bar{x}$ (min-max)<br>CV (%) | $\bar{x}$ (min-max)<br>CV (%) | $\bar{x}$ (min-max)<br>CV (%) | $\bar{x}$ (min-max)<br>CV (%) | N     | $\bar{x}$ (min-max)<br>CV (%) |  |      |  |
| pAS3M/hap+ | ICR  | 8 - 25 | 17                            | 76 (73 – 80)*<br>3.28         | 89 (86 – 91)*<br>1.50         | 6.30 (4.93 – 8.73)*<br>17.6   | 7.90 (5.30 – 9.70)*<br>15.1   | 2     | 54.5 (49.3 – 59.6)            |  |      |  |
|            | 2KB  | 8 – 9  | 3                             | 67 (65 – 70)<br>4.9           | 89 (89 – 90)<br>0.8           | 2.96 (2.58 – 3.57)<br>17.9    | 8.61 (7.92 – 9.21)<br>7.50    |       |                               |  |      |  |
| pAS3M/hap- | 3AN  | 8 - 24 | 4                             | 30 (26 – 32)<br>9.85          | 65 (65 – 68)<br>3.78          | 0.90 (0.70 – 0.92)<br>12.9    | 1.85 (1.64 – 2.13)<br>11.0    | 3     | 67.1 (58.5 – 75.8)            |  |      |  |
|            | 4MVG | 8 - 25 | 17                            | 42 (32 – 50)*<br>10.6         | 72 (58 – 80)*<br>7.66         | 1.40 (1.22 – 1.67)*<br>9.35   | 2.62 (1.39 – 4.10)*<br>26     |       |                               |  |      |  |

CV - coefficient of variation; n - number of obtained urine samples from 8 – 25<sup>th</sup> day; \* statistical significant difference ( $p < 0.05$ ) between patients ICR and 4MVG; PMI calculated as MMA/ (AsIII + AsV), SMI as DMA/MMA and TMI as DMA+ MMA/ (AsIII + AsV); S-As – serum As



**Figure 2:** As serum levels of two APL patients (ICR and 3AN) in pre-infusion samples from 2<sup>nd</sup> week of the therapy on (I – induction phase, B – brake, C- consolidation phase)



### 3.3 *AS3MT* expression levels in patients and controls according to the presence or absence of *AS3MT* haplotype

In order to assess relationships of *AS3MT* expression with “protective” *AS3MT* haplotype and As<sub>2</sub>O<sub>3</sub> treatment, *AS3MT* mRNA levels were followed during treatment in peripheral blood of four APL patients, one haplotype carrier (1CR) and three non-carriers (3AN, 4MVG, 5BH). Expression was also performed for 14 healthy controls to obtain a proximal background levels for general population. Controls were stratified based on the gender and the presence/absence of *AS3MT* haplotype (**Table 5**) and in such way appropriately compared to each patient.

Results of gene expression for each patient are presented in **Figure 3**. Due to the small number of patients, differences in number and collection time of samples between patients, and responsiveness of both housekeeping genes to As treatment (explained in material and methods), only the general conclusions regarding *AS3MT* expression in APL patients could be made.

1CR patient, identified as p*AS3MThap*<sup>+</sup> with more efficient As methylation (**Figure 1; Table 4**), showed significantly lower *AS3MT* expression levels when compared to p*AS3MThap*<sup>-</sup> patients (AN and MVR) (average *AS3MT/HPRT1* mRNA level 8 - 25<sup>th</sup> day: 0.21 vs. 1.12 – 1.30 ng/μL, respectively). Similarly, a slight trend in higher expression levels in men and women carrying *AS3MT* haplotype versus those without haplotype was observed among controls (**Table 5**). Such results are in accordance with findings by (Engström et al., 2013;2011) on reduced *AS3MT* expression in peripheral blood associated with “protective” *AS3MT* haplotype.

Furthermore, basal *AS3MT* expression levels of patients estimated by the expression in pre-therapeutic sample of 1<sup>st</sup> day, except for 1CR for whom we used post-therapeutic sample (14<sup>th</sup> day after the termination of therapy), was compared to the average expression of gender and haplotype matched controls. Differences were specific for each patient (**Figure 3**). 1CR showed significantly lower expression than controls. Among p*AS3MThap*<sup>-</sup> patients, AN showed similar expression and BH slightly higher expression than controls. Such result, together with high variability in expression among controls (high SD; **Figure 3**), indicate that in addition to genetic variations, other factors also influence *AS3MT* expression. For example, in CR patient her overweight and consequently enhanced metabolism rate could lead to increased *AS3MT* activity and As methylation efficiency (Šlejkovec et al., 2016) and consequently decreased *AS3MT* expression.

For 3AN patient, non-carrier with less efficient As methylation, the results were different. *AS3MT* expression levels were available for the first and last day of therapy. Their comparison showed statistically significantly reduced expression at the end of therapy (*AS3MT/HPRT1* mRNA level: 2.26 vs. 0.87, respectively;  $p < 0.0501$ ). We assume that this could be explained by the saturation and/or inhibition of As methylation as an effect of high AsIII levels in therapy (Fowler et al., 2015). On contrary, for a 1CR patient with more efficient As methylation, the expression increased close to the end of the therapy (day 50) (**Figure 3**).

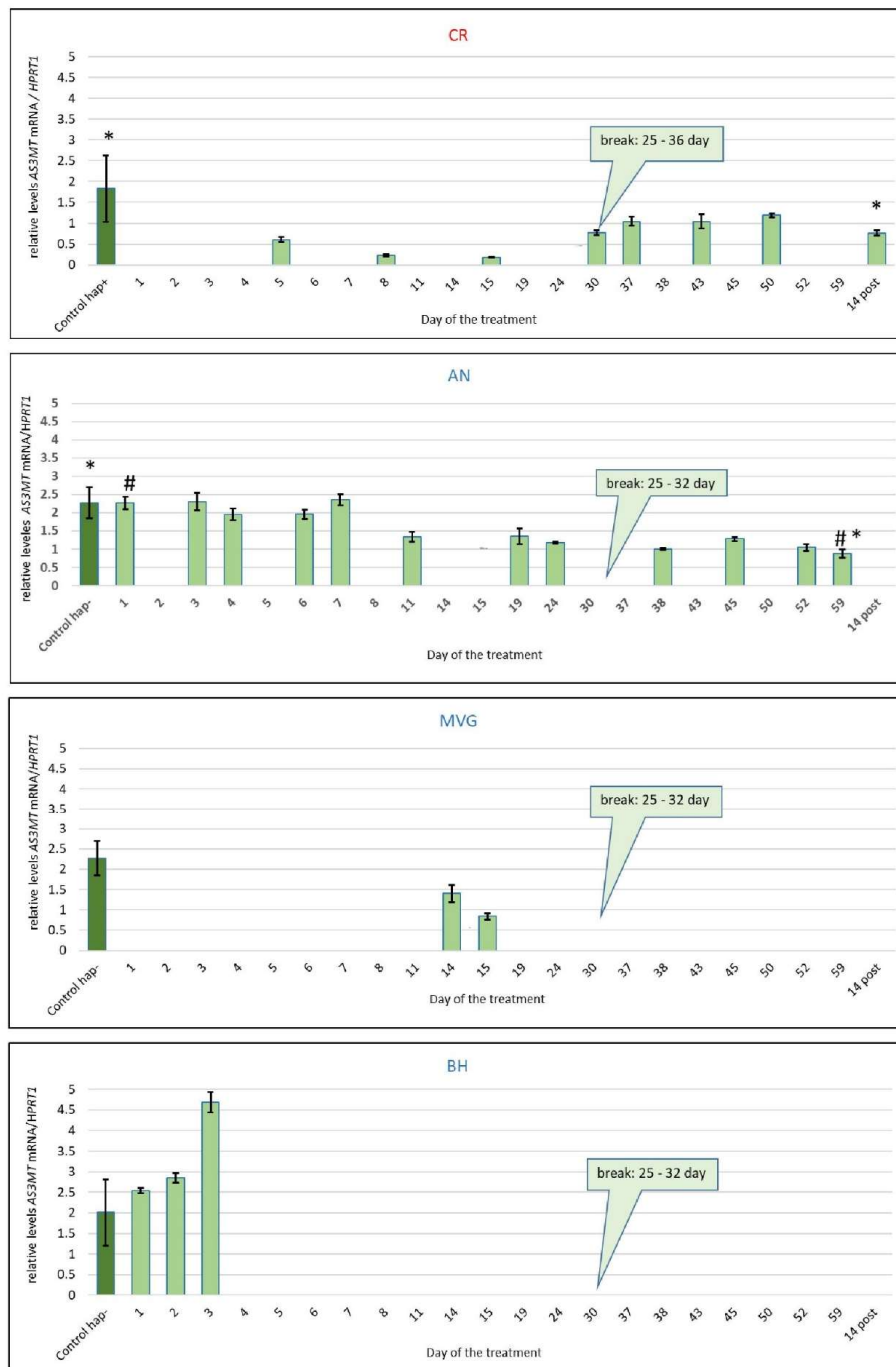
Taken all together, *AS3MT* expression in the present study was shown to be affected by the presence of p*AS3MThap*, BMI of patient and by the As<sub>2</sub>O<sub>3</sub> therapy. Such results could indicate



that pAS3MThap genotyping in relation to urine As metabolites rather than AS3MT expression analysis, might be a good predictor of As<sub>2</sub>O<sub>3</sub> therapeutic efficiency.

**Table 5:** Relative levels of AS3MT mRNA/HPRT1 mRNA in controls stratified by gender and presence of pAS3MThap.

|             | ALL |                                | MEN |                                | WOMEN |                                |
|-------------|-----|--------------------------------|-----|--------------------------------|-------|--------------------------------|
|             | N   | $\bar{x} \pm (\text{min-max})$ | N   | $\bar{x} \pm (\text{min-max})$ | N     | $\bar{x} \pm (\text{min-max})$ |
| pAS3MThap + | 3   | 1.80 (1.27- 2.39)              | 1   | 1.75                           | 2     | 1.83 (1.27 - 2.39)             |
| pAS3MThap - | 9   | 2.10 (1.28 - 3.48)             | 6   | 2.00 (1.28 - 3.48)             | 5     | 2.27 (1.67 - 2.68)             |



**Figure 3:** Comparison of *AS3MT* expression in pre-infusion blood samples of APL patients during As treatment, one pAS3MT<sup>hap</sup> and tree pAS3MT<sup>hap</sup> with added expression of gender matched controls (statistically significant difference ( $p < 0.5$ ), # between first and last day of treatment, and \* between control and last day of treatment)

#### 4. CONCLUSIONS

Comparison of the urine arsenic metabolite pattern (iAs, MMA and DMA) between five As<sub>2</sub>O<sub>3</sub> treated cancer patients showed interindividual variability, which was in line with the presence or absence of “protective” AS3MT haplotype. Two patients identified as a carrier of haplotype expressed more efficient As methylation identified through percentage of DMA and secondary methylation index (DMA/MMA). Correspondingly, the results for one haplotype carrier compared to three non-carriers showed lower AS3MT gene expression. Results are pointing on the influence of AS3MT genetic variations on its gene expression. Besides, by genetic variations AS3MT, methylation efficiency was most probably affected also by observed variability in BMI and Se status. Therefore, genotyping of pAS3MT<sub>hap</sub> might be a good predictor for therapeutic efficiency in patients treated with As<sub>2</sub>O<sub>3</sub>.

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## Chapter 4

# Conclusions

The focus of the present PhD dissertation lied on the human association studies of TE with biomarkers of exposure, kidney function and susceptibility/adaptability at low exposure levels. Such associations are well investigated and established at occupational and high environmental exposures while at lower environmental and/or diet-related exposures they are questionable and poorly assessed – especially in the presence of “specific” physiological states such as pregnancy, lactation, childhood. In this matter we estimated the appropriateness of studied associations of urine and blood Cd levels with renal function biomarkers. With association studies of As (As urine metabolites), Pb, Cd and Se levels with selected gene polymorphisms we estimated the importance of genetic background and identified a few SNPs as possible susceptibility or adaptability biomarkers. The main studied populations were Slovenian men and lactating women in their childbearing years (1st Slovenian HBM), Slovenian and Croatian pregnant/non-pregnant women and children (PHIME-CROME project) – both with very low to moderate diet/environmental exposure. Additionally we investigated two experimental cases: Kyrgyzstani population with moderate environmental exposure and cancer patients with pharmacological As exposure. The main conclusions from four manuscripts, their supportive materials (see Section 3.1 – 3.4) and five working reports (App. A.1 – A.5) are pointed out below, following the hypotheses of the dissertation.

1. Associations studied in Slovenian-Croatian population with low environmental exposure, concentrated on Cd (HBM survey) and As and Pb (PHIME-CROME), were – as hypothesized – in general weak, in some cases absent and considerably influenced by methodological uncertainty and physiological variability. Physiological variability resulted from non-genetic changes related to “special” physiological states (pregnancy, lactation and childhood), gender, age, or from normal changes in individual’s diet (seafood intake) and habits (smoking).

### *Normalization and spot urine biomarkers*

- We confirmed that the normalization of spot urine biomarkers for inter-individual variations in diuresis, especially by creatinine, is inappropriate at low levels of exposure. As the difference in the measurement sensitivity of creatinine and those of trace elements is too high (e.g. LOD: 2 µgCrea/mL versus 0.03 ngCd/mL) the normalization is introducing methodological uncertainty into association studies, resulting in conflicting and inconsistent results, as observed in our study on Cd. In such cases, TE determined in blood (plasma) represent a better biomarker of exposure. However, when studying urine metabolites of TE (e.g. As) we can use their proportions to avoid normalization.

### *Cd and urine renal function biomarkers*

- We observed associations between Cd and four urine renal function biomarkers, commonly used biomarkers of Cd effect, namely, A1M, Alb, IgG and NAG. They were all far below critical values, and assessed at low Cd exposure (GM <0.3 ng/mL for blood and urine). We strongly believe that associations were driven by the normal physiological changes in kidney function rather than by the pathological processes related to Cd exposure. Such conclusion was based on the observed positive associations of all four studied renal function biomarkers with urine Cd and their absence with blood Cd. Both, protein bound – Cd (mainly as Cd-metallothioneins) and renal functional biomarkers use similar excretion pathways and are consequently affected in the same way by the kidney function, leading to their co-excretion and observed positive associations in urine. Such results could be, as also pointed out by other research groups, misinterpreted as causal.
- Non-causal associations between Cd and renal function biomarkers were further supported by the observed influence of other physiological factors unrelated to kidney function. For example, the presence of current or past smoking, beside expected increase in blood Cd, also increased A1M levels and modified Cd-A1M associations. This points to the response of A1M to oxidative stress and inflammation related to smoking, and supports its proposed anti-oxidative and anti-inflammatory role. Moreover, also NAG associations with blood Cd – observed among women – were most probably a reflection of post-pregnancy and/or lactation status (increased oxidative stress). Accordingly, the usefulness of studied proteins as biomarkers of renal function and biomarkers of Cd renal effects is at their normal (low) physiological levels questionable and calls for the identification of more specific biomarkers of kidney function.

### *SNP-TE interactions*

- SNP-TE interactions studied in Slovenian-Croatian women population with low environmental (iAs in water  $\leq$  1  $\mu$ g/L) or diet exposure (GMs: blood and urine Cd < 0.5 ng/ml, urine iAs < 3.3 ng/ml, blood Pb  $\leq$  12 ng/ml) showed that non-genetic physiological variability probably has a bigger impact on TE levels genetic factors. Associations were clearly influenced by the pregnancy status, which either overwhelmed or enhanced the SNPs effect in comparison with the non-pregnant status. Nevertheless, significant influences of: “protective” *AS3MT* haplotype, SNP of *INMT*, *AQP9* and *4*, *ALAD* and *SELENOP* and two SNPs of *MT2A* gene, on selected TE levels were identified as summarized in Table 2.

Table 4.1: Genes with SNPs significantly or marginally associated with selected TE (Cd, As, Pb and Se) levels in urine or blood of Slovenian-Croatian pregnant and/or non-pregnant women.

|                                   | Cd<br>(urine) |   | As, As<br>metabolites<br>(urine) |   | Pb<br>(blood) |   | Se<br>(urine) |   |
|-----------------------------------|---------------|---|----------------------------------|---|---------------|---|---------------|---|
|                                   | NP            | P | NP                               | P | NP            | P | NP            | P |
| »protective«<br><i>AS3MT</i> hap* |               |   | +                                | - |               |   | -             | - |
| <i>INMT</i><br>rs6970396          |               |   | +                                | - |               |   | +             | - |
| <i>AQP9</i><br>rs2414539          |               |   | +                                | - |               |   | +             | - |
| <i>AQP4</i><br>rs9951307          |               |   | -                                | + |               |   | -             | - |
| <i>SELENOP</i><br>rs7579          |               |   | +                                | - |               |   | -             | - |
| <i>MT2A</i><br>rs28366003         | -             | - | +                                | + |               |   | -             | - |
| <i>MT2A</i><br>rs10636            | -             | + |                                  |   |               |   |               |   |
| <i>ALAD</i><br>rs1805313          |               |   |                                  |   | +             | - |               |   |

NP – non-pregnant; P – pregnant; +, significant influence of SNP ( $p < 0.05$ ), +’ marginally significant influence of SNP ( $p \leq 0.1$ ), – non-significant influence of SNP ( $p > 0.1$ ), blank, interaction was not assessed. \*, Protective” *AS3MT* haplotype sequence: variant alleles of SNPs in 5’-3’ order, based on their position in the gene: rs7085104 (G), rs3740400 (G), rs3740393 (C), rs3740390 (T), rs10748835 (A), rs1046778 (C).

- *AS3MT* haplotype associations with very low iAs levels were to our knowledge studied for the first time in the present work. We confirmed “protective” role of *AS3MT* haplotype: carriers showed higher DMA % and SMI – both indicating higher As methylation efficiency – when compared to non-carriers.

- AQPs are known to facilitate As transition, but their SNPs’ have not yet been associated with As levels. We observed significant impact of *AQP9* (rs2414539) and *AQP4* (rs9951307) on urine As metabolite concentrations and methylation efficiency. Both may be used as susceptibility or adaptability biomarkers, if the observed associations are confirmed in populations/individuals with higher environmental, occupational or therapeutic As exposures.

- Moreover, *AQP9* (rs2414539) and *INMT* (rs6970396) SNPs were both significantly associated with higher urine excretion of both As and Se. Such concurrent influence of both SNPs could be explained by the use of same transport pathways by As and Se, but should be further investigated also for their possible co-excretion related to As-Se complexes.

- We have confirmed genetic influences on blood Pb variability. *ALAD* (rs1805313) was weakly negatively associated with blood Pb of Slovenian non-pregnant women (GM  $\leq 12$  ngPb/ml) and when tested in population of Kyrgyzstan with higher blood Pb (GM 21 ngPb/g), the effect was stronger. Moreover, on a subgroup of Kyrgyzstan with even higher Pb levels (GM 27 ngPb/g, Pb contaminated Sumsar region) the additional influence of rs1800435 SNP was observed, showing the same trend.

- In association studies, we tested and used several confounding factors (modifiers) such as generally used age, BMI, gender, etc., but also less common ones like the status of essential trace elements. Status of Se in blood or plasma was identified as a significant modifying factor in all above-mentioned association studies. In a study on *ALAD*-Pb interactions in Kyrgyzstan population, we used a set of essential elements Se, Mn, Fe, and Cu in blood as confounders, and the modifying effect was observed for the first three. Status of essential TE represents a measurable reflection of the physiological variability, because their levels can be influenced by individual's health condition, diet, age, gender, physical activity, life style (e.g. smoking, alcohol) etc. Accordingly, and additionally due to the known interactions between essential and non-essential TE, the inclusion of essential TE rather than questionnaire's data could better explain the variability and improve the strength of statistical association models.

2. The present dissertation *provides data on allele frequency distribution of 35 SNPs in 20 genes* (*AS3MT*, *MTHFR*, *AQP4*, *AQP9*, *INMT*, *SELENOP*, *MT1A*, *MT1B*, *MT1E*, *MT1F*, *MT1G*, *MT1X*, *MT2A*, *MT3*, *MT4*, *MTF1*, *DMT1*, *LRP2*, *LRP8*, and *ALAD*) and of "protective" *AS3MT* haplotype for the Slovenian-Croatian population (PHIME-CROME) and 3 SNPs in *ALAD* gene for the Kyrgyzstan population. Their frequencies were compared with European and other worldwide populations:

- For the Slovenian-Croatian population minor allele frequencies of the majority of studied SNPs were – as expected – similar to those reported for European populations and significantly different from those of Asian (east and/or south) and especially African populations. Anyway, they differed from those in European population for 7 SNPs:

*ALAD* rs18005313 (35 vs. 28 %)

*MT3* rs28754593 (35 vs. 29 %)

*LRP8* rs3820198 (35 vs. 28 %)

*MT1X* rs2301234 (45 vs. 32 %)

*AQP9* rs2414539 (17 vs. 9 %)

*DMT1* rs224589 (22 vs. 31 %)

*MTHFR* rs1801133 (35 vs 46 %)

- In case of "protective" *AS3MT* haplotype we observed a clear frequency difference between the Slovenian-Croatian population with historically low iAs exposure and populations genetically adapted to historically high iAs exposure such as: San Antonio de los Cobres, Argentina (SAC), specific regions of Chile and Peru (10% vs 70%, 48-68% and 51%, respectively) (Apata et al., 2017; Engström et al., 2011; Schlebusch et al., 2013).

- Among all SNPs, three (*ALAD* rs1805313, rs818708, and rs1800435) were determined in the Slovenian-Croatian population and the population of Kyrgyzstan (Central Asia). Interestingly, minor allele frequencies of all three SNPs were similar for both populations. Within the Kyrgyzstan population, two *ALAD* SNPs (rs1805313 and rs1800435) considerably varied among three studied areas (Kadji Sai, Mailuu-Suu and Sumsar). However, observed differences should be confirmed by a higher number of participants per region in order to draw some conclusion on the possible role of different geology and environmental pollution of studied areas in Kyrgyzstan. We expect that similar differences could be found for Slovenia after genotyping the HBM population from 12



geographical regions including metal polluted hot spots (Mežica valley with former Pb mine and smelting).

3. In  $\text{As}_2\text{O}_3$ -treated cancer patients arsenic therapy influenced the expression of various genes of selenoproteins involved in oxidative stress and/or As binding (*TXNRD1*, *TXNRD2*, *CAT*, *SELENOP*, *GPX1*, *GPX3* and *GPX4*) and genes involved in As transport and methylation such as *AQP9* and *AS3MT*, respectively. For *AS3MT* – as a main gene of As methylation – we also confirmed relations between  $\text{As}_2\text{O}_3$  treatment, urine As metabolites patterns and the presence or absence of "protective" *AS3MT* haplotype.

- Methylation efficiency (DMA % and SMI) was higher with the presence of "protective" *AS3MT* haplotype.

- The number of patients and samples, and quantity of samples were too small to get 'strong' results for *AS3MT* mRNA expression. Results were not clear enough for reliable estimation of influence of *AS3MT* haplotype on its mRNA levels. Anyway, a clear difference in patients' basal mRNA levels, regardless of the presence of *AS3MT* haplotype, suggests influence of other factors on *AS3MT* expression.

- Genotyping of "protective" *AS3MT* haplotype in relation to urine As metabolites might be a good predictor for therapeutic efficiency in patients treated with  $\text{As}_2\text{O}_3$ .

*The findings of the present dissertation represent a good foundation for our future gene-environmental studies:*

- All SNPs/TE interactions from the present dissertation will be retested on the population from the 1st Slovenian HBM survey.

- Moreover, *ALAD* SNPs could represent additional information about possible higher or lower vulnerability to Pb exposure, however more studies are needed in order to overcome the observed inconsistencies between studies; as such it is planned to be retested in population with higher Pb levels from the Mežica region.

- In relation to As exposure we will test SNPs of other *AQPs* (e.g. *AQP3* and *AQP7*) which are proposed to be related with As uptake and cellular influx and efflux pathways (Mukhopadhyay et al., 2014).

- We will attempt to apply the haplotype approach in relation to TE also in the case of *ALAD* and *AQPs*.

- Enzymes/proteins involved in the TE kinetics/dynamics, which are encoded by various genes, have overlapping metabolic pathways. Therefore, we will investigate the presence of combined effect of SNPs in two or more genes (gene-gene interactions); in particular: combination of *AS3MT*, *AQP9* and *INMT* in relation to As; *AQP9* and *INMT* in relation to Se; and combination of various *MT* genes in relation to different TE;

- We will try to identify and assess *AS3MT* SNPs with possible negative effect on As metabolism and test them as possible susceptibility biomarkers for APL patients treated with  $\text{As}_2\text{O}_3$ .



## Appendix A

### Working Reports

A.1 Hemoglobin and Post-delivery Time Had no  
Confounding Effect on Cd Associations with Renal  
Function Biomarkers (First National HBM Survey)



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**IJS – Delovno poročilo**  
*IJS - Report*

**IJS DP – 12825**

***Haemoglobin and post-delivery time had no  
confounding effect on Cd  
associations with renal function biomarkers  
(first National HBM survey)***

Stajnko Anja, Snoj Tratnik Janja, Mazej Darja, Jagodic Marta, Krsnik Mladen,  
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**Ljubljana, 15.2.19**

## 1. BACKGROUND:

In our previous study “Low cadmium exposure in males and lactating females – estimation of biomarkers” (Stajniko et al., 2017) we estimated the appropriateness and reliability of Cd in urine and blood as biomarkers of exposure and four urine renal proteins (A1M, Alb, IgG and NAG) as biomarkers of effect, and their associations, at low Cd exposure (<1 ng/mL). Study was conducted on a general population consisting of 533 lactating women and 548 men in childbearing age, which participated in a Slovenian Human Biomonitoring survey (2007-2015). Associations of Cd levels with urine renal proteins were performed for men and women separately and were tested for possible confounding of age, BMI, smoking, nutritional status of Se, co-exposure to blood Pb, Hg and As – their selection depended on the studied association. The detailed information on study population, methodology and statistics of the study are presented in Stajniko et al., 2017.

Some metal(loid)s, such as Cd, Pb, Hg and Se, are in blood mainly bound to the red blood cells, whose amount varies between individuals. As this could influence their blood levels and their studied associations, blood biomarkers should be normalised to account for such inter-individual variations. Furthermore, during the post-pregnancy (lactation) period women go through physiological and behavioural changes (Moya et al., 2014). Because such changes can vary within post-pregnancy period, the time of sampling can influence the status of studied biomarkers. Therefore, in the present working report we additionally tested associations between Cd and renal function biomarkers - from above mentioned study by Stajniko (2017) - for the possible confounding of inter-individual difference in haemoglobin status (**Table 1**) and for women difference in post-delivery time of sampling (days after delivery) (**Table 2**).



Table A1: Association between urine renal proteins and blood Cd normalized by haemoglobin (estimated by multiple linear regression models).

| Gender | Dependent variable | Independent variable  | Un-normalised urine data    |              | Crea-normalised urine data  |                  | SG-normalised urine data    |                  |
|--------|--------------------|-----------------------|-----------------------------|--------------|-----------------------------|------------------|-----------------------------|------------------|
|        |                    |                       | $\beta$ (95% CI)            | p-value      | $\beta$ (95% CI)            | p-value          | $\beta$ (95% CI)            | p-value          |
| MALE   | IgG                | B-Cd/Hb               | 0.01 (-0.06, 0.08)          | 0.812        | 0.03 (-0.07, 0.13)          | 0.576            | 0.05 (-0.03, 0.13)          | 0.188            |
|        |                    | BMI                   | <b>0.02 (-0.00, 0.03)</b>   | <b>0.094</b> | -                           | -                | -                           | -                |
|        |                    | Current smoking       | -                           | -            | 0.26 (-0.08, 0.59)          | 0.125            | -                           | -                |
| FEMALE | IgG                | B-Cd/Hb               | -0.01 (-0.14, 0.12)         | 0.833        | -0.07 (-0.21, 0.06)         | 0.292            | -0.05 (-0.18, 0.09)         | 0.513            |
|        |                    | B-As                  | -0.07 (-0.10, 0.03)         | 0.141        | -                           | -                | -                           | -                |
|        |                    | B-Pb/Hb               | 0.06 (-0.08, 0.13)          | 0.514        | 0.05 (-0.14, 0.25)          | 0.586            | 0.05 (-0.14, 0.25)          | 0.628            |
|        |                    | BMI                   | <b>-0.03 (-0.02, 0.00)</b>  | <b>0.012</b> | <b>-0.04 (-0.06, -0.02)</b> | <b>&lt;0.001</b> | <b>-0.03 (-0.05, -0.01)</b> | <b>0.003</b>     |
|        |                    | Age                   | -                           | -            | <b>0.04 (0.02, 0.06)</b>    | <b>&lt;0.001</b> | <b>0.03 (0.01, 0.05)</b>    | <b>0.001</b>     |
| MALE   | Alb                | B-Cd/Hb               | 0.02 (-0.11, 0.15)          | 0.733        | 0.02 (-0.06, 0.11)          | 0.626            | 0.03 (-0.05, 0.14)          | 0.539            |
|        |                    | B-Se/Hb               | <b>-0.65 (-1.11, -0.20)</b> | <b>0.005</b> | -                           | -                | -                           | -                |
|        |                    | BMI                   | 0.02 (-0.01, 0.04)          | 0.218        | -                           | -                | -                           | -                |
|        |                    | Current smoking       | -0.30 (-0.67, 0.09)         | 0.131        | -                           | -                | -                           | -                |
| FEMALE | Alb                | B-Cd/Hb               | 0.03 (-0.19, 0.18)          | 0.780        | -0.03 (-0.19, 0.13)         | 0.681            | -0.01 (-0.21, 0.01)         | 0.894            |
|        |                    | BMI                   | <b>-0.03 (-0.06, -0.01)</b> | <b>0.014</b> | <b>-0.05 (-0.07, -0.02)</b> | <b>&lt;0.001</b> | <b>-0.04 (-0.07, -0.02)</b> | <b>0.001</b>     |
| MALE   | AIM                | B-Cd/Hb               | -0.01 (-0.10, 0.09)         | 0.888        | 0.03 (-0.07, 0.12)          | 0.586            | 0.05 (-0.03, 0.15)          | 0.290            |
|        |                    | B-As                  | -0.03 (-0.13, 0.06)         | 0.520        | -0.01 (-0.12, 0.09)         | 0.832            | -0.01 (-0.10, 0.09)         | 0.937            |
|        |                    | B-Pb/Hb               | <b>0.12 (0.01, 0.25)</b>    | <b>0.063</b> | <b>0.11 (-0.01, 0.26)</b>   | <b>0.124</b>     | <b>0.13 (0.00, 0.25)</b>    | <b>0.052</b>     |
|        |                    | B-Hg/Hb               | -0.07 (-0.14, 0.01)         | 0.081        | -0.05 (-0.13, 0.03)         | 0.177            | <b>-0.06 (-0.14, 0.01)</b>  | <b>0.088</b>     |
|        |                    | Age                   | 0.00 (-0.01, 0.02)          | 0.445        | <b>0.01 (-0.002, 0.03)</b>  | <b>0.019</b>     | 0.01 (-0.003, 0.02)         | 0.133            |
|        |                    | Current smoking       | <b>0.29 (0.02, 0.56)</b>    | <b>0.036</b> | <b>0.40 (0.11, 0.66)</b>    | <b>0.007</b>     | <b>0.26 (-0.04, 0.50)</b>   | <b>0.070</b>     |
| FEMALE | AIM                | B-Cd/Hb               | -0.02 (-0.05, 0.08)         | 0.693        | -0.06 (-0.19, 0.05)         | 0.287            | -0.04 (-0.15, 0.06)         | 0.411            |
|        |                    | B-As                  | -                           | -            | -                           | -                | 0.01 (-0.07, 0.09)          | 0.749            |
|        |                    | B-Pb/Hb               | 0.12 (-0.03, 0.29)          | 0.144        | 0.07 (-0.08, 0.25)          | 0.408            | 0.08 (-0.07, 0.21)          | 0.302            |
|        |                    | B-Hg/Hb               | -                           | -            | 0.03 (-0.05, 0.11)          | 0.449            | 0.04 (-0.04, 0.12)          | 0.310            |
|        |                    | B-Se/Hb               | <b>0.30 (-0.07, 0.68)</b>   | <b>0.038</b> | <b>0.26 (0.04, 0.74)</b>    | <b>0.094</b>     | 0.21 (-0.13, 0.55)          | 0.214            |
|        |                    | BMI                   | -                           | -            | <b>-0.02 (-0.04, 0.003)</b> | <b>0.029</b>     | -0.01 (-0.01, 0.03)         | 0.117            |
|        |                    | Age                   | -                           | -            | <b>0.04 (0.004, 0.02)</b>   | <b>&lt;0.001</b> | <b>0.04 (0.03, 0.06)</b>    | <b>&lt;0.001</b> |
|        |                    | Pre-pregnancy smoking | <b>0.17 (0.06, 0.28)</b>    | <b>0.040</b> | 0.13 (-0.09, 0.34)          | 0.242            | 0.13 (-0.04, 0.32)          | 0.165            |
| MALE   | NAG                | B-Cd/Hb               | -0.02 (-0.11, 0.05)         | 0.579        | -0.02 (-0.10, 0.07)         | 0.703            | 0.01 (-0.09, 0.08)          | 0.850            |
|        |                    | B-Pb/Hb               | -                           | -            | 0.07 (-0.07, 0.21)          | 0.297            | -                           | -                |
|        |                    | B-Hg/Hb               | -                           | -            | <b>0.06 (0.006, 0.15)</b>   | <b>0.083</b>     | <b>0.06 (-0.02, 0.13)</b>   | <b>0.084</b>     |
|        |                    | B-Se/Hb               | <b>-0.41 (-0.78, -0.03)</b> | <b>0.032</b> | -                           | -                | -                           | -                |
|        |                    | Age                   | -                           | -            | <b>0.01 (0.003, 0.03)</b>   | <b>0.026</b>     | <b>0.01 (-0.00, 0.02)</b>   | <b>0.015</b>     |
|        |                    | BMI                   | <b>0.03 (0.01, 0.05)</b>    | <b>0.013</b> | <b>0.02 (-0.002, 0.04)</b>  | <b>0.073</b>     | <b>0.02 (-0.01, 0.04)</b>   | <b>0.077</b>     |
| FEMALE | NAG                | B-Cd/Hb               | <b>0.15 (0.00, 0.29)</b>    | <b>0.034</b> | 0.05 (-0.11, 0.20)          | 0.480            | 0.11 (-0.049, 0.25)         | 0.145            |
|        |                    | B-Pb/Hb               | -                           | -            | 0.07 (-0.13, 0.27)          | 0.503            | 0.09 (-0.11, 0.29)          | 0.343            |
|        |                    | Age                   | <b>-0.02 (-0.04, -0.00)</b> | <b>0.027</b> | -0.2 (-0.007, 0.04)         | 0.242            | 0.01 (-0.12, 0.03)          | 0.300            |
|        |                    | Pre-pregnancy smoking | 0.11 (-0.13, 0.35)          | 0.757        | -                           | -                | -                           | -                |

bolded are significant or marginally significant results ( $p \leq 0.1$ );  $\beta$ —beta coefficient, Alb—albumin, AIM— $\alpha$ -1-microglobulin, nag—N-acetyl- $\beta$ -glucosaminidase, IgG—immunoglobulin G.

**Table A2:** Association between renal proteins and Cd estimated by linear regression model with added post-pregnancy sampling time, among women.

| Dependent variable | Independent variable  | Un-normalised urine data    |                  | Crea-normalised urine data  |                  | SG-normalised urine data     |                  |
|--------------------|-----------------------|-----------------------------|------------------|-----------------------------|------------------|------------------------------|------------------|
|                    |                       | $\beta$ (95% CI)            | p-value          | $\beta$ (95% CI)            | p-value          | $\beta$ (95% CI)             | p-value          |
| IgG                | <b>U-Cd</b>           | <b>0.32 (0.23, 0.40)</b>    | <b>&lt;0.001</b> | <b>0.17 (0.02, 0.30)</b>    | <b>0.021</b>     | <b>0.16 (0.04, 0.29)</b>     | <b>0.016</b>     |
|                    | B-As                  | -0.04 (-0.13, 0.05)         | 0.416            | -                           | -                | -                            | -                |
|                    | B-Pb                  | -0.03 (-0.17, 0.18)         | 0.745            | 0.01 (-0.19, 0.21)          | 0.956            | 0.005 (-0.19, 0.20)          | 0.957            |
|                    | BMI                   | -0.02 (-0.04, -0.00)        | 0.109            | <b>-0.03 (-0.05, -0.01)</b> | <b>0.007</b>     | <b>-0.02 (-0.05, -0.003)</b> | <b>0.023</b>     |
|                    | Age                   | -                           | -                | <b>0.043 (0.01, 0.05)</b>   | <b>0.002</b>     | <b>0.03 (0.01, 0.05)</b>     | <b>0.004</b>     |
|                    | Sampling time (days)  | 0.00 (-0.01, 0.01)          | 0.161            | 0.00 (-0.00, 0.00)          | 0.871            | 0.00 (-0.00, 0.01)           | 0.793            |
|                    | <b>B-Cd</b>           | <b>-0.01 (-0.14, 0.13)</b>  | <b>0.908</b>     | <b>-0.07 (-0.21, 0.07)</b>  | <b>0.303</b>     | <b>-0.05 (-0.19, 0.08)</b>   | <b>0.442</b>     |
|                    | B-As                  | -0.06 (-0.16, 0.03)         | 0.185            | -                           | -                | -                            | -                |
|                    | B-Pb                  | 0.002 (-0.14, 0.24)         | 0.981            | 0.04 (-0.16, 0.24)          | 0.685            | 0.02 (-0.19, 0.22)           | 0.875            |
|                    | BMI                   | <b>-0.02 (-0.05, -0.01)</b> | <b>0.050</b>     | <b>-0.04 (-0.06, -0.02)</b> | <b>&lt;0.001</b> | <b>-0.03 (-0.05, -0.01)</b>  | <b>0.009</b>     |
| Alb                | Age                   | -                           | -                | <b>0.04 (0.02, 0.06)</b>    | <b>&lt;0.001</b> | <b>0.04 (0.01, 0.06)</b>     | <b>0.001</b>     |
|                    | Sampling time (days)  | 0.00 (-0.00, 0.01)          | 0.170            | 0.00 (-0.00, 0.00)          | 0.997            | 0.00 (-0.00, 0.00)           | 0.940            |
|                    | <b>U-Cd</b>           | <b>0.61 (0.49, 0.73)</b>    | <b>&lt;0.001</b> | <b>0.22 (0.06, 0.38)</b>    | <b>0.008</b>     | <b>0.29 (0.13, 0.44)</b>     | <b>&lt;0.001</b> |
|                    | BMI                   | -0.02 (-0.05, -0.00)        | 0.077            | <b>-0.04 (-0.07, -0.02)</b> | <b>0.003</b>     | <b>-0.03 (-0.06, -0.01)</b>  | <b>0.013</b>     |
|                    | Sampling time (days)  | 0.00 (-0.00, 0.01)          | 0.196            | 0.00 (-0.00, 0.01)          | 0.442            | 0.00 8-0.00, 0.01)           | 0.130            |
|                    | <b>B-Cd</b>           | <b>-0.00 (-0.19, 0.18)</b>  | <b>0.994</b>     | <b>-0.05 (-0.21, 0.11)</b>  | <b>0.552</b>     | <b>-0.03 (-0.21, 0.01)</b>   | <b>0.719</b>     |
|                    | BMI                   | <b>-0.03 (-0.06, -0.01)</b> | <b>0.047</b>     | <b>-0.04 (-0.07, -0.02)</b> | <b>0.001</b>     | <b>-0.04 (-0.07, -0.02)</b>  | <b>0.005</b>     |
|                    | Sampling time (days)  | 0.00 (-0.00, 0.01)          | 0.195            | 0.00 (-0.00, 0.01)          | 0.598            | 0.00 (-0.00, 0.01)           | 0.172            |
|                    | <b>U-Cd</b>           | <b>0.10 (0.08, 0.15)</b>    | <b>&lt;0.001</b> | <b>0.08 (-0.04, 0.19)</b>   | <b>0.186</b>     | <b>-0.04 (-0.13, 0.05)</b>   | <b>0.347</b>     |
|                    | B-As                  | -                           | -                | -                           | -                | 0.01 (-0.07, 0.09)           | 0.796            |
| A1M                | B-Pb                  | 0.04 (-0.03, 0.14)          | 0.413            | 0.06 (-0.11, 0.19)          | 0.503            | 0.08 (-0.07, 0.22)           | 0.312            |
|                    | B-Hg                  | -                           | -                | 0.03 (-0.06, 0.11)          | 0.5033           | 0.04 (-0.03, 0.12)           | 0.320            |
|                    | B-Se                  | <b>0.29 (0.14, 0.52)</b>    | <b>0.003</b>     | <b>0.38 (-0.04, 0.75)</b>   | <b>0.066</b>     | <b>0.20 (0.15, 0.54)</b>     | <b>0.272</b>     |
|                    | BMI                   | -                           | -                | <b>-0.02 (-0.04, 0.00)</b>  | <b>0.063</b>     | <b>-0.01 (-0.03, 0.003)</b>  | <b>0.137</b>     |
|                    | Age                   | -                           | -                | <b>0.04 (0.02, 0.06)</b>    | <b>&lt;0.001</b> | <b>0.04 (0.02, 0.06)</b>     | <b>&lt;0.001</b> |
|                    | Pre-pregnancy smoking | <b>0.16 (0.07, 0.28)</b>    | <b>0.003</b>     | <b>0.14 (-0.08, 0.35)</b>   | <b>0.228</b>     | <b>0.15 (0.04, 0.33)</b>     | <b>0.117</b>     |
|                    | Sampling time (days)  | 0.00 (-0.00, 0.00)          | 0.189            | <b>-0.00 (-0.00, 0.00)</b>  | <b>0.485</b>     | <b>-0.00 (-0.00, 0.00)</b>   | <b>0.715</b>     |
|                    | <b>B-Cd</b>           | <b>0.02 (-0.05, 0.08)</b>   | <b>0.627</b>     | <b>-0.07 (-0.19, 0.06)</b>  | <b>0.295</b>     | <b>-0.05 (-0.16, 0.05)</b>   | <b>0.331</b>     |
|                    | B-As                  | -                           | -                | -                           | -                | 0.01 (-0.07, 0.09)           | 0.828            |
|                    | B-Pb                  | 0.03 (-0.03, 0.14)          | 0.473            | 0.08 (-0.08, 0.25)          | 0.351            | 0.07 (-0.07, 0.21)           | 0.348            |
|                    | B-Hg                  | -                           | -                | 0.03 (-0.05, 0.12)          | 0.470            | 0.04 (-0.04, 0.12)           | 0.206            |
|                    | B-Se                  | <b>0.21 (0.01, 0.42)</b>    | <b>0.038</b>     | <b>0.37 (0.04, 0.74)</b>    | <b>0.073</b>     | <b>0.21 (-0.13, 0.55)</b>    | <b>0.128</b>     |

|     |                       |                             |                   |                   |                           |                  |                          |                  |
|-----|-----------------------|-----------------------------|-------------------|-------------------|---------------------------|------------------|--------------------------|------------------|
|     | BMI                   | -                           | -                 | -                 | -0.02 (-0.04, 0.003)      | 0.034            | -0.01 (-0.01, 0.03)      | 0.110            |
|     | Age                   | -                           | -                 | -                 | <b>0.04 (0.004, 0.02)</b> | <b>&lt;0.001</b> | <b>0.04 (0.03, 0.06)</b> | <b>&lt;0.001</b> |
|     | Pre-pregnancy smoking | <b>0.16 (0.04, 0.27)</b>    | <b>0.008</b>      | <b>0.008</b>      | 0.12 (-0.09, 0.34)        | 0.245            | 0.15 (-0.04, 0.32)       | 0.107            |
|     | Sampling time (days)  | 0.00 (0.00, 0.00)           | 0.134             | 0.134             | -0.00 (-0.00, 0.00)       | 0.553            | -0.00 (0.00, 0.00)       | 0.730            |
| NAG | U-Cd                  | <b>0.39(0.30, 0.48)</b>     | <b>&lt; 0.001</b> | <b>&lt; 0.001</b> | <b>0.37 (0.24, 0.49)</b>  | <b>&lt;0.001</b> | <b>0.27 (0.15, 0.40)</b> | <b>&lt;0.001</b> |
|     | B-Pb                  | -                           | -                 | -                 | 0.11 (-0.10, 0.29)        | 0.297            | 0.10 (-0.10, 0.29)       | 0.329            |
|     | Age                   | -0.01 (-0.03, 0.01)         | 0.325             | 0.325             | 0.01 (-0.01, 0.03)        | 0.516            | 0.01 (-0.02, 0.03)       | 0.532            |
|     | Pre-pregnancy smoking | 0.05 (-0.18, 0.328)         | 0.678             | 0.678             | -                         | -                | -                        | -                |
|     | Sampling time (days)  | -0.00 (-0.01, 0.00)         | 0.101             | 0.101             | -0.00 (-0.01, 0.00)       | 0.431            | -0.00 (-0.00, 0.00)      | 0.580            |
|     | B-Cd                  | <b>0.14 (0.01, 0.28)</b>    | <b>0.052</b>      | <b>0.052</b>      | 0.06 (-0.08, 0.20)        | 0.433            | 0.13 (-0.09, 0.27)       | 0.100            |
|     | B-Pb                  | -                           | -                 | -                 | 0.09 (-0.10, 0.28)        | 0.391            | 0.09 (-0.11, 0.29)       | 0.355            |
|     | Age                   | <b>-0.02 (-0.04, -0.00)</b> | <b>0.034</b>      | <b>0.034</b>      | -0.1 (-0.007, 0.04)       | 0.240            | 0.01 (-0.12, 0.03)       | 0.437            |
|     | Pre-pregnancy smoking | 0.07 (-0.18, 0.31)          | 0.598             | 0.598             | -                         | -                | -                        | -                |
|     | Sampling time (days)  | -0.00 (-0.00, 0.00)         | 0.246             | 0.246             | -0.00 -0.00, 0.00)        | 0.602            | -0.00 (-0.00, 0.00)      | 0.952            |

bolded are significant or marginally significant results ( $p \leq 0.1$ );  $\beta$ —beta coefficient, Alb—albumin, AIM— $\alpha$ -1-microglobulin, nag - N-acetyl- $\beta$ -glucosaminidase, IgG - immunoglobulin G

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## A.2 *MTs, MTF1, DMT1, LRP2 and LRP8* Polymorphisms and Cd levels in Slovenian-Croatian Population



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### ***MTs, MTF1, DMT1, LRP2 and LRP8 polymorphisms and Cd levels in Slovenian-Croatian population***

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**Ljubljana, 23/3/2019**

## STUDY POPULATION AND METHODS

Study was conducted on Slovenian non-pregnant women (n = 177, aged 30 – 51 years; residents of Ljubljana and its surroundings) and Croatian pregnant women (n = 205, aged 19 – 44, residents of Rijeka and its surroundings). Participants were recruited between 2006 and 2017 within the wider EU-funded PHIME-FP6 project and its follow-up EU-funded CROME-LIFE+ project. Information regarding basic characteristics, socioeconomic status, lifestyle, and nutritional habits were acquired through interviewer-administered questionnaires.

Cd in blood and urine was determined using octapole reaction system inductively coupled plasma mass spectrometer (ORS ICP-MS). Measurements were performed at the Jožef Stefan institute, Department of Environmental Sciences, Slovenia (ORS ICP-MS; 7500ce, Agilent Technologies) and at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre Ljubljana, Slovenia (ORS ICP-MS 7700x, Agilent Technologies).

DNA was isolated from peripheral blood and used for SNPs genotyping by pre-designed TaqMan assays as described in details by [Stajniko et al., 2019a](#). Genotyped were 12 SNP in genes coding for 9 metallothionein (MT) sub-isoforms (*MT1A*, *MT1B*, *MT1E*, *MT1F*, *MT1G*, *MT1X*, *MT2A*, *MT3* and *MT4*), 2 SNPs in gene coding for metal regulatory transcription factor 1 (MTF1) and LDL Receptor Related Protein 8 (LRP8), and one SNP in divalent metal transporter 1 (DMT1) and LDL Receptor Related Protein 2 (LRP2). The basic characteristics on studied SNPs are reported in [Stajniko et al., 2019b](#).

For the statistical analysis was used STATA12/SE software. Performed were non-parametrical Kruskal-Wallis rank test for the distribution of Cd levels between carriers and non-carriers of SNPs variant allele. Observed significant associations were further tested using multivariable linear regression model considering adjustment for possible influencing factors age, BMI, ever smoking, and plasma Se. The level of statistical significance was set at  $p < 0.05$  (two-sided).



**Table 1:** Basic characteristics and Cd levels in Slovenian non-pregnant and Croatian pregnant women.

|                              | SLO: non-pregnant women (n = 176) | CRO-pregnant women (N =205)    |
|------------------------------|-----------------------------------|--------------------------------|
| Age (N) years                | 38, 30 – 51 (176)                 | 30, 19 – 44 (192)              |
| BMI (N) kg/m <sup>2</sup>    | 25, 18 – 44 (177)                 | 23, 17 – 41 (203) <sup>a</sup> |
| B-Cd (N) ng/g*, ng/mL        | 0.41, 0.37 – 0.46 (169)           | 0.47, 0.42 – 0.53 (198)        |
| U-Cd <sub>SG</sub> (N) ng/MI | 0.13, 0.11 – 0.15 (173)           | 0.31, 0.28 – 0.35 (157)        |

<sup>a</sup>, pre-pregnancy BMI;  $\bar{x}$ ; min-max for Age and BMI and GM (95-CI) for B-Cd and U-Cd.

**Table 2:** Data distribution of Cd levels between carriers and non-carriers of variant alleles of studied SNPs in Slovenian non-pregnant women and Croatian pregnant women.

| GENE        | SNP        | allele  | SLO: non-pregnant women |                  |                             |                  | CRO: pregnant women |                  |                             |                  |
|-------------|------------|---------|-------------------------|------------------|-----------------------------|------------------|---------------------|------------------|-----------------------------|------------------|
|             |            |         | B-Cd (ng/mL)            |                  | U-Cd (ng/mL <sub>SG</sub> ) |                  | B-Cd (ng/mL)        |                  | U-Cd (ng/mL <sub>SG</sub> ) |                  |
|             |            |         | n                       | GM (95CI)        | n                           | GM (95CI)        | n                   | GM (95CI)        | n                           | GM (95CI)        |
| <i>MT1A</i> | rs11076160 | A-      | 91                      | 0.40 (0.34-0.46) | 93                          | 0.12 (0.10-0.15) | 121                 | 0.47 (0.41-0.55) | 97                          | 0.29 (0.25-0.34) |
|             |            | A+      | 78                      | 0.43 (0.36-0.52) | 80                          | 0.13 (0.10-0.18) | 77                  | 0.47 (0.37-0.58) | 60                          | 0.35 (0.29-0.41) |
|             |            | p-value |                         | 0.85             |                             | 0.71             |                     | 0.82             |                             | 0.06             |
| <i>MT1B</i> | rs2070839  | T-      | 61                      | 0.41 (0.34-0.49) | 65                          | 0.11 (0.08-0.15) | 94                  | 0.48 (0.40-0.58) | 76                          | 0.29 (0.25-0.34) |
|             |            | T+      | 108                     | 0.41 (0.35-0.48) | 108                         | 0.13 (0.11-0.17) | 104                 | 0.46 (0.40-0.54) | 81                          | 0.33 (0.29-0.39) |
|             |            | p-value |                         | 0.74             |                             | 0.33             |                     | 0.61             |                             | 0.42             |
|             | rs8052334  | T-      | 44                      | 0.43 (0.34-0.53) | 48                          | 0.11 (0.08-0.17) | 69                  | 0.50 (0.39-0.63) | 55                          | 0.29 (0.24-0.35) |
|             |            | T+      | 125                     | 0.41 (0.36-0.47) | 125                         | 0.13 (0.10-0.16) | 129                 | 0.46 (0.40-0.53) | 102                         | 0.32 (0.28-0.37) |
|             |            | p-value |                         | 0.45             |                             | 0.44             |                     | 0.439            |                             | 0.60             |
| <i>MT1E</i> | rs2070836  | C-      | 153                     | 0.42 (0.37-0.47) | 156                         | 0.12 (0.10-0.15) | 168                 | 0.46 (0.41-0.53) | 132                         | 0.31 (0.27-0.35) |
|             |            | C+      | 16                      | 0.36 (0.24-0.54) | 17                          | 0.14 (0.07-0.28) | 30                  | 0.52 (0.38-0.72) | 25                          | 0.34 (0.27-0.44) |
|             |            | p-value |                         | 0.57             |                             | 0.94             |                     | 0.39             |                             | 0.29             |
| <i>MT1F</i> | rs2291956  | T-      | 138                     | 0.41 (0.36-0.47) | 141                         | 0.13 (0.11-0.15) | 160                 | 0.47 (0.42-0.54) | 127                         | 0.30 (0.17-0.34) |
|             |            | T+      | 31                      | 0.40 (0.30-0.53) | 32                          | 0.12 (0.07-0.20) | 38                  | 0.46 (0.34-0.62) | 30                          | 0.36 (0.28-0.45) |
|             |            | p-value |                         | 0.93             |                             | 0.61             |                     | 0.83             |                             | 0.13             |
| <i>MT1G</i> | rs12315    | T-      | 154                     | 0.42 (0.37-0.47) | 160                         | 0.12 (0.10-0.15) | 177                 | 0.48 (0.42-0.55) | 141                         | 0.32 (0.28-0.36) |
|             |            | T+      | 15                      | 0.37 (0.24-0.56) | 13                          | 0.20 (0.11-0.36) | 21                  | 0.42 (0.33-0.54) | 16                          | 0.27 (0.19-0.38) |
|             |            | p-value |                         | 0.59             |                             | 0.14             |                     | 0.43             |                             | 0.32             |
| <i>MT1X</i> | rs2301234  | T-      | 61                      | 0.41 (0.33-0.51) | 62                          | 0.13 (0.09-0.17) | 55                  | 0.48 (0.37-0.61) | 47                          | 0.32 (0.27-0.39) |
|             |            | T+      | 108                     | 0.41 (0.36-0.47) | 111                         | 0.13 (0.10-0.16) | 143                 | 0.47 (0.41-0.54) | 110                         | 0.31 (0.27-0.35) |
|             |            | p-value |                         | 0.84             |                             | 0.89             |                     | 0.95             |                             | 0.60             |
| <i>MT2A</i> | rs10636    | C-      | 88                      | 0.42 (0.36-0.49) | 90                          | 0.12 (0.10-0.15) | 100                 | 0.50 (0.43-0.58) | 75                          | 0.35 (0.30-0.42) |
|             |            | C+      | 81                      | 0.41 (0.34-0.49) | 83                          | 0.13 (0.10-0.17) | 98                  | 0.46 (0.37-0.54) | 82                          | 0.28 (0.24-0.32) |
|             |            | p-value |                         | 0.56             |                             | 0.88             |                     | 0.36             |                             | 0.03             |
|             | rs28366003 | G-      | 153                     | 0.42 (0.37-0.48) | 157                         | 0.13 (0.11-0.15) | 175                 | 0.46 (0.41-0.53) | 136                         | 0.31 (0.28-0.35) |
|             |            | G+      | 16                      | 0.32 (0.23-0.44) | 16                          | 0.11 (0.06-0.21) | 23                  | 0.53 (0.35-0.78) | 21                          | 0.31 (0.24-0.40) |
|             |            | p-value |                         | 0.15             |                             | 0.68             |                     | 0.86             |                             | 0.75             |
| <i>MT3</i>  | rs11644094 | G-      | 59                      | 0.38 (0.32-0.45) | 62                          | 0.12 (0.09-0.16) | 93                  | 0.52 (0.43-0.62) | 70                          | 0.35 (0.29-0.41) |
|             |            | G+      | 110                     | 0.43 (0.37-0.50) | 111                         | 0.13 (0.10-0.16) | 105                 | 0.44 (0.37-0.51) | 87                          | 0.29 (0.25-0.33) |
|             |            | p-value |                         | 0.37             |                             | 0.77             |                     | 0.14             |                             | 0.01             |
|             | rs28754593 | C-      | 87                      | 0.41 (0.34-0.50) | 86                          | 0.14 (0.11-0.19) | 66                  | 0.49 (0.40-0.59) | 55                          | 0.34 (0.28-0.41) |
|             |            | C+      | 82                      | 0.41 (0.36-0.47) | 87                          | 0.11 (0.09-0.14) | 132                 | 0.46 (0.40-0.54) | 102                         | 0.30 (0.26-0.34) |
|             |            | p-value |                         | 0.64             |                             | 0.18             |                     | 0.82             |                             | 0.31             |
| <i>MT4</i>  | rs396230   | C-      | 121                     | 0.42 (0.36-0.48) | 123                         | 0.13 (0.11-0.16) | 127                 | 0.44 (0.38-0.51) | 100                         | 0.29 (0.26-0.34) |
|             |            | C+      | 48                      | 0.40 (0.33-0.50) | 50                          | 0.11 (0.08-0.15) | 71                  | 0.54 (0.45-0.65) | 57                          | 0.34 (0.29-0.41) |
|             |            | p-value |                         | 0.93             |                             | 0.18             |                     | 0.11             |                             | 0.19             |
| <i>MTF1</i> | rs3748682  | C-      | 90                      | 0.44 (0.38-0.51) | 92                          | 0.14 (0.11-0.18) | 109                 | 0.45 (0.38-0.52) | 85                          | 0.30 (0.26-0.35) |
|             |            | C+      | 79                      | 0.38 (0.32-0.46) | 81                          | 0.11 (0.09-0.14) | 89                  | 0.51 (0.42-0.61) | 72                          | 0.32 (0.27-0.39) |
|             |            | p-value |                         | 0.11             |                             | 0.21             |                     | 0.11             |                             | 0.61             |
|             | rs4653329  | T-      | 93                      | 0.39 (0.34-0.46) | 98                          | 0.12 (0.10-0.16) | 123                 | 0.45 (0.39-0.52) | 100                         | 0.31 (0.27-0.36) |
|             |            | T+      | 76                      | 0.44 (0.36-0.53) | 75                          | 0.13 (0.10-0.17) | 75                  | 0.51 (0.41-0.63) | 57                          | 0.31 (0.26-0.37) |
|             |            | p-value |                         | 0.32             |                             | 0.65             |                     | 0.32             |                             | 0.91             |
| <i>DMT1</i> | rs224589   | T-      | 102                     | 0.40 (0.34-0.46) | 107                         | 0.12 (0.10-0.15) | 121                 | 0.47 (0.40-0.54) | 98                          | 0.30 (0.26-0.34) |
|             |            | T+      | 67                      | 0.43 (0.36-0.53) | 66                          | 0.13 (0.09-0.17) | 77                  | 0.48 (0.40-0.58) | 59                          | 0.33 (0.28-0.41) |

|      |           | p-value | 0.504                | 0.884                | 0.734                | 0.261               |
|------|-----------|---------|----------------------|----------------------|----------------------|---------------------|
| LRP2 | rs2544390 | T-      | 58 0.37 (0.31-0.46)  | 58 0.11 (0.08-0.16)  | 84 0.49 (0.41-0.59)  | 62 0.30 (0.24-0.37) |
|      |           | T+      | 110 0.43 (0.37-0.50) | 114 0.13 (0.11-0.16) | 114 0.46 (0.39-0.54) | 95 0.32 (0.28-0.36) |
|      |           | p-value | 0.238                | 0.204                | 0.904                | 0.793               |
| LRP8 | rs5174    | T-      | 60 0.36 (0.30-0.42)  | 61 0.13 (0.10-0.18)  | 64 0.31 (0.25-0.37)  | 58 0.31 (0.25-0.39) |
|      |           | T+      | 107 0.44 (0.38-0.52) | 110 0.12 (0.10-0.15) | 92 0.32 (0.28-0.36)  | 98 0.31 (0.28-0.36) |
|      |           | p-value | 0.158                | 0.734                | 0.264                | 0.741               |
|      | rs382198  | C-      | 68 0.39 (0.33-0.47)  | 72 0.12 (0.09-0.17)  | 79 0.43 (0.36-0.52)  | 64 0.31 (0.25-0.37) |
|      |           | C+      | 100 0.43 (0.36-0.50) | 100 0.13(0.10-0.16)  | 118 0.50(0.42-0.58)  | 92 0.32 (0.28-0.36) |
|      |           | p-value | 0.890                | 0.662                | 0.608                | 0.776               |

in bold are significant results ( $p < 0.05$ ) and in italic bold marginally significant ( $p < 0.1$ ).

**Table 3:** Influence of rs11076160 (*MT1A*), rs10636 (*MT2A*) and rs11644094 (*MT3*) SNPs on U-Cd levels in Croatian pregnant women (multiple linear regression models).

|                                |                           | rs11076160<br>variant allele A |         |                                | rs10636<br>variant allele C |              |                                | rs11644094<br>variant allele G |         |                                |
|--------------------------------|---------------------------|--------------------------------|---------|--------------------------------|-----------------------------|--------------|--------------------------------|--------------------------------|---------|--------------------------------|
| U-Cd<br>(ng/mL <sub>SC</sub> ) | Independent variable      | $\beta$                        | p-value | N<br>R <sup>2</sup><br>p-value | $\beta$                     | p-value      | N<br>R <sup>2</sup><br>p-value | $\beta$                        | p-value | N<br>R <sup>2</sup><br>p-value |
|                                | SNPs variant allele       | 0.17                           | 0.144   |                                | <b>-0.34</b>                | <b>0.003</b> |                                | -0.08                          | 0.505   |                                |
|                                | Age (years)               | 0.04                           | 0.006   | 145                            | 0.04                        | 0.002        | 145                            | 0.04                           | 0.007   | 145                            |
|                                | BMI (kg/m <sup>2</sup> )  | 0.00                           | 0.923   | 0.11                           | -0.00                       | 0.943        | 0.15                           | 0.00                           | 0.975   | 0.10                           |
|                                | Ever smoking (yes vs. no) | 0.32                           | 0.009   | 0.007                          | 0.33                        | 0.006        | <0.001                         | 0.31                           | 0.013   | 0.014                          |
|                                | P-Se (ng/mL)              | 0.25                           | 0.347   |                                | 0.24                        | 0.366        |                                | 0.24                           | 0.384   |                                |

BMI, body mass index

## LITERATURE

- Stajanko, A., Špirić, Z., BRIŠKI, A. S., Prpić, I., Šlejkovec, Z., Falnoga, I., ... Mazej, D., 2019a. Arsenic metabolites; selenium; and AS3MT, MTHFR, AQP4, AQP9, SELENOP, INMT, and MT2A polymorphisms in Croatian-Slovenian population from PHIME-CROME study. *Environmental Research*, 170, 301–319. <https://doi.org/10.1016/j.envres.2018.11.045>
- Stajanko, A., Runkel A. A., , M., Trdin, A., Horvat, M., Falnoga, I., 2019b. Allele frequency distribution of selected SNPs in the Slovenian-Croatian population in comparison with other worldwide populations. IJS working report No. 12814

### A.3 Allele Frequency Distribution of Selected SNPs in the Slovenian-Croatian Population in Comparison with Other Worldwide Populations

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**IJS – Delovno poročilo**  
***IJS - Report***

**IJS DP – 12814**

#### ***Allele frequency distribution of selected SNPs in the Slovenian-Croatian population in comparison with other worldwide populations***

Stajenko Anja, Agneta Annika Runkel, Ajda Trdin, Milena Horvat, Ingrid Falnoga

**Ljubljana, 13.4.2019**

## STUDY GROUP AND METHODS

Study was conducted on Slovenian-Croatian woman population (n= 383, aged 19-51); 178 residents of Ljubljana and its surroundings recruited between 2013 and 2017 within the follow-up EU-funded CROME-LIFE+ project, and 205 residents of Rijeka and its surroundings recruited between 2006 and 2011 within the wider EU-funded PHIME-FP6 project. Residential areas—Rijeka and Ljubljana—were not far apart (<150 km) and before 1991, they both belonged to the former Yugoslavia. Therefore, in the present study they presented one population. Nevertheless, we tested allele frequency distribution of studied SNPs between areas and observed no differences. All participants provided signed informed consent, and the research protocol of the study was approved by the National Ethics Committee of Croatia and Slovenia.

For all participants DNA was isolated from peripheral blood and used for SNP genotyping as described in details in [Stajanko et al., 2019](#). Basic information on genotyped 21 SNPs in 15 genes are given in **Table 1**.

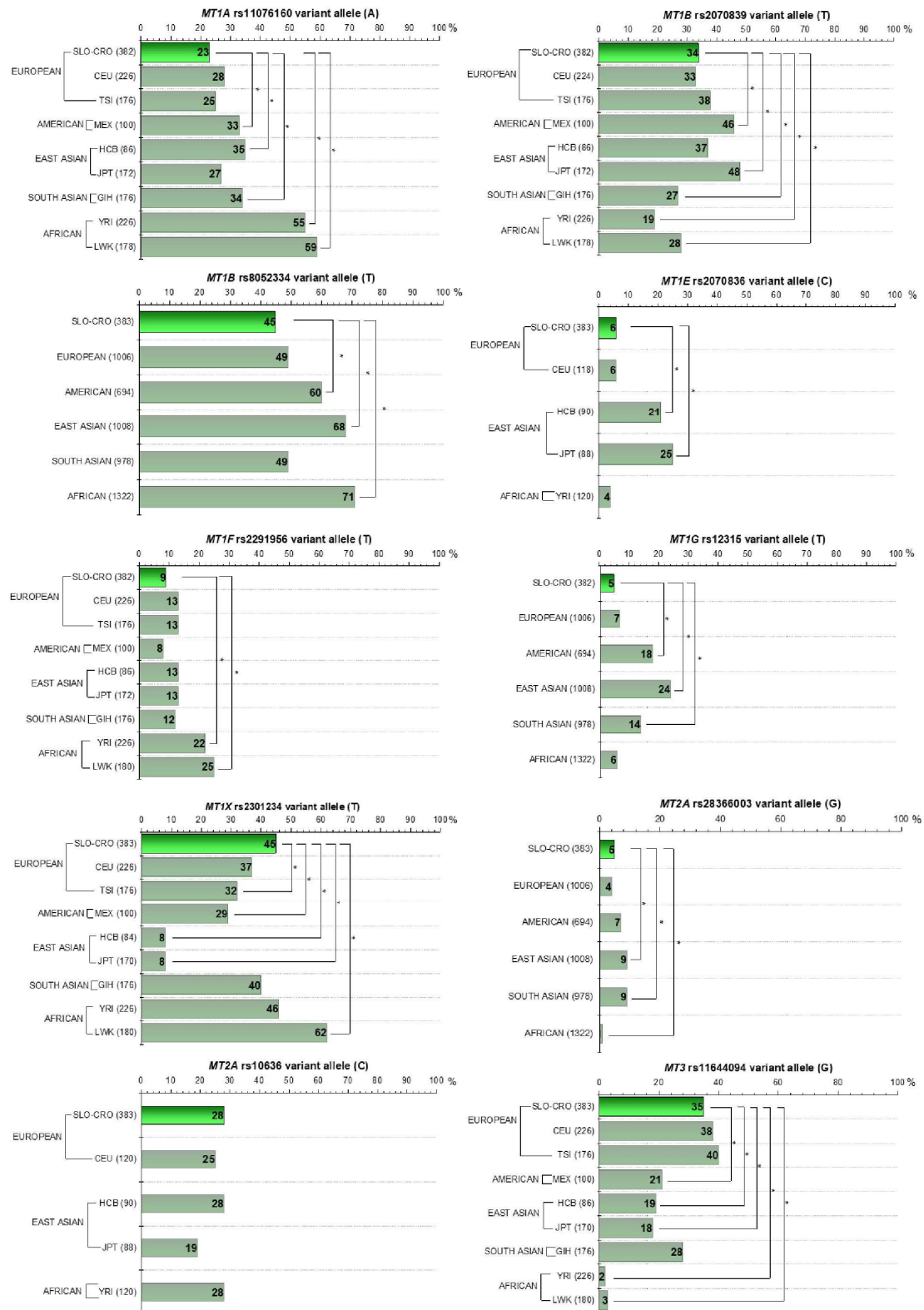
Consistency of genotype frequencies with the Hardy-Weinberg equilibrium (HWE,  $p > 0.05$ , consistent all SNPs except rs12315) and comparisons of the SNP allelic frequencies between populations were performed using Pearson's chi-squared test.

Variant allele frequencies obtained for Slovenian-Croatian population were compared with those reported for populations from SNP database established by National Center for Biotechnology Information (dbSNP, NCBI). For the comparison preferentially were chosen smaller specified populations from HapMap project (e.g. CEU, TSI, HCB, JPT etc.) and if those were not available used were larger populations from 1000Genome project (e.g. EUR, SAS, EAS, AFR, AMR) (**Figure 1**).

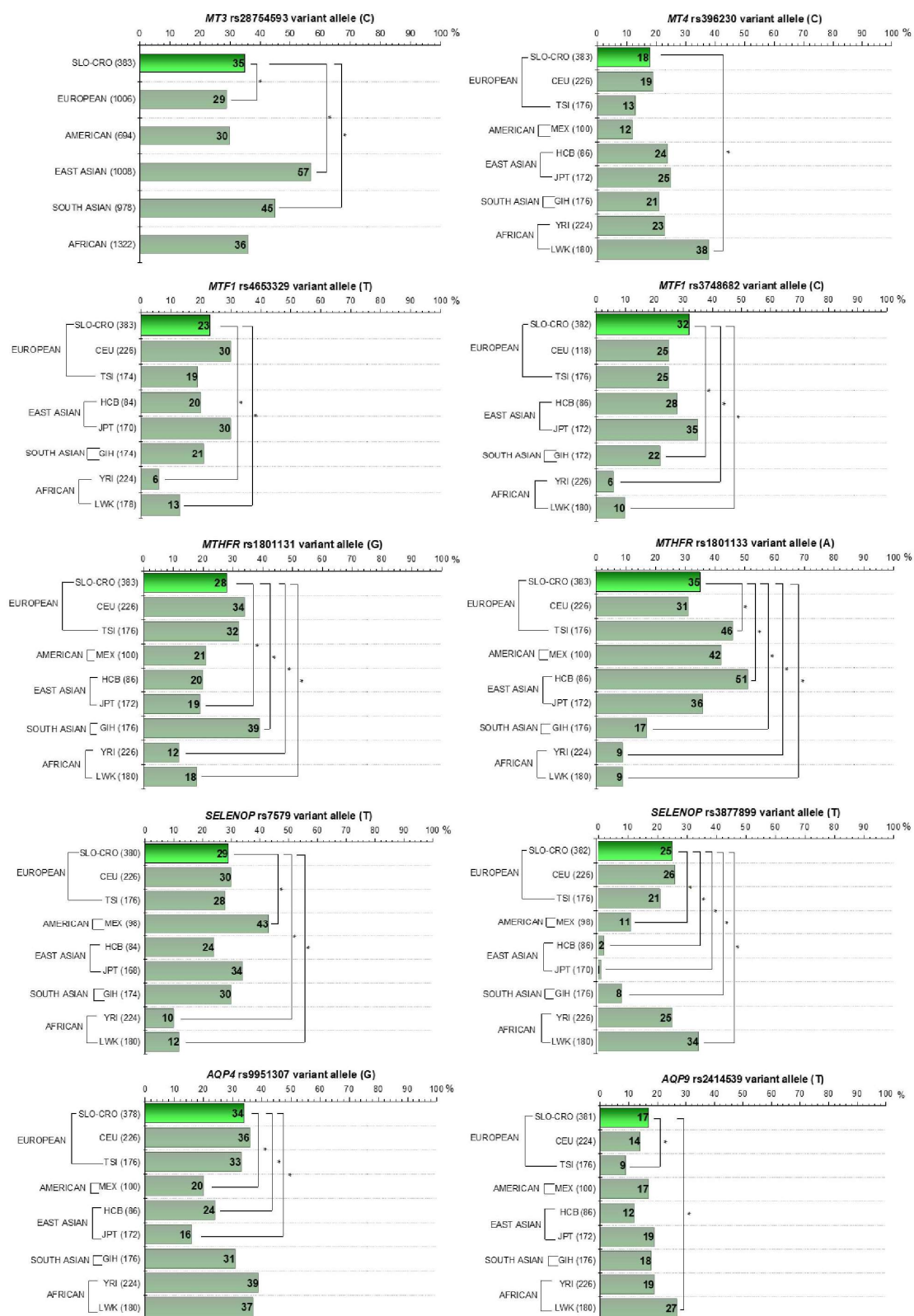
**Table 1:** Information on studied SNPs

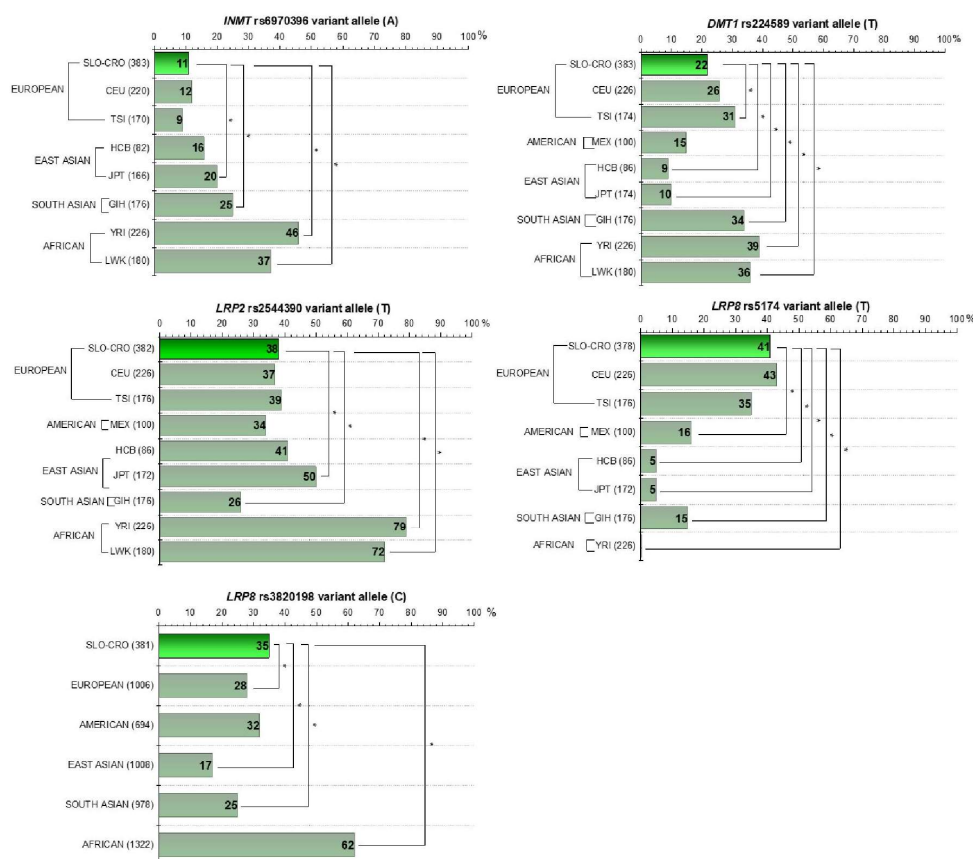
| Gene name                               | Gene ID        | dbSNP NCBI ID | Chromosome | Location | Nucleotide change | Amino acid change | TaqMan assay ID |
|-----------------------------------------|----------------|---------------|------------|----------|-------------------|-------------------|-----------------|
| Metallothioneins                        | <i>MT1A</i>    | rs11076161    | 16         | intron   | G > A             |                   | C_25996927_10   |
|                                         | <i>MT1B</i>    | rs8052334     | 16         | intron   | C > T             |                   | C_1430037_10    |
|                                         |                | rs2070839     | 16         | intron   | C > T             |                   | C_1430032_20    |
|                                         | <i>MT1E</i>    | rs2070836     | 16         | intron   | G > C             |                   | C_1429985_20    |
|                                         | <i>MT1F</i>    | rs2291956     | 16         | intron   | C > T             |                   | C_1430047_20    |
|                                         | <i>MT1G</i>    | rs12315       | 16         | intron   | G > T             |                   | C_11718154_10   |
|                                         | <i>MT1X</i>    | rs2301234     | 16         | intron   | G > T             |                   | C_15756069_10   |
|                                         | <i>MT2A</i>    | rs10636       | 16         | 3'UTR    | G > C             |                   | C_1402094_10    |
|                                         |                | rs28366003    | 16         | 5'UTR    | A > G             |                   | C_60284591_10   |
|                                         | <i>MT3</i>     | rs11644094    | 16         | intron   | A > G             |                   | C_31759126_10   |
|                                         |                | rs28754593    | 16         | intron   | A > C             |                   | C_60284513_10   |
|                                         | <i>MT4</i>     | rs396230      | 16         | intron   | T > C             |                   | C_2853838_10    |
|                                         | <i>MTF1</i>    | rs3748682     | 1          | 3'UTR    | T > C             |                   | C_1156514_20    |
|                                         |                | rs4653329     | 1          | intron   | G > C             |                   | C_27950314_20   |
|                                         |                | rs1801131     | 1          | Exon     | T > G             | Glu > Ala         | C_850486_20     |
| Metal regulatory transcription factor 1 | <i>MTHFR</i>   | rs1801133     | 1          | Exon     | G > A             | Ala > Val         | C_1202883_20    |
|                                         | <i>SELENOP</i> | rs7579        | 5          | 3'UTR    | C > T             |                   | C_8806056_10    |
|                                         |                | rs3877899     | 5          | missense | C > T             | Ala > Thr         | C_2841533_10    |
|                                         | <i>AQP4</i>    | rs9951307     | 18         | 3'UTR    | A > G             |                   | C_29992536_20   |
|                                         | <i>AQP9</i>    | rs2414539     | 15         | Intron   | A > T             |                   | C_15799601_10   |
|                                         | <i>INMT</i>    | rs6970396     | 7          | 3'UTR    | G > A             |                   | C_29402264_10   |
|                                         | <i>DMT1</i>    | rs224589      | 12         | intron   | G > T             |                   | C_2967992_1_    |
|                                         | <i>LRP2</i>    | rs2544390     | 2          | intron   | C > T             |                   | C_8822318_10    |
|                                         |                | rs5174        | 1          | missense | C > T             | Arg > Gln         | C_190754_10     |
|                                         | <i>LRP8</i>    | rs3820198     | 1          | missense | A > C             | Asp > Glu         | C_25620871_20   |

## RESULTS









**Figure 1:** Variant allele frequency distribution of selected SNPs in Slovenian-Croatian population (SLO-CRO) and their comparison with other worldwide populations; **dbSNP, NCBI**; from project 1000Genome or *HapMap*: CEU – Utah residents with ancestry from northern and western Europe; TSI – Tuscany, Italy; MEX – Mexican Ancestry from Los Angeles, USA; HCB – Beijing, China; JPT – Tokyo, Japan; GIH – Gujarati Indian from Houston, Texas; YRI – Yoruba in Ibadan, Nigeria; LWK – Luhya in Webuye, Kenya). (\* – significant difference,  $p < 0.05$ ; in the brackets are added number of individuals in population).

## REFERENCES:

Stajanko, A., Špirić, Z., BRIŠKI, A. S., Prpić, I., Šlejkovec, Z., Falnoga, I., ... Mazej, D. (2019). Arsenic metabolites; selenium; and AS3MT, MTHFR, AQP4, AQP9, SELENOP, INMT, and MT2A polymorphisms in Croatian-Slovenian population from PHIME-CROME study. *Environmental Research*, 170, 301–319. <https://doi.org/10.1016/j.envres.2018.11.045>

#### **A.4 *ALAD* SNPs Frequency Distribution and its Impact on Pb Levels in Slovenian-Croatian Population (PHIME-CROME Study)**



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*IJS - Report*

**IJS DP – 12666**

### ***ALAD* SNPs frequency distribution and its impact on Pb levels in Slovenian-Croatian population (PHIME-CROME study)**

Stajnko Anja, Jagodic Marta, Krušič Žiga, Mazej Darja, Horvat Milena,  
Falnoga Ingrid

**Ljubljana, 20.3.19**

## 1. INTRODUCTION:

Delta-aminolevulinic acid dehydratase also known as porphobilinogen synthase is a cytoplasmic enzyme that catalyses the second step in the porphyrin and heme biosynthetic pathway and is also well-known to influence Pb toxicity (Skerfving and Bergdahl, 2016). The enzyme is encoded by the *ALAD* gene. Previous research has shown that the single nucleotide polymorphisms (SNPs) in the gene may have an impact on the individual response to Pb toxicity and on the variability in internal Pb levels (Warrington, 2015; Skerfving and Bergdahl, 2016).

The aim of present study was to assess the possible differences in minor allele frequencies of three *ALAD* SNPs (rs1805313, rs818708, and rs1800435) between Slovenian-Croatian population and other worldwide populations. Additionally, we assessed possible influence of SNPs on blood and/or urine Pb levels within Slovenian non-pregnant women and Croatian pregnant women.

## 2. MATERIAL AND METALS:

### 2.1 Study population

Study was conducted on 2 populations:

1) Slovenian non-pregnant women (n = 176, mean age 39 years, range 30 – 51 years; residents of Ljubljana and its surroundings) recruited between 2013 and 2017 within the follow-up EU-funded CROME-LIFE+ project,

2) Croatian pregnant women (n = 205, mean aged 30 years, range 19 – 44 years, residents of Rijeka and its surroundings) recruited between 2006 and 2011 within the wider EU-funded PHIME-FP6 project.

Information regarding basic characteristics, socioeconomic status, lifestyle, and nutritional habits were acquired through interviewer-administered questionnaires.

### 2.2 Determination of metal(loid)s

Blood Pb, Zn, Se, Mn, Cu and urine Pb in Slovenian and Croatian women were measured using octapole reaction system inductively coupled plasma mass spectrometer (ORS ICP-MS). Measurements were performed at the Jožef Stefan institute, Department of Environmental Sciences, Slovenia (ORS ICP-MS; 7500ce, Agilent Technologies) and at the laboratory of the Clinical Chemistry and Biochemistry Institute of the University Medical Centre Ljubljana, Slovenia (ORS ICP-MS 7700x, Agilent Technologies) as described by (Miklavčič et al., 2013).

### 2.3 DNA isolation and genotyping

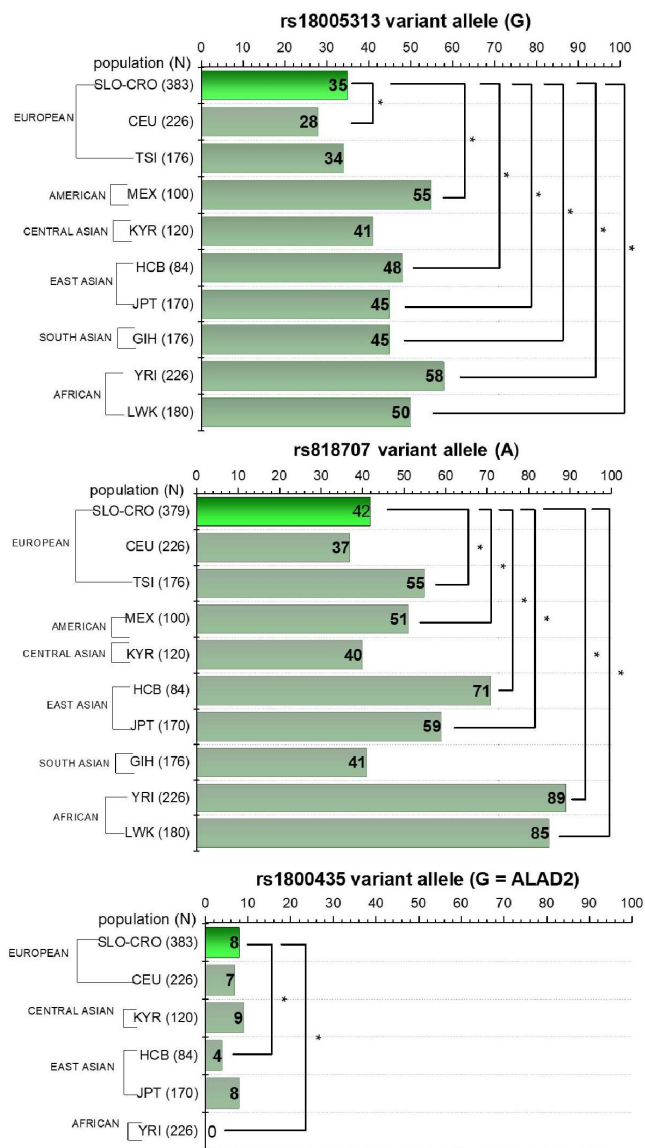
DNA was extracted from peripheral blood samples and genotyped for SNPs rs1805313 (A > G), rs818708 (G > A) and rs1800435 (C > G; Lys > Asn) located in the *ALAD* gene on chromosome 9 as described in detail by Stajniko et al., 2019.

### 2.4 Statistical analysis

For the statistical analysis was used STATA12/SE software. Performed were: Pearson's chi square test for the comparison of SNP minor allele frequencies (MAF) among populations; non-parametrical tests: Mann-Whitney U test and Kruskal-Wallis rank test for the distribution of blood and urine Pb levels among carriers of SNP allele or genotype; and multivariable linear regression model for the assessment of possible impact of SNP on Pb variability considering adjustment for possible influencing factors (age, BMI, smoking, levels of essential trace elements, parity, seafood intake). The level of statistical significance was set at  $p < 0.05$  (two-sided). OriginPro 2017 software was used for visualization of the results.

### 3. RESULTS

#### 3.1 ALAD SNPs allele frequency comparison



**Figure 1.** Comparison of *ALAD* SNPs minor allele frequencies (%) obtained in Slovenian-Croatian population (combined pregnant and non-pregnant women) with those of other worldwide populations reported in SNP database (dbSNP, NCBI) or [Stajanko et al., 2019b](#). (\* - significant difference  $p < 0.05$ ; CAS - Central Asian (KIR- Kyrgyzstan); EAS - East Asian; HCB - Beijing, China; JPT - Tokyo, Japan; SAS - South Asian; GIH - Gujarati Indian from Houston, Texas; EUR - European; CEU - Utah residents with ancestry from northern and western Europe; SLO-CRO - Ljubljana, Slovenia and Rijeka, Croatia; TSI - Tuscany, Italy; AMR - American; MEX - Mexican Ancestry from Los Angeles, USA; AFR - African, YRI - Yoruba in Ibadan, Nigeria; LWK - Luhya in Webuye, Kenya)



### 3.2 Impact of ALAD SNP on Pb levels

**Table 1:** Levels of Pb in study group with comparison to Kyrgyzstan population<sup>a</sup>.

|                                 |           | Slovenian<br>non-pregnant women | Croatian<br>pregnant women | Kyrgyzstan<br>non-pregnant women <sup>a</sup> |
|---------------------------------|-----------|---------------------------------|----------------------------|-----------------------------------------------|
| <b>B-Pb</b> ng/g                | GM, 95 CI | 10.9, 10.2 – 11.6               | 12.0, 11.2 – 12.9          | 18.4, 16.7 – 20.3                             |
|                                 | P5 – P95  | 5.62 – 21.7                     | 5.54 – 25.8                | 9.05 – 36.5                                   |
| <b>U-Pb</b> ng/mL <sub>SG</sub> | GM, 95 CI | 0.17, 0.14 – 0.21               | 0.56, 0.47 – 0.67          | -                                             |
|                                 | P5 – P95  | 0.06 – 1.70                     | 0.05 – 2.73                | -                                             |

<sup>a</sup> Stajanko et al., 2019 (submitted to Science of the Total Environment)

**Table 2.** Data distribution of Pb levels between carriers and non-carriers of ALAD SNPs variant alleles (Mann-Whitney test) and genotypes (Kruskal-Wallis test) in Slovenian non-pregnant women (n=176) and Croatian pregnant women (n=198); for comparisons are added data on Kyrgyzstan non-pregnant women<sup>a</sup> (aged > 20 y; n = 80)

| Allele or genotype (N) |                       | Slovenian<br>non-pregnant women |                                 | Croatian<br>pregnant women |                                 | Kyrgyzstan<br>non-pregnant women <sup>a</sup> |                                 |
|------------------------|-----------------------|---------------------------------|---------------------------------|----------------------------|---------------------------------|-----------------------------------------------|---------------------------------|
|                        |                       | Blood<br>(ng/g)                 | Urine<br>(ng/mL <sub>SG</sub> ) | Blood<br>(ng/g)            | Urine<br>(ng/mL <sub>SG</sub> ) | Blood<br>(ng/g)                               | Urine<br>(ng/mL <sub>SG</sub> ) |
| <b>rs1805313</b>       |                       |                                 |                                 |                            |                                 |                                               |                                 |
| <b>allele</b>          | G- (82), (90), (25)   | 11.8                            | 0.16                            | 11.8                       | 0.64                            | 22.2                                          | NA                              |
|                        | G+ (55), (108), (55)  | 10.3                            | 0.18                            | 12.2                       | 0.50                            | 17.3                                          |                                 |
|                        | p-value               | <b>0.048</b>                    | 0.835                           | 0.754                      | 0.121                           | <b>0.009</b>                                  |                                 |
| <b>genotype</b>        | AA (82) (90), (25),   | 11.8                            | 0.16                            | 11.8                       | 0.64                            | 22.2                                          | NA                              |
|                        | AG (62), (86), (40),  | 10.7                            | 0.19                            | 12.3                       | 0.46                            | 17.7                                          |                                 |
|                        | GG (23), (22), (15),  | 9.00                            | 0.13                            | 12.0                       | 0.81                            | 16.3                                          |                                 |
|                        | p-value               | <b>0.020</b>                    | 0.241                           | 0.937                      | 0.127                           | <b>0.046</b>                                  |                                 |
| <b>rs818708</b>        |                       |                                 |                                 |                            |                                 |                                               |                                 |
| <b>allele</b>          | A- (57), (69), (32)   | 10.8                            | 0.17                            | 11.3                       | 0.57                            | 18.2                                          |                                 |
|                        | A+ (121), (129), (48) | 10.9                            | 0.17                            | 12.5                       | 0.56                            | 19.0                                          |                                 |
|                        | p-value               | 0.793                           | 0.756                           | 0.319                      | 0.807                           | 0.327                                         |                                 |
| <b>genotype</b>        | GG (57) (69), (32),   | 10.8                            | 0.17                            | 11.3                       | 0.57                            | 18.2                                          | NA                              |
|                        | GA (81), (100), (37), | 10.7                            | 0.16                            | 13.0                       | 0.54                            | 19.6                                          |                                 |
|                        | AA (40), (29), (11),  | 11.3                            | 0.18                            | 11.0                       | 0.61                            | 17.2                                          |                                 |
|                        | p-value               | 0.956                           | 0.940                           | 0.113                      | 0.848                           | 0.418                                         |                                 |
| <b>rs1800435</b>       |                       |                                 |                                 |                            |                                 |                                               |                                 |
| <b>allele</b>          | G- (149), (173), (68) | 10.9                            | 0.17                            | 12.2                       | 0.56                            | 18.4                                          |                                 |
|                        | G+ (27), (31), (13)   | 11.2                            | 0.16                            | 11.0                       | 0.55                            | 19.0                                          | NA                              |
|                        | p-value               | 0.450                           | 0.930                           | 0.180                      | 0.755                           | 0.989                                         |                                 |
| <b>genotype</b>        | CC (149) (173), (68), |                                 |                                 |                            |                                 |                                               |                                 |
|                        | CG (25), (30), (13),  |                                 |                                 |                            |                                 |                                               |                                 |
|                        | GG (2), (1), (0),     |                                 |                                 |                            |                                 |                                               |                                 |
|                        | p-value               |                                 |                                 |                            |                                 |                                               |                                 |

NA, not available; <sup>a</sup> Stajanko et al., 2019 (under review in Chemosphere); in bold are statistically significant associations (p < 0.05)



**Table 3.** Influence of *ALAD* SNP rs1805313 minor allele (G) on blood Pb in Slovenian non-pregnant women, Croatian pregnant woman) assessed by multivariable linear regression model; for comparison are added data for Kyrgyzstan non-pregnant women (older than 20y)

| Dependent Variable (Pb)                                | Independent variable | Slovenian non-pregnant women |              |                          |     | Croatian pregnant women     |              |                          |     | Kyrgyzstan non-pregnant women <sup>a</sup> |              |                          |    |
|--------------------------------------------------------|----------------------|------------------------------|--------------|--------------------------|-----|-----------------------------|--------------|--------------------------|-----|--------------------------------------------|--------------|--------------------------|----|
|                                                        |                      | $\beta$ (95% CI)             | p-value      | R <sup>2</sup> (p-value) | N   | $\beta$ (95% CI)            | p-value      | R <sup>2</sup> (p-value) | N   | $\beta$ (95% CI)                           | p-value      | R <sup>2</sup> (p-value) | N  |
| <b>Model 1</b>                                         | rs1805313            | <b>-0.13 (-0.27, 0.01)</b>   | <b>0.055</b> |                          |     | 0.09 (-0.04, 0.23)          | 0.181        |                          |     | <b>-0.23 (-0.43, -0.05)</b>                | <b>0.021</b> |                          |    |
|                                                        | age                  | 0.0078 (-0.008, 0.02)        | 0.413        |                          | 168 | -0.005 (-0.02, 0.01)        | 0.459        |                          | 186 | 0.00 (-0.005, 0.01)                        | 0.565        |                          | 80 |
|                                                        | B-Se                 | <b>0.54 (0.01, 1.06)</b>     | <b>0.044</b> | 0.07 (0.030)             |     | <b>0.56 (0.24, 0.88)</b>    | <b>0.001</b> | 0.19 (<0.001)            |     | <b>0.44 (0.03, 0.86)</b>                   | <b>0.037</b> | 0.28 (<0.001)            |    |
|                                                        | B-Mn                 | -0.001 (-0.20, 0.20)         | 0.871        |                          |     | <b>0.41 (0.21, 0.61)</b>    | <b>0.001</b> |                          |     | <b>-0.35 (-0.58, -0.11)</b>                | <b>0.004</b> |                          |    |
|                                                        | B-Cu                 | <b>-0.35 (-0.73, 0.03)</b>   | <b>0.072</b> |                          |     | <b>-0.41 (-0.79, -0.04)</b> | <b>0.032</b> |                          |     | 0.26 (-0.25, 0.73)                         | 0.290        |                          |    |
| <b>Model 2</b><br>(Model 1+<br>Fe)                     | rs1805313            |                              |              |                          |     |                             |              |                          |     | <b>-0.22 (-0.42, -0.03)</b>                | <b>0.030</b> |                          |    |
|                                                        | age                  |                              |              |                          |     |                             |              |                          |     | -0.001 (-0.01, 0.01)                       | 0.666        |                          |    |
|                                                        | B-Se                 |                              |              |                          |     |                             |              |                          |     | <b>0.38 (-0.13, 0.74)</b>                  | <b>0.072</b> | 0.30 (<0.001)            | 79 |
|                                                        | B-Mn                 |                              |              |                          |     |                             |              |                          |     | <b>-0.36 (-0.59, -0.12)</b>                | <b>0.003</b> |                          |    |
|                                                        | B-Cu                 |                              |              |                          |     |                             |              |                          |     | 0.26 (-0.26, 0.71)                         | 0.298        |                          |    |
|                                                        | B-Fe                 |                              |              |                          |     |                             |              |                          |     | 0.20 (-0.22, 0.55)                         | 0.296        |                          |    |
| <b>Model 3</b><br>(Model 1+Zn<br>Hb)                   | rs1805313            | <b>-0.12 (-0.25, 0.016)</b>  | <b>0.084</b> |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | age                  | 0.006 (-0.009, 0.02)         | 0.404        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Zn                 | -0.27 (-0.68, 0.14)          | 0.195        |                          | 166 |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Se                 | <b>0.58 (0.05, 1.11)</b>     | <b>0.032</b> | 0.010 (0.020)            |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Mn                 | 0.05 (-0.15, 0.25)           | 0.615        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Cu                 | <b>-0.37 (-0.75, 0.005)</b>  | <b>0.053</b> |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | Hb                   | <b>0.67 (-0.04, 1.37)</b>    | <b>0.065</b> |                          |     |                             |              |                          |     |                                            |              |                          |    |
| <b>Model 4</b><br>(Model 1+Zn<br>BMI,<br>ever smoking) | rs1805313            | -0.10 (-0.23, 0.04)          | 0.161        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | age                  | 0.007 (-0.008, 0.02)         | 0.401        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | BMI                  | 0.004 (-0.008, 0.02)         | 0.455        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | ever smoking         | <b>0.24 (0.10, 0.37)</b>     | <b>0.001</b> | 0.15 (<0.001)            | 168 |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Zn                 | -0.14 (-0.54, 0.27)          | 0.508        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Se                 | <b>0.62 (0.11, 1.13)</b>     | <b>0.018</b> |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Mn                 | -0.02 (-0.21, 0.17)          | 0.841        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Cu                 | -0.33 (-0.73, 0.07)          | 0.104        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | rs1805313            | -0.09 (-0.23, 0.04)          | 0.165        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | age                  | 0.006 (-0.008, 0.02)         | 0.409        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | BMI                  | 0.002 (-0.011, 0.01)         | 0.795        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | ever smoking         | <b>0.22 (0.08, 0.35)</b>     | <b>0.002</b> | 0.15 (<0.001)            | 166 |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Zn                 | -0.17 (-0.58, 0.24)          | 0.420        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Se                 | <b>0.61 (0.08, 1.13)</b>     | <b>0.022</b> |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Mn                 | -0.02 (-0.17, 0.22)          | 0.819        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | B-Cu                 | -0.30 (-0.70, 0.10)          | 0.135        |                          |     |                             |              |                          |     |                                            |              |                          |    |
|                                                        | Hb                   | 0.51 (-0.20, 1.22)           | 0.160        |                          |     |                             |              |                          |     |                                            |              |                          |    |

|                                                                                                 |              |  |  |  |
|-------------------------------------------------------------------------------------------------|--------------|--|--|--|
| <b>Model 6</b><br>( <b>Model 1+Zn</b><br><b>BMI,</b><br><b>Ever smoking,</b><br><b>parity</b> ) | rs1805313    |  |  |  |
|                                                                                                 | age          |  |  |  |
|                                                                                                 | BMI          |  |  |  |
|                                                                                                 | ever smoking |  |  |  |
|                                                                                                 | parity       |  |  |  |
|                                                                                                 | B-Zn         |  |  |  |
|                                                                                                 | B-Se         |  |  |  |
|                                                                                                 | B-Mn         |  |  |  |
|                                                                                                 | B-Cu         |  |  |  |
|                                                                                                 |              |  |  |  |

in bold are significant results ( $p < 0.05$ ) and in bold italic marginally significant results ( $p < 0.1$ ); <sup>a</sup> Stajniko et al., 2019 (in preparation)

|                             |              |
|-----------------------------|--------------|
| 0.08 (-0.05, 0.21)          | 0.244        |
| 0.007 (-0.01, 0.02)         | 0.381        |
| 0.003 (-0.01, 0.02)         | 0.637        |
| <b>0.13 (0.003, 0.27)</b>   | <b>0.050</b> |
| -0.12 (-0.26, 0.03)         | 0.122        |
| <b>0.41 (-0.03, 0.85)</b>   | <b>0.068</b> |
| 0.25 (-0.15, 0.65)          | 0.221        |
| <b>0.36 (0.16, 0.57)</b>    | <b>0.001</b> |
| <b>-0.39 (-0.76, -0.01)</b> | <b>0.042</b> |

183  
0.24 (<0.001)

**Table 4.** Influence of ALAD SNP rs1805313 minor allele (G) on urine Pb in Slovenian non-pregnant women and Croatian pregnant women assessed by multivariable linear regression model

| Dependent Variable (Pb) | Independent variable | Slovenian non-pregnant women |         |     |                          | Croatian pregnant women |         |     |                          |
|-------------------------|----------------------|------------------------------|---------|-----|--------------------------|-------------------------|---------|-----|--------------------------|
|                         |                      | $\beta$ (95% CI)             | p-value | N   | R <sup>2</sup> (p-value) | $\beta$ (95% CI)        | p-value | N   | R <sup>2</sup> (p-value) |
| <b>Model 1</b>          | rs1805313            | 0.70 (-0.38, 1.79)           | 0.205   |     |                          | -0.35 (-0.74, 0.03)     | 0.215   |     |                          |
|                         | age                  | 0.08 (-0.04, 0.21)           | 0.225   |     |                          | 0.02 (-0.02, 0.07)      | 0.274   |     |                          |
|                         | BMI                  | 0.03 (-0.07, 0.13)           | 0.572   | 165 | 0.02 (0.578)             | 0.00 (-0.04, 0.05)      | 0.989   | 145 | 0.05 (0.264)             |
|                         | Ever smoking         | -0.05 (-1.12, 1.03)          | 0.931   |     |                          | 0.23 (0.17, 0.64)       | 0.261   |     |                          |
|                         | P-Se                 | -0.46 (-4.17, 3.26)          | 0.809   |     |                          | 0.44 (-0.45, 1.33)      | 0.337   |     |                          |
| <b>Model 2</b>          | rs1805313            | 0.64 (-0.43, 1.71)           | 0.239   |     |                          | -0.23 (-0.62, 0.17)     | 0.261   |     |                          |
|                         | age                  | 0.07 (-0.05, 0.20)           | 0.255   |     |                          | 0.02 (-0.02, 0.07)      | 0.285   |     |                          |
|                         | BMI                  | 0.03 (-0.07, 0.13)           | 0.547   | 173 | 0.02 (0.576)             | 0.00 (-0.04, 0.05)      | 0.987   |     |                          |
|                         | Ever smoking         | -0.12 (-1.18, 0.92)          | 0.817   |     |                          | 0.27 (0.17, 0.69)       | 0.191   |     |                          |
|                         | Seafood intake       | 0.61 (-1.77, 2.98)           | 0.616   |     |                          | 0.19 (-0.48, 0.88)      | 0.578   |     |                          |
| <b>Model 3</b>          | rs1805313            |                              |         |     |                          | -0.37 (-0.75, 0.01)     | 0.059   |     |                          |
|                         | age                  |                              |         |     |                          | 0.03 (-0.02, 0.08)      | 0.244   |     |                          |
|                         | BMI                  |                              |         |     |                          | 0.00 (-0.05, 0.05)      | 0.991   | 144 | 0.05 (0.32)              |
|                         | Ever smoking         |                              |         |     |                          | 0.21 (-0.20, 0.62)      | 0.311   |     |                          |
|                         | parity               |                              |         |     |                          | -0.07 (-0.50, 0.36)     | 0.735   |     |                          |
|                         | P-Se                 |                              |         |     |                          | 0.44 (-0.46, 1.33)      | 0.334   |     |                          |

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**A.5 *SELENOP, GPX1, GPX3, GPX4, TXNRD1, TXNRD2, CAT and AQP9* Expression in Two As<sub>2</sub>O<sub>3</sub> Treated APL Patients**



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**IJS DP – 12826**

***SELENOP, GPX1, GPX3, GPX4, TXNRD1,  
TXNRD2, CAT, and AQP9 expression  
in two As<sub>2</sub>O<sub>3</sub> treated APL patients***

Anja Stajnko, Zdenka Šlejkovec, Helena Podgornik, Černelč Franc, Janja Zupan,  
Marta Jagodic, Janja Marc, Ingrid Falnoga

**Ljubljana, 20.5.19**

## 1. INTRODUCTION:

In our previous work, we studied associations between “protective” arsenic(3+) methyltransferase (*AS3MT*) haplotype, As methylation efficiency and *AS3MT* expression in APL patients treated with As<sub>2</sub>O<sub>3</sub> and healthy controls ([PhD dissertation, section 3.4](#)). We observed clear influence of haplotype on As methylation efficiency.

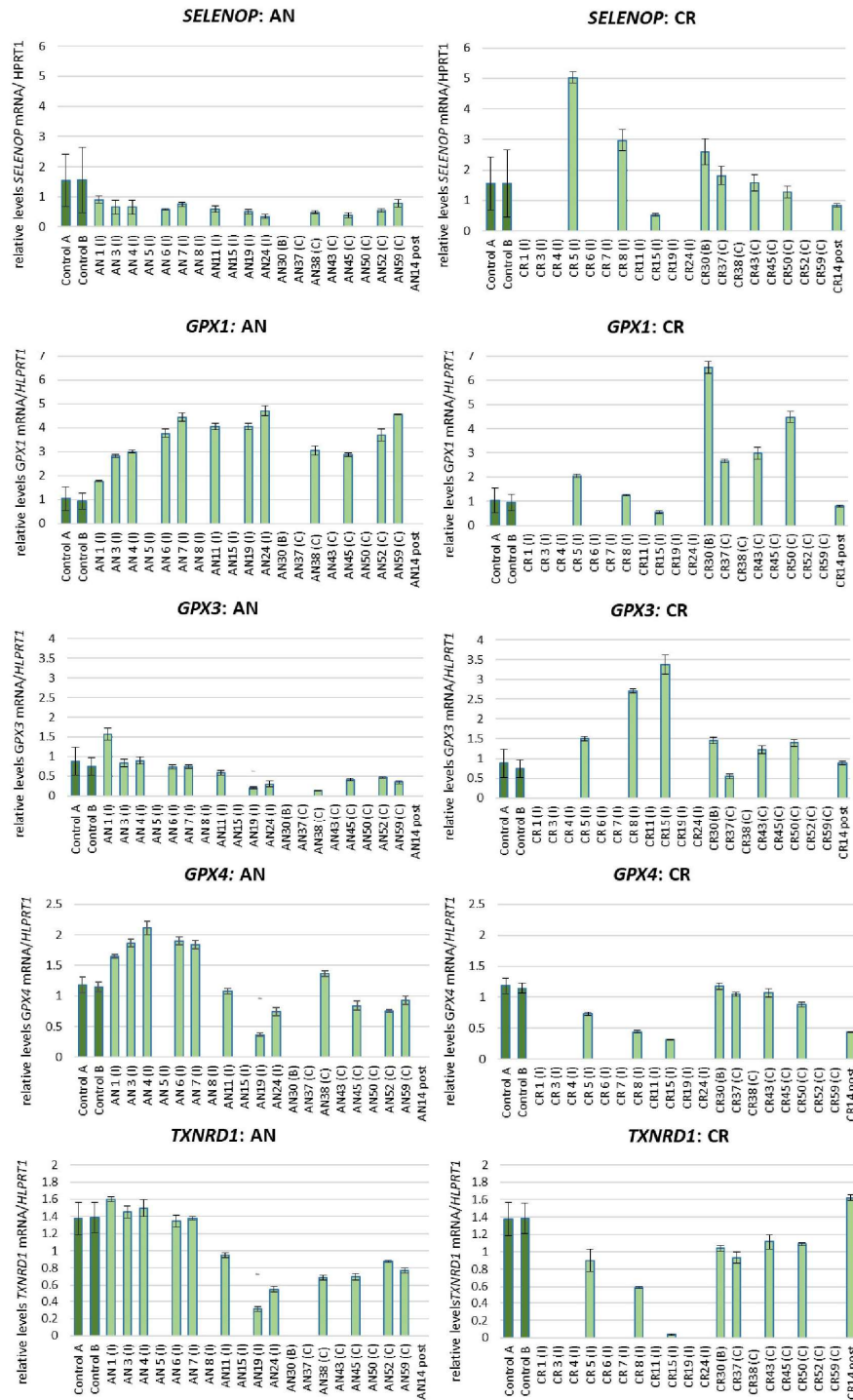
“Protective” *AS3MT* haplotype was constructed out of 6 *AS3MT* SNPs with the sequence rs7085104(G), rs3740400(G), rs3740393(C), rs3740390(T), rs10748835(A) and rs1046778(C) in 5’-3’ order, based on their position in the gene; described in details by ([Stajanko et al., 2019](#)).

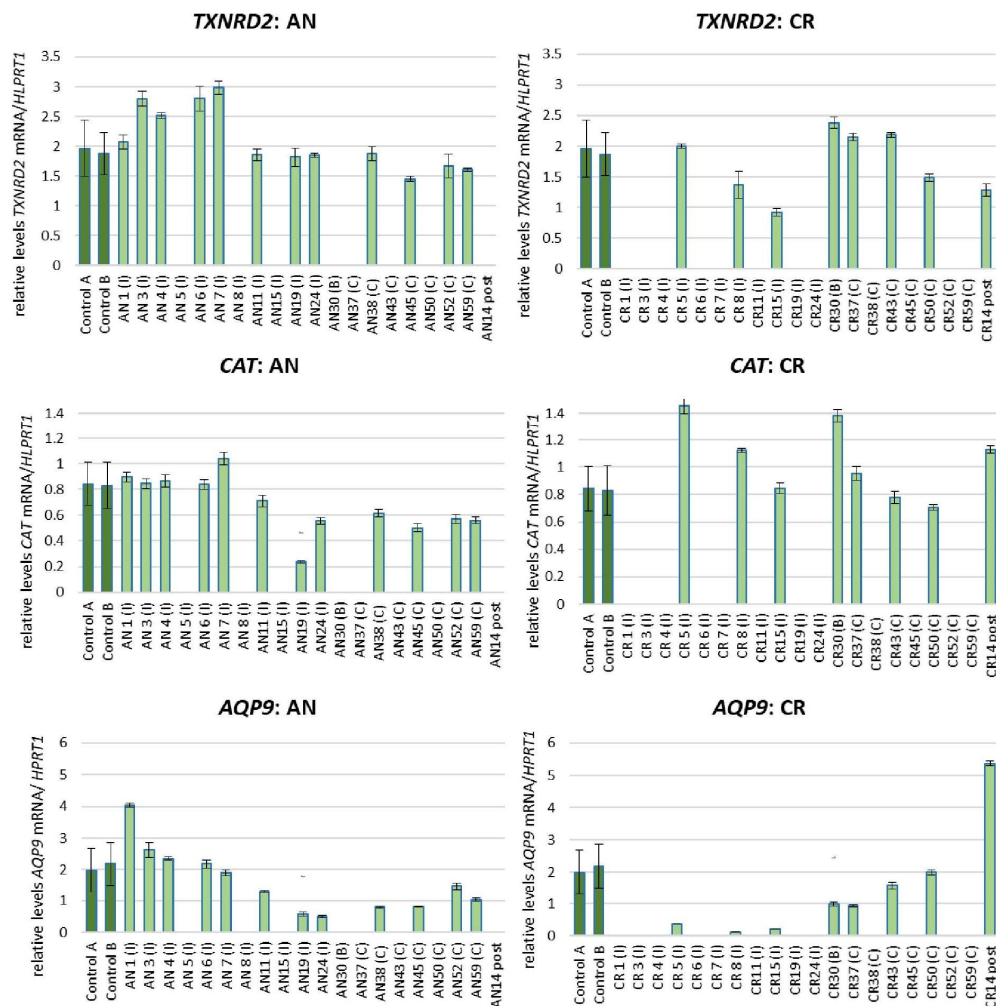
In addition to that study in present working report, we selected two patients with acute promyelocytic leukemia (APL); one carrier of protective *AS3MT* haplotype and one non-carrier (AN *AS3MT* hap- and CR *AS3MT*hap+, both women). We followed blood mRNA levels for 8 genes, which are involved in As metabolism and/or oxidative stress (aquaporin 9 - *AQP9*, selenoprotein P - *SELENOP*, glutathione peroxidase 1, 3 and 4 - *GPX1*, 3, and 4, thioredoxin reductase 1 and 2 - *TXNRD1* and 2, catalase - *CAT*). For the comparison, expression was followed also in 7 healthy women controls (control A), which were additionally stratified based on the presence (n = 2) or absence (n = 5) of protective *AS3MT* haplotype (control B), in order to see possible connection of haplotype with other metabolic pathways.

Expression of *GPX1*, 3 and 4, *TXNRD1*, *TXNRD2* and *SELENOP*, genes for patient CR was already determined in year 2013 and presented on the 11<sup>th</sup> International Symposium on Selenium in biology and medicine ([Falnoga, et al., 2013](#)). Trends were similar to those presented below; however, some observed differences were most probably due to the use of different housekeeping genes for the normalisation of expression (geometric mean of *RPLP0*, *ACTB* and *GAPDH* in study from 2013 and only *HPRT1* in present study).



## 2. RESULTS:





**Figure 1:** mRNA levels of various genes (normalised by housekeeping gene *HPRT1*) followed in pre-infusion blood samples of two APL patients (AN as *AS3MThap*<sup>-</sup> and CR as *AS3MThap*<sup>+</sup>) during the therapy with As<sub>2</sub>O<sub>3</sub>, and compared with average expression of healthy controls (A = all women, n=7; B = *AS3MThap*<sup>+</sup> (n=2) when compared to CR patient or *AS3MThap*<sup>-</sup> (n=5) when compared to AN patient).

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## Publications Related to the Thesis

### Journal Articles

- Snoj Tratnik, J., Falnoga, I., Mazej, D., Kocman, D., Fajon, V., Jagodic, M., ... Horvat, M. (2019). Results of the first national human biomonitoring in Slovenia: Trace elements in men and lactating women, predictors of exposure and reference values. *International Journal of Hygiene and Environmental Health*, 222(3), 563–582. <https://doi.org/10.1016/j.ijheh.2019.02.008>
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### Conference Paper

- Stajniko, A., Falnoga, I., Mazej, D., Horvat, M. (2017) Metallothioneins polymorphism and trace elements in pregnant females (PHIME study). In *Journal of trace elements in medicine and biology*.



- Stajanko, A., Horvat, M., Falnoga, I. (2017) Urine biomarkers at low-level exposure: study of cadmium and renal function proteins. In: *Proceedings of Uniromics nephrology*. Lisbon, Portugal.
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# Biography

The author of this thesis Anja Stajnko was born on March 24th, 1988 in Rakičan, Slovenia. She finished primary school in Križevci, Slovenia, and high school at Gymnasium Franca Miklošiča Ljutomer, Slovenia. In 2007, she enrolled in the undergraduate studies “Microbiology” at the Biotechnical Faculty of University of Ljubljana and received her BSc degree in microbiology in 2010. In the same year, she enrolled in the master program “Molecular Biology” at the same faculty. Following the defense of her master’s thesis entitled “Speciation of arsenic in tissue of selected cartilaginous fish from the Northern Adriatic” she was awarded the MSc of Molecular Biology degree in 2013. During her bachelor and master studies, she was a scholarship holder from the Department of Environmental Sciences at the Jožef Stefan Institute, Ljubljana. In 2014, she did 2 months of traineeship at the Plant and Soil Science department of the University of Aberdeen, Scotland, where she broadened her theoretical and practical knowledge on molecular biology techniques. In the same year, she started her PhD studies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Asst. Prof. Ingrid Falnoga.

The focus of her PhD research is the investigation of gene – environment interactions. More precisely, the estimation of biomarker appropriateness and estimation of influences of genetic variability (single nucleotide polymorphisms and gene expression) on the internal dose of non-essential and essential trace elements in populations with either low to moderate exposure or individuals with acute promyelocytic leukemia. For this purpose, she used different molecular techniques for DNA and RNA isolation, genotyping and gene expression analyses. During her studies, she attended various workshops and various national and international conferences related to her work. In 2018, she did two months of traineeship at the Institute of Environmental Medicine (IMM), Karolinska Institutet in Stockholm, Sweden, where she broadened her theoretical and practical knowledge in the field of epigenetics, which will play an important role in her future research.



